Ogataea neixiangensis sp. nov. and Ogataea paraovalis f.a., sp. nov., two methanol-assimilating yeast species isolated from rotting wood

Yun-Feng Lu, Min Wang, Jun Zheng and Feng-Li Hui*

Abstract

Four yeast strains were isolated from rotting wood samples collected in the Baotianman Nature Reserve in Henan Province, central China. The sequences of the D1/D2 domains of the large subunit rRNA gene and the internal transcribed spacer regions showed that these four strains represent two different undescribed yeast species belonging to the Ogataea clade. Ogataea neixiangensis sp. nov. produces two to four hat-shaped ascospores per ascus, and its closest relative among recognized species is Candida nitratophila. Ogataea paraovalis f.a., sp. nov. is closely related to Candida ovalis but the formation of ascospores was not observed on various sporulation media. The type strain of O. neixiangensis sp. nov. (MycoBank number MB 820697) is NYNU 16951T (=CICC 33166=CBS 14695T), and the type strain of O. paraovalis f.a., sp. nov. (MycoBank number MB 820698) is NYNU 167106T (=CICC 33168=CBS 14697T).

The genus Ogataea was proposed by Yamada et al. [1] based on partial sequence analysis of small subunit (SSU) and large subunit (LSU) rRNA genes. At that time, the genus contained five species and two varieties, which were formerly assigned in the genus Pichia [1]. Because of the relatively small number of species compared in this study, the new genus was not immediately accepted [2]. In recent years, the proposal of the genus Ogataea has been supported by single and multigene analyses including more species of methanol-assimilating yeasts and some related taxa [2–7]. The quickly expanding genus Ogataea currently comprises more than 40 recognized yeast species, which produce ascospores that may be hat-shaped, allantoid or spherical with a ledge [7, 8]. There are more than 20 species of the genus Candida recognized as members of the Ogataea clade based on phylogenetic analysis of rRNA gene sequences [7, 9]. The vast majority of the members of the Ogataea clade share the ability to grow with methanol as a sole carbon source, which is primarily formed as a by-product of plant cell-wall metabolism [10]. Indeed, numerous yeasts in the Ogataea clade have been recovered from plants or plant-related substrates [5].

During a study of the yeast communities associated with rotting wood collected in the Baotianman Nature Reserve, four novel methanol-assimilating yeasts were found. Sequence analysis of the D1/D2 domains of the LSU rRNA gene and internal transcribed spacer (ITS) regions showed that these strains represent two novel species belonging to the Ogataea clade. In this work, we describe these two species as Ogataea neixiangensis sp. nov. and Ogataea paraovalis f.a., sp. nov., respectively.

The strains considered in this study are shown in Table 1. The rotting wood samples were collected from the Baotianman Nature Reserve (33°27′N 111°48′E) in Henan Province, central China. Yeast strains were isolated from rotting wood samples in accordance with the method described by Hui et al. [11]. Each sample (1 g) was added to 20 ml sterile yeast extract-malt extract (YM) broth (1 % glucose, 0.5 % peptone, 0.3 % yeast extract and 0.3 % malt extract; pH 5.4) supplemented with 0.02 % chloramphenicol in a 150 ml Erlenmeyer flask and then incubated at 25°C for 3 days on a rotary shaker. Subsequently, 0.1 ml enrichment culture and appropriate decimal dilutions were spread on YM agar plates supplemented with 0.02 % chloramphenicol and then incubated at 25°C for 3–4 days. Different yeast morphotypes were purified at least twice and then stored on YM agar slants at 4°C or in 15 % (v/v) glycerol at −80°C.

The yeast isolates were characterized by standard procedures described by Kurtzman et al. [12]. All tests were performed by replica plating on solid and in liquid media, and...
the results were read after 5 and 21 days of incubation. Ascosporulation was investigated by inoculating the yeast strains individually or in pairs on YM agar, 5% malt extract agar (MEA), corn meal agar (MEA) 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) at 17 and 25 °C for up to 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 sequenced using primers NL1 and NL4 [13] and ITS1 and ITS4 [14], respectively. Each 50µl PCR mixture included 21 µl PCR-grade water, 1 µl DNA template, 1.5 mM each primer and 1 µl PCR Master Mix (2×) (0.05 U Taq DNA polymerase µl⁻¹, reaction buffer, 4 mM MgCl₂, 0.4 mM each dNTP; Sangon Biotech). PCR reactions were carried out in a S1000 thermal cycler (Bio-Rad). The amplified products were purified with a QIAquick purification kit (Sangon Biotech) according to the manufacturer’s instructions. Sangon Biotech (China) performed direct sequencing of the purified LSU rRNA gene and ITS PCR products using primers NL1 and NL4, and ITS1 and ITS4 with a Taq Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer’s protocol. Purified sequencing reaction mixtures were separated with a 3730XL automated DNA analyser (Applied Biosystems).

