Four new yeasts in the *Pichia anomala* clade

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INTRODUCTION

In an effort to provide a rapid molecular-based system for yeast identification, Kurtzman & Robnett (1995, 1997, 1998) characterized all currently recognized ascomycetous yeasts from nucleotide divergence in the variable D1/D2 domain of large subunit (26S) rDNA. The extent of divergence in this domain appears to be sufficient to resolve nearly all species of ascomycetous yeasts. Several closely related species have proven to be exceptions and would be unresolved, but strains with greater than 1% substitution have been shown to be separate species from genetic crosses and from comparisons of nuclear DNA relatedness (Kurtzman & Robnett, 1998; Peterson & Kurtzman, 1991); thus providing a reliable means for strain identification when used in the context discussed above.

In the present study, four new ascomycetous yeasts were detected from their unique D1/D2 rDNA sequences. These strains are maintained in the Agricultural Research Service (ARS) Culture Collection (NRRL) but were not recognized as novel from sequence comparisons of all media cited in this report are given by Wickerham (1951) and Yarrow (1998). In the course of the work, additional strains for one of the species were detected from sequence similarity and these proved to be complementary mating types, which allowed production of the ascoporic state. Phylogenetic analysis of the sequences placed the four species in the *Pichia anomala* clade as recognized in the study of Kurtzman & Robnett (1998). Consequently, two of the species, which are ascosporic, were assigned to the genus *Pichia*, whereas the other two, in which no ascospores were detected, were placed in the genus *Candida*. The *Pichia* species are heterothallic and are of particular interest because they form spherical ascospores and show limited interspecific conjugation.

METHODS

Organisms, fermentation and assimilation tests, single ascospore isolations and scanning electron microscopy (SEM). Strains of the new species described (Table 1), as well as reference strains, are maintained in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, Illinois, USA. Fermentation and assimilation tests and the morphological examination of cultures are as described by Wickerham (1951) and Yarrow (1998), except that assimilation tubes were agitated on a reciprocal shaker at 25 °C for the 4 week incubation period. Protocols for strain isolation are unavailable, with the exception of NRRL Y-5377, NRRL Y-5381, NRRL YB-1545, NRRL YB-2116 and NRRL YB-2694, which were isolated by L. J. Wickerham. The first four strains were from direct streaking of samples onto YM agar plates followed by incubation at 25 °C. The sample providing NRRL YB-2694 was first incubated in liquid 20D medium (Wickerham, 1969) following by streaking onto a YM agar plate. The compositions of all media cited in this report are given by Yarrow (1998).

To determine whether the ascosporogenous strain NRRL Y-5377 was homothallic or heterothallic, single ascospore isolates were obtained with a de Fonbrune micro-manipulator using standard procedures as described by Fowell (1969). Asci of this species are persistent and...
Table 1. Strains of the newly described *Pichia* and *Candida* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain designation*</th>
<th>GenBank accession no. for D1/D2 sequences</th>
<th>Number of D1/D2 nucleotide differences with type strain</th>
<th>Ploidy or mating type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. maclurae</em></td>
<td>Y-5377&lt;sup&gt;T&lt;/sup&gt;</td>
<td>8671&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AF017405</td>
<td>Diploid</td>
<td>Decaying fruit of an osage orange, Peoria, Illinois, USA</td>
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<tr>
<td></td>
<td>Y-5377-1</td>
<td>8672</td>
<td>a</td>
<td>Single-ascospore isolate from NRRL Y-5377&lt;sup&gt;T&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td>Y-5377-3</td>
<td>8673</td>
<td>z</td>
<td>Single-ascospore isolate from NRRL Y-5377&lt;sup&gt;T&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Y-5381</td>
<td>8674</td>
<td>0</td>
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<td><em>P. misumaiensis</em></td>
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<td>8062&lt;sup&gt;T&lt;/sup&gt;</td>
<td>U73581</td>
<td>a</td>
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</tr>
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<td>a</td>
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<td></td>
<td>Y-27069</td>
<td>1784</td>
<td>0</td>
<td>α</td>
<td>Sweet apple must, Switzerland</td>
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<tr>
<td></td>
<td>Y-27080</td>
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<td>μ</td>
<td>Cider, UK</td>
</tr>
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<td></td>
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<td>a</td>
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</tr>
<tr>
<td></td>
<td>YB-3520</td>
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<td>μ</td>
<td>Soil, Ontario, Canada</td>
</tr>
<tr>
<td><em>C. mycetangii</em></td>
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<td>8675&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AF017241</td>
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<td>Mycetangium, unidentified ambrosia beetle, Kansas, USA</td>
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<td></td>
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<td>8676</td>
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<td>?</td>
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<tr>
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<td>Y-6846</td>
<td>8677</td>
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<tr>
<td><em>C. ulmi</em></td>
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<td>8668</td>
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<td>?</td>
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<tr>
<td></td>
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<td>?</td>
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<td></td>
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<td>8670&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AF017249</td>
<td>?</td>
<td>Insect frass, elm tree, Peoria, Illinois, USA</td>
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</tbody>
</table>

