Four new yeasts in the *Pichia anomala* clade

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Four new yeasts are described that were recognized as novel from nucleotide substitutions in domain D1/D2 of 26S rDNA, a region that is sufficiently divergent to allow resolution of most ascomycetous yeast species. The new species and their type strains are as follows: *Pichia macluriae* NRRL Y-5377^T (= CBS 8671^T); *Pichia misumaiensis* NRRL Y-17389^T (= CBS 8062^T); *Candida mycetangii* NRRL Y-6843^T (= CBS 8675^T); and *Candida ulmi* NRRL YB-2694^T (= CBS 8670^T). The two *Pichia* species form spherical ascospores and are heterothallic. Phylogenetic analysis of domain D1/D2 sequences placed the four new species in the *Pichia anomala* clade.

Keywords: *Pichia*, *Candida*, new yeast species, rDNA, molecular systematics

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 standard yeast identification methods that use diagnostic responses on physiological tests (Payne *et al.*, 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 asco-

sporic 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 inter-specific 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^T, NRRL Y-5381, NRRL YB-1545, NRRL YB-2116 and NRRL YB-2694^T, 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^T was first incubated in liquid 20D medium (Wickerham, 1969) followed 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^T was homothallic or heterothallic, single ascospore isolates were obtained with a de Fonbrune micro-manipulator using standard procedures as described by Powell (1969). Asci of this species are persistent and

Abbreviations: ARS, Agricultural Research Service; SEM, scanning electron microscopy.

The GenBank accession numbers for the 26S rDNA D1/D2 sequences of *Pichia macluriae*, *Pichia misumaiensis*, *Candida mycetangii* and *Candida ulmi* are AF017405, U73581, AF017241, and AF017249, respectively.

Table 1. Strains of the newly described *Pichia* and *Candida* species

Species	Strain designation*		GenBank accession no. for D1/D2 sequences	Number of D1/D2 nucleotide differences with type strain	Ploidy or mating type	Source
	NRRL	CBS				
<i>P. macluriae</i>	Y-5377 ^T	8671 ^T	AF017405	0	Diploid	Decaying fruit of an osage orange, Peoria, Illinois, USA
	Y-5377-1	8672			a	Single-ascospore isolate from NRRL Y-5377 ^T
	Y-5377-3	8673			α	Single-ascospore isolate from NRRL Y-5377 ^T
	Y-5381	8674			Diploid	Decaying fruit of an osage orange, Peoria, Illinois USA
<i>P. misumaiensis</i>	Y-17389 ^T	8062 ^T	U73581	0	a	Orchard soil, Japan
	Y-17390	8063			a	Orchard soil, Japan
	Y-27069	1784			α	Sweet apple must, Switzerland
	Y-27080	4149			α	Cider, UK
	Y-27082	4148			a	Cider, UK
	YB-3520	8549			α	Soil, Ontario, Canada
<i>C. mycetangii</i>	Y-6843 ^T	8675 ^T	AF017241	2	?	Mycetangium, unidentified ambrosia beetle, Kansas, USA
	Y-6845	8676			?	Mycetangium, unidentified ambrosia beetle, Kansas, USA
	Y-6846	8677			?	Mycetangium, unidentified ambrosia beetle, Kansas, USA
<i>C. ulmi</i>	YB-1545	8668	AF017249	0	?	Soil, Peoria, Illinois, USA
	YB-2116	8669			?	Insect frass, elm tree, Peoria, Illinois, USA
	YB-2694 ^T	8670 ^T			?	Insect frass, elm tree, Peoria, Illinois, USA

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

ascospores were released by digestion of ca 0.5 mm³ 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 accession 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

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.

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 ascosporegous

Fig. 2. *Pichia macluriae* NRRL Y-5377^T. (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) asci 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.

species are assigned to the genus *Pichia* and the two anascosporogenous species are placed in the genus *Candida*.