The program BLAST [15] from the National Centre of Biotechnology Information was used to compare the sequences from the D1/D2 domains of the LSU rRNA gene and ITS regions with available data present in GenBank (www.ncbi.nlm.nih.gov) for assignment of the most closely related taxa. The sequences were aligned using CLUSTAL X version 1.81 [16]. A phylogenetic tree was reconstructed based on D1/D2 sequences with MEGA software version 5.0 [17]. The evolutionary distance data were calculated using Kimura’s two-parameter model [18] in the neighbour-joining analyses. Pichia membranifaciens NRRL Y-2026T was employed as the outgroup in this analysis. Confidence limits were estimated from bootstrap analysis (1000 replicates) [19], and only values above 50% were recorded on the resulting trees. Sequences from this study and reference sequences obtained from GenBank are listed on the tree.

### Table 1. Yeasts isolated from rotting wood in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source of isolation</th>
</tr>
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<tbody>
<tr>
<td>O. neixiangensis sp. nov.</td>
<td>14695T</td>
<td>CBS</td>
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<tr>
<td>O. paraovalis sp. nov.</td>
<td>14697T</td>
<td>CBS</td>
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<tr>
<td></td>
<td>33166T</td>
<td>CICC</td>
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<tr>
<td></td>
<td>33168T</td>
<td>NYNU</td>
</tr>
<tr>
<td></td>
<td>16951T</td>
<td>Rotting wood, September 2016</td>
</tr>
<tr>
<td></td>
<td>16958</td>
<td>Rotting wood, September 2016</td>
</tr>
<tr>
<td></td>
<td>167106T</td>
<td>Rotting wood, July 2016</td>
</tr>
<tr>
<td></td>
<td>16855</td>
<td>Rotting wood, August 2016</td>
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</tbody>
</table>

CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CICC, China Centre of Industrial Culture Collection, Beijing, PR China; NYNU, Microbiology Lab, Nanyang Normal University, Henan, PR China.

### SPECIES DELINEATION, CLASSIFICATION AND ECOLOGY

Phylogenetic analysis of D1/D2 sequences showed that the four novel strains were in two separate clusters in the Ogataea clade (Fig. 1). Strains NYNU 16951T and NYNU 16958, which had identical D1/D2 and ITS sequences, were clustered in a clade containing Ogataea ramenticola, Ogataea methylivora, Ogataea nagamishii and seven related anamorphic species of the genus Candida (Fig. 1). However, the position of the novel species represented by these strains within the clade remained unclear as evidenced by the low bootstrap value. These two strains differed in the D1/D2 domain by 11 or more substitutions from their closest relatives, Candida sp. NRRL YB-1238 and Candida nitratophila NRRL YB-3654T. The ITS sequences of the two strains differed by 26 substitutions from that of the type strain of C. nitratophila but could not be aligned successfully with the sequence of Candida sp. NRRL YB-1238.

The phenotypic profile of strains NYNU 16951 and NYNU 16958 fit the description of the genus Ogataea. Two to four hat-shaped ascospores were produced in a deliquescent ascus. Additionally, the two strains of this novel species could be differentiated physiologically from the most closely related known species, C. nitratophila [9], in terms of growth at 30 °C, ability to ferment trehalose and inability to assimilate D-glucosamine or D-ribose. Hence, we concluded that the two strains represent a novel species of the genus Ogataea. The name Ogataea neixiangensis sp. nov. is proposed to categorize these two strains.

The D1/D2 and ITS sequences of the other strains, NYNU 167106T and NYNU 16855, were also identical. They formed a statistically well-supported clade with Candida ovalis, Candida sithepensis and Candida arabinofermentans in a tree reconstructed from the D1/D2 sequences (Fig. 1). They differed from the type strain of C. ovalis, the closest species in term of pairwise sequence similarities, by six substitutions in the D1/D2 domain. Analysis of the ITS sequence showed that these two strains differed by 49 substitutions from the latter species.

Physiological differences were found between strains NYNU 167106T and NYNU 16855 and their nearest phylogenetic relatives. Specifically, strains NYNU 167106T and
Fig. 1. Phylogenetic tree derived from neighbour-joining analysis based on the sequences of the D1/D2 domains of the LSU rRNA gene, showing the placement of the two novel species in the Ogataea clade. *Pichia membranifaciens* NRRL Y-2026\(^1\) was used as an outgroup. Bootstrap values of above 50\% are given at nodes based on 1000 replications. Bar, 0.02 substitutions per site.
NYNU 16855 could be differentiated from their sister species, *C. ovalis* [9], by the ability to grow at 37°C, and the inability to assimilate L-sorbose, DL-lactate, succinate or citrate. On the basis of the evidence from the molecular and other taxonomic criteria obtained during this study, we concluded that the two strains represent a novel species of the genus *Ogataea* although formation of ascospores was not observed. The name *Ogataea paraovalis* f.a., sp. nov. is proposed to accommodate strains NYNU 167106\(^T\) and NYNU 16855.

Members of the *Ogataea* clade have been found associated with plant material including tree bark, tree exudate, flowers, leaves, gall on a leaf, rotten wood, insects and insect frass [3–9]. Among these, a few strains of the recognized species were reported to be isolated from rotten wood, i.e. *Candida hungarica*, *Ogataea methylichroma*, *Ogataea nitratotransversa* and *Ogataea salutana* [4, 8, 9]. This study increases the number of species of the genus *Ogataea* isolated from rotten wood.