*T, Type strain. NRRL: ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA. CBS: Centraalbureau voor Schimmelcultures, Delft, The Netherlands.*

Ascosporases were released by digestion of ca 0.5 mm sporulating cells for 1-5 h at 25 °C in 20 µl of the enzyme preparation Glusulase (Endo Laboratories). For SEM, ascospores were critical-point dried using the procedures of Kurtzman et al. (1974). Specimens were observed in a JEOL JSM-6400V SEM.

**rDNA sequencing and sequence analysis.** Methods for nuclear DNA isolation, amplification of the 600 nt 26S rDNA domain D1/D2 by PCR and sequencing with the ABI TaqDyeDeoxy Terminator Cycle Sequencing kit and the ABI model 377 automated DNA sequencer (Applied Biosystems) were previously described (Kurtzman & Robnett, 1997).

Sequence data were visually aligned with QEDIT 2.15 (SemWare). Phylogenetic relationships were calculated with a Power Macintosh 8500/120 by the maximum-parsimony program of PAUP* 4.0 (written by D. L. Swofford; test version distributed by Sinauer Associates) with the simple heuristic search option. Relationships were further analysed by the neighbour-joining program of PAUP* 4.0 with the Jukes–Cantor distance measure. *Schizosaccharomyces pombe* was the designated outgroup in all analyses. Confidence limits for phylogenetic trees were estimated from bootstrap analysis (1000 replications). The nucleotide sequences for the new species reported in this study have been deposited with GenBank under the accessions numbers shown in Table 1; GenBank accession numbers for reference species were reported by Kurtzman & Robnett (1998).

**RESULTS**

The 26S domain D1/D2 nucleotide sequences of the four proposed new species were phylogenetically analysed in a database containing D1/D2 sequences from all currently recognized ascomycetous yeasts. The
New Pichia and Candida species analysis showed that the four taxa represent new species that are allied in a broad assemblage of species termed the Pichia anomala clade (Kurtzman & Robnett, 1998). Each of these new species shows greater than 1% nucleotide substitution with its nearest neighbour (Fig. 1). The two ascosporogenous

Fig. 1. Phylogenetic tree showing placement of the four proposed new species in the Pichia anomala clade as represented by one of six most parsimonious trees derived from maximum parsimony analysis of 26S rDNA domain D1/D2. Branch lengths are proportional to nucleotide differences as indicated on the bar. Numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replicates. Frequencies under 50% are not given. Tree length, 931; consistency index, 0.427; retention index, 0.619; rescaled consistency index, 0.265; homoplasy index, 0.573; parsimony-informative characters, 176. Schizosaccharomyces pombe served as outgroup species for the analysis.
species are assigned to the genus *Pichia* and the two anascosporogenous species are placed in the genus *Candida*.

**Latin diagnosis of *Pichia maclurae* Kurtzman sp. nov.**


**Description of *Pichia maclurae* Kurtzman sp. nov.**

*Pichia maclurae* (ma.clur.ae. L. gen. fem. adj. maclurae of Maclura, referring to the genus name of the osage orange).