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

In agaro malti post dies 3 ad 25 °C, cellulae vegetativae globosae (2.5–5.0 µm) aut ellipsoideae (1.8–5.0 × 3.0–8.0 µm), singulae vel binae. In agaro morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, hebes, butyrosus; centrum coloniae sublatum; margo glabro vel undulato. Pseudohyphae fiunt; hyphae verae non fiunt. Species heterothallica. Asci liberi, 2–3 ascosporeae globosae, non liberi. Glucosum, sucrosus et raffinosis (infirme) fermentantur. Galactosum, maltosum, lactosum et trehalosum non fermentantur. Assimilantur glucosum, sucrosus, maltosum, cellobiosum, trehalosum, raffinosis, meleztosum, inulinum (variabile), ethanolum, glycerolum, D-mannitolum, D-glucitolum, methyl α, D-glucosidum, salicinum, D-gluconas, DL-acidum lacticum (variabile), acidum succinicum, acidum citricum et cadaverinum. Non assimilantur galactosum, L-sorbosum, lactosum, melibiosum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, erythritolum ribitolum, galac-

titolum, 2-keto-D-gluconas, 5-keto-D-gluconas, saccharatas, inositolum, hexadecanum et potassii nitras. Amyllum non formatur. Vitaminae externae ad crescentiam necessaria sunt. Gelatinum non liquescit; esteres fiunt (infirme); pellicula fiunt. Augmentum non fiunt in temperatura 37 °C. Typus: NRRL Y-5377^T (= CBS 8671^T), designat stirpem typicum. Isolata a pomum *Maclura pomifera*, Peoria, Illinois, USA, depositata in Collectione Culturarum ARS (NRRL), Peoria, Illinois.

Description of *Pichia macluriae* Kurtzman sp. nov.

Pichia macluriae (ma.clur.ae. L. gen. fem. adj. *macluriae* 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 × 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

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 agars, asci with ascospores were detected after 2 weeks at 25 °C, and after 4–5 weeks at 15 °C. Ascosporeulation 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. macluriae* 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 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: $\alpha = 12$, $a = 16$. The failure of asci to form tetrads did not markedly affect the distribution of mating types. NRRL Y-5377-1 (α) and NRRL Y-5377-3 (a), 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, lactose 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, variable; soluble starch, –; D-xylose, –; 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 α ,D-glucoside, +; salicin, +; D-gluconate, +; 2-keto-D-gluconate, –; 5-keto-D-gluconate, –; saccharate, –; DL-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 $\mu\text{g ml}^{-1}$, +; 37 °C, –. Source of cultures: NRRL Y-5377^T and NRRL Y-5381 were isolated by L. J. Wickerham in early December 1960, from fallen, decaying fruits of the osage orange (hedge apple), *Maclura pomifera* (Raf.) Schneid, which were collected near the National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois, USA. Type: NRRL

Y-5377^T (= CBS 8671^T) 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 agar malti post dies 3 ad 25 °C, cellulae vegetativae globosae (2.0–5.6 μm), ellipsoideae aut elongatae (2.0–4.0 $\times$ 2.3–6.0 μm), singulae et binae. In agar morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, nitens, butyrosus; centrum coloniae sublatum; margo glabro vel undulato. Pseudohyphae et hyphae verae non fiunt. Species heterothallica. Asci liberi, 2–4 ascosporae globosae, non liberi. Glucosum fermentatur. Galactosum, sucrosus, maltosus, lactosus, raffinosis et trehalosis non fermentantur. Assimilantur glucosum, cellobiosum, D-xylosum, ethanol, glycerolum, D-mannitol, D-glucitol, salicin, D-gluconas, DL-acidum lacticum, acidum succinicum, acidum citricum, cadaverinum et potassii nitrates. Non assimilantur galactosum, L-sorbosus, sucrosus, maltosus, trehalosis, lactosus, melibiosus, raffinosis, melezitosus, inulin, amyllum solubile, L-arabiosus, D-arabiosus, D-ribosus, L-rhamnosus, D-glucosaminus, N-acetyl-D-glucosaminus, methanol, erythritolum, ribitol, galactitol, methyl α ,D-glucosidum, 2-keto-D-gluconas, 5-keto-D-gluconas, saccharatas et inositol. Amyllum non formatur. Vitaminae externae ad crescentiam necessariae sunt. Gelatinum non liquescit; pellicula non fiunt. Augmentum non fiunt in temperatura 37 °C. Typus: NRRL Y-17389^T (= CBS 8062^T), designat stirpem typicam. Isolata a terra, Japonia, depositata in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

History of '*Hansenula misumaiensis*' Sasaki & Yoshida (1958) nom. nud.