**DESCRIPTION OF *Ogataea neixiangensis* HUI AND ZHENG SP. NOV.**

*Ogataea neixiangensis* (nei.xiang.en’esis. N.L. fem. adj. neixiangensis of or belonging to the county of Neixiang, Henan Province, China, the collection locality of the type strain).

In YM broth after 3 days at 25°C, cells are ovoid (2–5×3–6 µm) and occur singly or in pairs. Budding is multilateral (Fig. 2a). After 7 days at 25°C, compact sediment is present and a pellicle is absent. 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 or true hyphae are not formed. Ascii develop from conjugation between a cell and its bud. Two to four hat-shaped ascospores are formed at 25°C after 7 days on corn meal and YCBS agars (Fig. 2b). Ascospores are rapidly liberated from the asc and tend to agglutinate. Glucose, galactose and trehalose are fermented weakly but not sucrose, maltose, lactose, raffinose, inulin, cellobiose, xylose, methyl α-D-glucoside, melibiose, melezitose or soluble starch. Glucose, inulin (weakly), galactose, trehalose (weakly), salicin (weakly), L-rhamnose, D-xylose, L-arabinose, methanol, ethanol, glycerol, erithritol, ribitol, mannitol, glucitol, sucrose, xylitol, D-glucurono-1,5-lactone, arbutin (weakly) and L-arabinose are assimilated. No growth occurs with sucrose, raffinose, melibiose, lactose, maltose, melezitose, methyl α-D-glucoside, cellobiose, L-sorbose, D-arabinose, D-ribose, galactitol, myo-inositol, DL-lactate, citrate, D-gluconate, D-glucosamine, 2-keto-D-gluconate, 5-keto-D-gluconate or D-glucuronate. For the assimilation of nitrogen compounds, nitrate, nitrite, L-lysine, glucosamine and D-tryptophan are positive, whereas ethylamine, cadaverine, creatine, creatinine and imidazole are negative. Growth is observed at 40°C but not at 42°C. Growth in the presence of 0.1% cycloheximide is positive, but growth in the presence of 1% acetic acid or 10% NaCl plus 5% glucose is negative. Starch-like compounds are not produced. Urease activity and diazonium blue B reactions are also negative.

The type strain, NYNU 16951\(^T\), was isolated in September 2016 from rotten wood collected in the Baotianman Nature Reserve in Henan Province, central China. It has been deposited in the China Centre of Industrial Culture Collection (CICC), Beijing, China, as strain CICC 33166\(^T\) and is permanently preserved in a metabolically inactive state. Ex-type culture has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 14695\(^T\). The MycoBank number is MB 820697.

**DESCRIPTION OF *Ogataea paraovalis* HUI AND ZHENG SP. NOV.**

*Ogataea paraovalis* (pa.ra.o.va’lis. N.L. gen. n. paraovalis like ovalis, referring to its phylogenetic closeness to *C. ovalis*).

In YM broth after 3 days at 25°C, cells are ovoid to elliptical (2–4×3–5 µm) and occur singly or in pairs. Budding is multilateral (Fig. 2c). Sediment is formed after 7 days, but no pellicle is observed. 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 or true hyphae are not formed. Ascospores are not observed on YM, 5% malt extract, corn meal or YCBS agars in pure or mixed cultures at 17 or 25°C for up...
to 4 weeks. Glucose and trehalose are fermented but not galactose, sucrose, maltose, lactose, raffinose, methyl α-D-glucoside, cellobiose, xylose, melibiose, melezitose, inulin or soluble starch. Glucose, trehalose, cellobiose, salicin (weakly), D-xylose, L-arabinose, D-arabinose (weakly), D-ribose, methanol, ethanol, glycerol, erythritol, ribitol, mannitol, glucitol, D-gluconate (weakly), xylitol, D-glucono-1,5-lactone, arbutin and L-arabinitol are assimilated. No growth occurs with inulin, sucrose, raffinose, melibiose, galactose, lactose, maltose, methyl α-D-glucoside, melezitose, L-sorbose, L- rhamnose, galactitol, myo-inositol, Dl-lactate, succinate, citrate, D-glucosamine, 2-keto-D-gluconate, 5-keto-D-glucuronate or D-glucuronate. For the assimilation of nitrogen compounds, ethylamine, L-lysine, D-glucosamine and D-tryptophan are positive, whereas nitrate, nitrite, cadaverine, creatine, creatinine and imidazole are negative. Growth is observed at 40 °C but not at 42 °C. Growth in the presence of 0.1% cycloheximide is positive, but growth in the presence of 1% acetic acid or 10% NaCl plus 5% glucose is negative. Starch-like compounds are not produced. Urease activity and diazonium blue B reactions are also negative.

The type strain, NYNU 167106T, was isolated in July 2016 from rotten wood collected in the Baotianan Nature Reserve in Henan Province, central China. It has been deposited in the China Centre of Industrial Culture Collection (CICC), Beijing, China, as strain CICC 33168, and is permanently preserved in a metabolically inactive state. Ex-type culture has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 14697T. The MycoBank number is MB 820698.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References