After 3 d growth on 5 % malt extract agar at 25 °C, the cells are spherical (2–5–5–0 µm) to ellipsoidal or occasionally elongate (1–8–5–0 x 3–0–8–0 µm), and occur singly or sometimes in pairs (Fig. 2a). Cells often contain 1–4 distinct refractile droplets. Budding is multilateral. Growth is tannish-white, glistening and butyrous. Thin pellicles are formed on the surface of some assimilation media. After 7 d Dalmau plate culture on morphology agar at 25 °C, growth under the cover-slip shows pseudohyphae with sparse

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**Fig. 2. *Pichia maclurae* NRRL Y-5377T.** (a) Budding yeast cells, 3 d, 5 % malt extract agar, 25 °C; (b) pseudohyphae, 7 d, Dalmau plate culture on yeast morphology agar, 25 °C; (c) asc with ascospores, 2 weeks, YM agar, 25 °C; (d) ascospores by SEM; (e) conjugating cells, NRRL Y-5377-1 × NRRL Y-5377-3, 2 d, 5 % malt extract agar, 15 °C. Bars: a, b, c and e, 5 µm; d, 1 µm.
branching (Fig. 2b). Aerobic growth is dull, tannish-white, butyrous and somewhat raised, but with a depressed centre. The colony margin is entire to sparingly lobed. A faint, fragrant ester-like odour may be present. On YM and 5% malt extract agar, asci with ascospores were detected after 2 weeks at 25 °C, and after 4–5 weeks at 15 °C. Ascosporulation was markedly more abundant at 15 °C, and 5% malt extract agar was the better medium. Asci are unconjugated, persistent and form 2–3 spherical ascospores that appear slightly roughened under the light microscope (Fig. 2c). By SEM, ascospore walls are seen to be heavily ornamented with small protuberances and one or more irregularly placed, often circumfluent, ledges (Fig. 2d). To determine whether or not P. machaerum is heterothallic, single-ascospore isolates were obtained from 10 three-spored asci. Colonies developing from single ascospores were asporogenous, but the pairing of appropriate isolates resulted in abundant conjugations between cells (Fig. 2e) and the formation of appropriate isolates resulted in abundant con-jugations between cells (Fig. 2e) and the formation of 2–3 spherical ascospores that appear slightly roughened under the light microscope (Fig. 2e). By SEM, ascospore walls are seen to be heavily ornamented with small protuberances and one or more irregularly placed, often circumfluent, ledges (Fig. 2d). To determine whether or not P. machaerum is heterothallic, single-ascospore isolates were obtained from 10 three-spored asci. Colonies developing from single ascospores were asporogenous, but the pairing of appropriate isolates resulted in abundant con-jugations between cells (Fig. 2e) and the formation of ascospores, thus demonstrating the species to be heterothallic. Of the 30 single ascospores obtained, 28 were viable and gave the following distribution of mating types: a = 12, z = 16. The failure of asc to form tetrads did not markedly affect the distribution of mating types. NRRL Y-5377-1 (a) and NRRL Y-5377-3 (z), single-ascospore isolates derived from the same ascus, were selected as representative mating types. Mixtures of complementary mating types demonstrated sexual agglutination, which was apparent from the thickened consistency of growth as the cells were combined and from the clumping of cells as seen under the light microscope. All complementary pairs exhibited sexual agglutination and the reaction was strong for most pairs. Glucose, sucrose and raffinose (weak) are fermented; galactose, maltose, and fructose are not fermented. Assimilation of carbon compounds is as follows: glucose, +; galactose, −; l-sorbos, −; sucrose, +; maltose, +; cellobiose, +; trehalose, +; lactose, −; melibiose, −; raffinose, +; melezitose, +; inulin, variable; soluble starch, −; d-xylene, −; l-arabinose, −; d-arabinose, −; d-ribose, −; l-rhamnose, −; d-glucosamine, −; N-acetyl-d-glucosamine, −; methanol, −; ethanol, +; glycerol, +; erythritol, −; ribitol, −; galactitol, −; d-mannitol, +; d-glucitol, +; methyl x.d-glucoside, +; salicin, +; d-glucanate, +; 2-keto-dglucanate, −; 5-keto-d-glucanate, −; saccharate, −; di-lactate, weak/−; succinate, +; citrate, +; inositol, −; hexadecane, −. Assimilation of nitrogen compounds: nitrate, −; cadaverine, +. Growth or response in other tests: vitamin-free medium, −; 10% NaCl/5% glucose, −; starch formation, −; gelatin liquefaction, −; cycloheximide, 100 μg ml⁻¹, +; 37 °C, −. Source of cultures: NRRL Y-5377 and NRRL Y-5381 were isolated by L. J. Wickerham in early December 1960, from fallen, decaying fruits of the osage orange (hedge apple), Machura pomifera (Raf.) Schneid, which were collected near the National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois, USA. Type: NRRL Y-5377T (= CBS 8671T) is preserved as a lyophilized culture in the ARS Culture Collection (NRRL), Peoria, Illinois, USA.