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-17389^T 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 $\times$ 2.3–6.0 μm) and sometimes elongate (ca 2.0 $\times$ 9.0 μm), and

Fig. 3. *Pichia misumaiensis*. (a) Budding yeast cells, NRRL Y-17389^T, 3 d, 5% malt extract agar, 25 °C; (b) conjugation of complementary mating types, NRRL Y-17389^T × NRRL YB-3520, 5 d, 5% malt extract agar, 15 °C; (c) asci with ascospores, NRRL Y-17389^T × NRRL YB-3520, 5 weeks, 5% malt extract agar, 15 °C; (d) interspecific conjugations, *P. macluriae* NRRL Y-5377-1 × *P. misumaiensis* NRRL YB-3520, 5 d, 5% malt extract agar, 15 °C. Bars, 5 µm.

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, butyrous, 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, butyrous 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 observed. Asci are elongated, persistent and contain 2–4 spherical ascospores that appear to have smooth walls when viewed under the light microscope (Fig. 3c). 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 fer-

mented. 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, –; galactitol, –; D-mannitol, +; D-glucitol, +; methyl α -D-glucoside, –; salicin, +; D-gluconate, +; 2-keto-D-gluconate, –; 5-keto-D-gluconate, –; saccharate, –; DL-lactate, +; succinate, +; 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^T and NRRL Y-17390 were received from the Centraalbureau voor Schimmeltculturen (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^S (= CBS 8549^P) is designated the syntype.

Conjugation between *P. macluriae* and *P. misumaiensis*

Because of the morphological similarity between *P. macluriae* 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 malti post dies 3 ad 25 °C, cellulae vegetativae globosae (1.3–5.0 µm), ellipsoideae aut elongatae (1.2–4.3 × 1.8–7.0 µm), singulae et binae. In agaro morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, nitens, butyrosus; centrum coloniae sublatum; margo glabro vel undulato. Pseudohyphae fiunt; hyphae verae non fiunt. Ascospores non fiunt. Glucosum,

sucrosum et raffinoseum (infirme) fermentantur. Galactosum, maltosum, lactosum et trehalosum non fermentantur. Assimilantur glucosum, sucrosum, maltosum, cellobiosum, trehalosum, raffinoseum, melezitoseum, inulinum (variabile), amyllum solubile (variabile), D-xylosum, D-arabiosum (infirme, variabile), D-ribosum (variabile), L-rhamnosum, ethanolum, glycerolum, D-mannitolum, D-glucitolum, methyl α ,D-glucosidum, salicinum, D-gluconas (variabile), 5-keto-D-gluconas (variabile), DL-acidum lacticum, acidum succinicum, acidum citricum et cadaverinum. Non assimilantur galactosum, L-sorboseum, lactosum, melibiosum, L-arabiosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, erythritolum, ribitolum, galactitolum, 2-keto-D-gluconas, saccharatas, inositolum, hexadecanum et potassii nitras. Amyllum non formatur. Vitaminae externae ad crescentiam necessariae sunt. Gelatinum liquescit (infirme, variabile); esteres fiunt (infirme); pellicula fiunt. Augmentum fiunt in temperatura 37 °C. Typus: NRRL Y-6843^T (= CBS 8675^T) designat stirpem typicam. Isolata a mycetangium coleopterorum, Kansas, USA, depositata in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

Description of *Candida mycetangii* Kurtzman sp. nov.

Candida mycetangii [my.ce.tan.gii. L. gen. fem. adj. *mycetangii* from the fungus-inhabiting organ (mycetangium, mycangium) of beetles].