Latin diagnosis of Pichia misumaiensis Sasaki & Yoshida ex Kurtzman sp. nov.


In 1958, Y. Sasaki and T. Yoshida briefly described ‘Hansenula misumaiensis’ in an abstract entitled ‘Hansenula misumaiensis Sasaki et Yoshida’ for the Hokkaido Branch Meeting of the Agricultural and Chemical Society of Japan, but a valid description of this species was not published. The abstract included a drawing demonstrating spherical ascospores, but ascospores were no longer found in the representative strains deposited (NRRL Y-17389T and NRRL Y-17390).

Description of Pichia misumaiensis Sasaki & Yoshida ex Kurtzman sp. nov.

Pichia misumaiensis (misu.mai.ensis. L. nom. fem. adj. misumaiensis of Misumai, a region near Sapporo, Japan; the name applied by Y. Sasaki and T. Yoshida to ‘Hansenula misumaiensis’).

After 3 d growth on 5% malt extract agar at 25 °C, the cells are spherical (2.0–5.6 μm), ovoidal (2.0–4.0 μm × 2.3–6.0 μm) and sometimes elongate (ca 2.0 × 9.0 μm), and
occur singly or occasionally in pairs (Fig. 3a). Budding is multilateral. Cultures older than 2 weeks often have clusters of small spherical cells that superficially resemble aggregates of ascospores. Growth is thin, butyraceous, semi-glistening and tannish-white in colour. Pellicles are not formed on the surface of assimilation media. After 7 d Dalmau plate culture on morphology agar at 25 °C, growth under the cover-slip lacks hyphae or pseudohyphae. Aerobic growth is glistening to semi-glistening, finely striated, tannish-white, butyraceous and low convex with a depressed centre. The colony margin is entire to finely lobed. A faint acidic odour is present. None of the six strains listed in Table 1 formed ascospores on YM, 5% malt extract, V8 or RG agar media when incubated at 15 or 25 °C for up to 3 months. However, when certain strains were paired on 5% malt extract agar at 15 °C, conjugated cells developed after 3–7 d (Fig. 3b). Conjugation was sparse even for the most reactive strain pair (NRRL Y-17389 T × NRRL YB-3520) and sexual agglutination was not detected. After 5 weeks, a small number of ascospores were too few to be successfully viewed by SEM. The mating type designations of the six strains examined are given in Table 1. Glucose is fermented; galactose, sucrose, maltose, lactose, raffinose and trehalose are not fermented. Assimilation of carbon compounds is as follows: glucose, +; galactose, −; l-sorbose, −; sucrose, −; maltose, −; cellobiose, +; trehalose, −; lactose, −; melibiose, −; raffinose, −; melezitose, −; inulin, −; soluble starch, −; d-xylose, +; l-arabinose, −; d-arabinose, −; d-ribose, −; l-rhamnose, −; d-glucosamine, −; N-acetyl-d-glucosamine, −; methanol, −; ethanol, +; glycerol, +; erythritol, −; ribitol, −; lactitol, −; d-mannitol, +; d-glucitol, +; methyl d-glucoside, −; salicin, +; d-gluconate, +; 2-keto-d-gluconate, −; 5-keto-d-gluconate, −; saccharate, −; dl-lactate, +; sucinate, +; citrate, +; inositol, −; hexadecane, −. Assimilation of nitrogen compounds is as follows: nitrate, +; cadaverine, +. Growth or response in other tests: vitamin-free medium, −; 10% NaCl/5% glucose, −; starch formation, −; gelatin liquefaction, −; cycloheximide, 100 µg ml⁻¹, +; 37 °C, −. Source of cultures: NRRL Y-17389 and NRRL Y-17390 were received from the Centraalbureau voor Schimmelcultures (CBS), Delft, as the two strains cited in 1958 by Y. Sasaki and T. Yoshida in their description of 'Hansenula misumaiensis' (the strains were isolated from orchard soil in Japan); NRRL Y-27069 was received from CBS as Candida sp. and had been isolated from sweet apple must in Switzerland; NRRL Y-27080 and NRRL Y-27082 were received from CBS as Candida sp. and were from cider in the UK; and NRRL YB-3520 was received at NCAUR in 1953 as an unknown species from J. J. Miller and had been isolated from soil near Hamilton, Ontario, Canada. Type: NRRL Y-17389 T (= CBS 8062 T), preserved as a lyophilized culture in the ARS Culture Collection (NRRL), Peoria, Illinois, USA, has been designated the holotype. Because this species is heterothallic and usually haploid when isolated from nature, the complementary mating type NRRL YB-3520 a (= CBS 8549 a) is designated the syntype.

### Conjugation between P. maclurae and P. misumaiensis

Because of the morphological similarity between P. maclurae and P. misumaiensis, mating types of the two species were tested for a mating response. NRRL YB-3520/NRRL Y-5377-1 gave occasional conjugations between cells (Fig. 3d), but no mature ascospores were produced. One ascus was detected with two partial ascospore outlines. The other interspecific complementary pair, NRRL Y-17389 T/NRRL Y-5377-3, gave no conjugations or ascospores.

### Latin diagnosis of Candida mycetangii Kurtzman sp. nov.

*In agaro multi post dies 3 ad 25 °C, cellulae vegetativae globosae (1·3–5·0 µm), ellipsoidae aut elongatae (1·2–4·3 x 1·8–7·0 µm), singulae et binae. In agaro morphologicco post dies 7 ad 25 °C, incrementum fusce pallidum, nitens, butyrosum; centrum coloniae sublatum; margo glabro vel undulato. Pseudohyphae fiunt; hyphae verae non fiunt. Ascosporae non fiunt. Glucosum,*
Candida maritima related to margin is entire to slightly undulating. A faint, fragrant convex with a small central depression. The colony hyphae but readily separate at the septa. Aerobic pseudohyphae are morphologically similar to true the cover-slip shows moderately branched pseudo-myce- tangii. Thin pellicles form on the surface of white, glistening or semi-glistening and butyrous with (Fig. 4a). Budding is multilateral. Growth is tannish- cells are spherical (1 ± 3–5 µm), and occur singly or in pairs (Fig. 4b). Some pseudohyphae are morphologically similar to true hyphae but readily separate at the septa. Aerobic growth is butyrous, tannish-white, glistening and low convex with a small central depression. The colony margin is entire to slightly undulating. A faint, fragrant ester-like odour may be present. Ascospores were not detected in cultures of the three known strains of C. myceta- ngii, or in mixtures of the three, when grown for up to 2 months on YM, 5% malt extract agar, cornmeal or V8 agars at 15 and 25 °C. C. mycetangii is closely related to Candida maritima (Fig. 1; 9 nt differences) and for this reason, the type strain NRRL Y-17775T (= CBS 51075) was paired with each of the C. mycetangii strains on YM agar at 25 °C. The pairs were observed for 2 months, but there was no conjugation or ascosporation. Glucose, sucrose and raffinose (weak) are fermented; galactose, maltose, lactose and trehalose are not fermented. Assimilation of carbon compounds is as follows: glucose, +; galactose, −; l-sorbose, −; sucrose, +; maltose, +; cellobiose, +; trehalose, +; lactose, −; melibiose, −; raffinose, +; melizitose, +; inulin, variable; soluble starch, variable; d-xylene, +; l-arabinose, −; d-arabinose, weak/−; d-ribose, variable; l-rhamnose, +; d-glucosamine, −; N-acetyl-d-glucosamine, −; methanol, −; ethanol, +; glycerol, +; erythritol, −; ribitol, −; galactitol, −; d-mannitol, +; d-gluco-itol, +; methyl α-D-glucoside, +; salicin, +; d-gluconate, variable; 2-keto-d-gluconate, −; 5-keto-d-gluconate, variable; saccharate, −; D-lactate, +; succinate, +; citrate, +; inositol, −; hexadecane, −. Assimilation of nitrogen compounds: nitrate, −; cadaverine, +. Growth or response in other tests: vitamin-free me- dium, −; 10% NaCl, 5% glucose, −; starch formation, −; gelatin liquefaction, weak/−; cyclo- heximide, 10 µg ml⁻¹, variable, 100 µg ml⁻¹, variable; 37 °C, +. Source of cultures: NRRL Y-6843T (= LRB 69D³), NRRL Y-6845 (= LRB 70B4) and NRRL Y-6846 (= LRB 74) were received for identification at NCAUR in November 1965 and had been isolated by L. R. Batra from the mycetangia of unidentified ambrosia beetles collected in Kansas, USA. Type: NRRL Y-6843T (= CBS 8675³), pres-erved as a lyophilized preparation in the ARS Culture Collection (NRRL), Peoria, Illinois, USA.