After 3 d growth on 5 % malt extract agar at 25 °C, the cells are spherical (1.3–5.0 µm), ovoidal or elongate (1.2–4.3 × 1.8–7.0 µm), and occur singly or in pairs (Fig. 4a). Budding is multilateral. Growth is tannish-white, glistening or semi-glistening and butyrous with a mycelial fringe. Thin pellicles form on the surface of some assimilation media. After 7 d Dalmu plate culture on morphology agar at 25 °C, growth under the cover-slip shows moderately branched pseudohyphae with abundant blastoconidia (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. mycetangii*, or in mixtures of the three, when grown for up to 2 months on YM, 5 % malt extract, 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-17775^T (= CBS 5107^T) 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 ascosporeulation. 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, +; melezitose, +; inulin, variable; soluble

Fig. 4. (a, b) *Candida mycetangii* NRRL Y-6843^T: (a) budding yeast cells, 3 d, 5 % malt extract agar, 25 °C; (b) pseudohyphae, 7 d, Dalmu plate culture on yeast morphology agar, 25 °C. (c, d) *Candida ulmi* NRRL YB-2694^T: (c) budding yeast cells, 3 d, 5 % malt extract agar, 25 °C; (d) pseudohyphae, 7 d, Dalmu plate culture on yeast morphology agar, 25 °C. Bars, 5 µm.

starch, variable; D-xylose, +; 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-glucitol, +; methyl α ,D-glucoside, +; salicin, +; D-gluconate, variable; 2-keto-D-gluconate, –; 5-keto-D-gluconate, variable; 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, –; starch formation, –; gelatin liquefaction, weak/–; cycloheximide, 10 µg ml^{–1}, variable, 100 µg ml^{–1}, variable; 37 °C, +. Source of cultures: NRRL Y-6843^T (= LRB 69D^T), 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-6843^T (= CBS 8675^T), preserved as a lyophilized preparation in the ARS Culture Collection (NRRL), Peoria, Illinois, USA.

Latin diagnosis of *Candida ulmi* Kurtzman sp. nov.

In agar malti post dies 3 ad 25 °C, cellulae vegetativae globosae (1.8–7.0 µm), ellipsoideae aut elongatae (1.3–6.0 × 2.0–8.0 µm), singulae et binae. In agar morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, nitens et butyrosus; margo glabro vel serrulatus. Pseudohyphae fiunt; hyphae verae non fiunt. Ascospores non fiunt. Glucosum fermentatur. Galactosum, sucrosum, maltosum, lactosum, raffinsum et trehalosum non fermentantur. Assimilantur glucosum, sucrosum, maltosum, cellobiosum, trehalosum, melezitosum, D-xylosum, L-rhamnosum, ethanolum, glycerolum, D-mannitolum, D-glucitolum, methyl α ,D-glucosidum, salicinum, D-gluconas, DL-acidum lacticum, acidum succinicum, acidum citricum, cadaverinum et potassii nitras. Non assimilantur galactosum, L-sorbosum, lactosum, melibiosum, raffinsum, inulinum, amyllum solubile, L-arabiosum, D-arabiosum, D-ribosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, erythritolum, ribitolum, galactitolum, 2-keto-D-gluconas, 5-keto-D-gluconas, saccharatas, inositolum et hexadecanum. Amyllum non formatur. Vitaminae externa ad crescentiam necessaria sunt. Gelatinum non liquescit; esteres fiunt (variabile); pellicula fiunt. Augmentum fiunt in temperatura 37 °C. Typus: NRRL YB-2694^T (= CBS 8670^T), designat stirpem typicam. Isolata a dejectis coleopterorum in Ulmus sp., Peoria, Illinois, USA, depositata in Collectione Culturarum ARS (NRRL), Peoria, Illinois.

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 ascoporation 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-xylose, +; 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 α ,D-glucoside, +; 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. Wickerham. NRRL YB-1545 was from soil, and NRRL YB-2116 and NRRL YB-2694^T were from frass in tunnels made by unidentified insects in elm (*Ulmus* sp.) trees. Type: NRRL YB-2694^T (= CBS 8670^T), 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 pastorianus* 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. macluriae* 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. macluriae* 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. macluriae 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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