Latin diagnosis of Candida ulmi Kurtzman sp. nov.


Description of Candida ulmi Kurtzman sp. nov.

Candida ulmi (ul.mi. L. gen. fem. adj. ulmi from Ulmus, referring to the genus name of the elm).

After 3 d growth on 5% malt extract agar at 25 °C, the cells are spherical (1.8–7.0 μm), ellipsoidal or elongate (1.3–6.0 × 2.0–8.0 μm), and occur singly or in pairs (Fig. 4c). Budding is multilateral. Growth is butyrous, tannish-white and semi-glistening. Thin pellicles are formed on the surface of some assimilation media. After 7 d Dalmau plate culture on morphology agar at 25 °C, the type of growth under the cover-slip is strain-dependent and may show rudimentary pseudohyphae or well-branched pseudohyphae with abundant blastoconidia (Fig. 4d). True hyphae were not detected. Aerobic growth is tannish-white, butyrous, glistening and low convex. Colony margins are smooth to finely serrate. A faint, fragrant ester-like odour was produced by two of the strains. Ascospores were not detected in cultures of the three known strains of C. ulmi, or in mixtures of the three, when grown for up to 2 months on YM, 5% malt extract or RG agars at 15 and 25 °C. On the basis of domain D1/D2 sequence analysis, C. ulmi is most closely related to Pichia alni (Fig. 1; 30 nt differences), which is heterothallic. Isolates of C. ulmi were paired with P. alni mating types NRRL Y-11625 (= CBS 6986) and NRRL Y-11626 (= CBS 6987) on YM agar at 25 °C and observed for 2 months. There was no conjugation or ascosporation in interspecific pairings. Glucose is fermented; galactose, sucrose, maltose, lactose, raffinose and trehalose are not fermented. Assimilation of carbon compounds is as follows: glucose, +; galactose, –; L-sorbose, –; sucrose, +; maltose, +; cellobiose, +; trehalose, +; lactose, –; melibiose, –; raffinose, –; melezitose, +; inulin, –; soluble starch, –; D-xylene, +; L-arabinose, –; D-arabinose, –; D-ribose, –; L-rhamnose, +; D-glucosamine, –; N-acetyl-D-glucosamine, –; methanol, –; ethanol, +; glycerol, +; erythritol, –; ribitol, –; galactitol, –; D-mannitul, +; D-glucitol, +; methyl D-galactoside, +; salicin, +; D-gluconate, +; 2-keto-D-gluconate, –; 5-keto-D-gluconate, –; saccharate, –; DL-lactate, +; succinate, +; citrate, +; inositol, –; hexadecane, –. Assimilation of nitrogen compounds: nitrate, +; cadaverine, +. Growth or response in other tests: vitamin-free medium, –; 10% NaCl/5% glucose, variable; starch formation, –; gelatin liquefaction, –; cycloheximide, 10 μg ml⁻¹, –; 37 °C, +. All three strains were isolated in Peoria, Illinois, USA in 1950 and 1951 by L. J. Wickiser. NRRL YB-1545 was from soil, and NRRL YB-2116 and NRRL YB-2694T were from frass in tunnels made by unidentified insects in elm (Ulmus sp.) trees. Type: NRRL YB-2694T (= CBS 8670T), preserved as a lyophilized preparation in the ARS Culture Collection (NRRL), Peoria, Illinois, USA.

DISCUSSION

Identification of yeast species from cellular morphology and reactions in traditional growth tests has been problematic because results from these procedures may be strain variable and often do not reflect genetic relatedness, which results in a relatively high frequency of misidentifications (Kurtzman & Phaff, 1987; Price et al., 1978). For this reason, molecular methods are being increasingly used to identify yeasts. The variable 600 nt D1/D2 domain of large subunit rDNA has been sequenced for all currently recognized ascomycetous yeasts (Kurtzman & Robnett, 1995, 1997, 1998), and these studies have shown that strains with greater than 1% sequence divergence are separate species and that strains with less divergence are usually conspecific. The impact of this work has been to provide a rapid means for strain identification and detection of new species. However, a few exceptions to the preceding predictions have been detected and need to be taken into account when using the D1/D2 database for strain identification. Some closely related taxa, such as Saccharomyces bayanus/Saccharomyces pastorinus and Candida shehatae var. shehatae/var. lignosa, show no differences in domain D1/D2 and their genetic divergence from each other can, at present, only be resolved from nuclear DNA re-association. One further exception concerns mating types of Metschnikowia agaves, which differ from each other by nearly 1% substitution (5/600 nt).

The original description of ‘Hansenula misumaiensis’ by Y. Sasaki and T. Yoshida in 1958 reported that the isolates produced ascospores, but these strains now
represent haploid mating types. Haploidization of yeasts in culture followed by the loss of one mating type is not unusual and was previously reported for *Yarrowia lipolytica* (Wickerham et al., 1970) and *Issatchenkia terricola* (Kurtzman & Smiley, 1976).

The genus *Pichia* consists of nearly 100 species (Kurtzman, 1998), and various molecular analyses have shown that the genus is polyphyletic. Yamada *et al.* (1992, 1994a, b, 1995a, b) placed some of the species in new genera following comparisons of partial 18S and 26S sequences, but most of the lineages were not well supported from statistical analysis. Weak basal branch support is evident from the domain D1/D2 analysis presented in Fig. 1, and complete 18S sequence analyses are often similarly weak (Kurtzman & Robnett, 1998). In view of the uncertainty concerning division of *Pichia* into genera that are phylogenetically circumscribed, the two new ascosporic species described here are placed in *Pichia* as currently defined (Kurtzman, 1998).

Interspecific mating responses are often regarded as being indicative of close relatedness between yeast species. Thus, it was surprising to detect a mating response between *P. maclurae* and *P. misumaiensis* because these two taxa do not appear to be sister species, and they differ from one another by 96 nt in domain D1/D2. Heterothallic species in the *P. anomala* clade known for interspecific mating include the following species pairs: *Pichia euphorbiae*/*Pichia meyeriae*; *Pichia amylophila*/*Pichia mississippiensis*; and *Pichia americana*/*Pichia bimundalis* (Kurtzman, 1998 and references therein). None of these interspecific matings gave viable ascospores and, as seen in Fig. 1, members of each pair are closely related sister species. In view of the observed mating response between *P. maclurae* and *P. misumaiensis*, it would be of interest to determine if there is a response between some of the more distantly related heterothallic species of the *P. anomala* clade.

*P. maclurae* and *P. misumaiensis* produce spherical ascospores, which contrasts with the hat-shaped ascospores formed by other close relatives in the clade. Similar contrasts have been found among other groups of yeasts. Species of *Issatchenkia* produce spherical ascospores but appear to be members of the *Pichia membranifaciens* clade, in which most species have hat-shaped ascospores (Kurtzman & Robnett, 1998). Members of the genus *Saturnispora* form spherical ascospores with equatorial ledges as do species of *Williopsis*, which are only distantly related to *Saturnispora* (Kurtzman & Robnett, 1998). Consequently, heterogeneity of ascospore shape among closely related species appears to be a common theme among the yeasts.

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**REFERENCES**


