***Kodamaea nitidulidarum*, *Candida restingae* and *Kodamaea anthophila*, three new related yeast species from ephemeral flowers**

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Three new yeast species were discovered during studies of yeasts associated with ephemeral flowers in Brazil, Australia and Hawaii. Their physiological and morphological similarity to *Kodamaea (Pichia) ohmeri* suggested a possible relationship to that species, which was confirmed by rDNA sequencing. *Kodamaea nitidulidarum* and *Candida restingae* were found in cactus flowers and associated nitidulid beetles in sand dune ecosystems (*restinga*) of South-eastern Brazil. Over 350 strains of *Kodamaea anthophila* were isolated from *Hibiscus* and morning glory flowers (*Ipomoea* spp.) in Australia, and from associated nitidulid beetles and *Drosophila hibisci*. A single isolate came from a beach morning glory in Hawaii. Expansion of the genus *Kodamaea* to three species modified the existing definition of the genus only slightly. The type and isotype strains are as follows: *K. nitidulidarum* strains UFMG96-272^T (h⁺; CBS 8491^T) and UFMG96-394¹ (h[−]; CBS 8492¹); *Candida restingae* UFMG96-276^T (CBS 8493^T); *K. anthophila* strains UWO(PS)95-602.1^T (h⁺; CBS 8494^T), UWO(PS)91-893.2¹ (h[−]; CBS 8495¹) and UWO(PS)95-725.1¹ (h[−]; CBS 8496¹).

Keywords: *Kodamaea*, new yeast species, nitidulid beetles, *Hibiscus*, *Ipomoea*

INTRODUCTION

Pichia ohmeri (Etchells & Bell) Kreger-van Rij was first described as *Endomycopsis ohmeri* and later transferred to the genus *Pichia* Hansen (Kreger-van Rij, 1970). This yeast species is heterothallic, normally isolated in the haploid form, and, depending upon the mating types paired, ascospores may be either hat-shaped or spheroidal (Kurtzman, 1984). Billon-Grand (1989) introduced the new genus *Yamadazyma* Billon-Grand to accommodate 16 species from the genus *Pichia*, including *Pichia ohmeri* (*Yamadazyma ohmeri*). Yamada *et al.* (1995a), on the basis of rRNA sequences, concluded that *Y. ohmeri* was not closely related to any other species of either *Pichia* or *Yamadazyma*. Accordingly, the genus *Kodamaea* Yamada, Suzuki, Matsuda & Mikata was proposed for that species (Yamada *et al.*, 1995b).

In the course of studies of yeast communities as-

sociated with decaying cactus flowers in South-eastern Brazil, and *Hibiscus* and morning glory flowers in Australia and Hawaii, two heterothallic haploid and one anascosporogenous species were isolated that appeared physiologically similar to *Kodamaea ohmeri*. One species previously referred to as '*Pichia* sp. A' (Rosa *et al.*, 1994) was isolated from flowers of the cactus *Pilosocereus arrabidaei* and from an unidentified beetle in the family Nitidulidae that uses these flowers as feeding and breeding sites. The beetle was thought to be the principal vector of '*Pichia* sp. A' among cactus flowers in sand dune ecosystems in Brazil. Another new yeast species ('*Candida* sp. C'; Rosa *et al.*, 1995) was found in cactus flowers in the same area. This species was asporogenous but physiologically similar to *K. ohmeri* and '*Pichia* sp. A'. A third species was found in Australian *Hibiscus* and morning glory flowers, a Hawaiian morning glory, as well as in *Drosophila hibisci* and various nitidulid beetles found in the Australian flowers. In this paper, we describe *Kodamaea nitidulidarum*, *Candida restingae* and *Kodamaea anthophila*, and discuss their relatedness to *K. ohmeri*.

The GenBank accession numbers for the sequences in this paper are AF059665–AF059669.

METHODS

Isolation and characterization of yeasts. The origin of the strains considered in this study is described in Tables 1, 2 and 3. Strains of *K. nitidulidarum* and *Candida restingae* were isolated from decaying flowers of the cacti *P. arrabidaei* and *Cereus pernanbucensis*, and from tunnels of larvae of the moth *Sigelgaita* sp. in sand dune ecosystems of South-eastern Brazil in 1996. The methods used for yeast isolation were described by Rosa *et al.* (1994, 1995). The first isolations from Australian *Hibiscus* were performed in 1990 in a pilot study of the ecology of insects and yeasts associated with these flowers. Several hundred samples of *Hibiscus* and morning glory were collected in late spring 1995 over a 6-week period, across a 14° latitude gradient, mostly in the vicinity of the Australian East Coast. Beach morning glory (*Ipomoea pes-caprae*) flowers were collected in Hapuna Beach, near Waimea, Hawaii Island, in 1991. Yeasts were cultured the same day, usually within minutes of sampling. The nectary region of flowers was scraped gently with a sterile loop and streak-inoculated onto acidified YM agar (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar, acidified with HCl to pH 3.7). *Drosophila* or beetles were placed in plates of the same medium and kept for 15–60 min before being removed. The material deposited by the insects was spread evenly with a sterile loop. Cultures were maintained in YM slants until preserved in liquid nitrogen using the Microbank system of Pro-Lab diagnostics.

The yeasts were characterized by standard methods (van der Walt & Yarrow, 1984), with some additional tests (Lachance

et al., 1988). Identities were verified in the keys of Kreger-van Rij (1984) and Barnett *et al.* (1990). Phenetic similarity with other described yeasts was examined with the computer program IDENT (M.-A. Lachance, unpublished), which compares the nutritional characteristics of any yeast with those of known species. The database was initially taken from Barnett *et al.* (1990) and updated as appropriate. Sexual cross-reactivity was evaluated by mixing pairs of actively growing cultures on GY agar (1.0% glucose, 0.01% yeast extract and 2.0% agar), and observing microscopically for signs of conjugation or ascus formation daily during a week.

Scanning electron microscopy. Materials were suspended in sterile water and deposited over a Nuclepore membrane (0.45 µm) on agar. In the case of *K. nitidulidarum* the ascospores were released by digestion for 3 h with Mureinase (US Biochemical) in 10 mM sodium succinate buffer, pH 5.8, prior to fixation. The membrane and its content were fixed in 2.5% glutaraldehyde in 0.3 M cacodylate buffer, pH 7.0, for at least 15 min, rinsed twice in cacodylate buffer and dehydrated for 15 min in 2,2-dimethoxypropane lightly acidified with HCl. The material was then critical point dried, sputter-coated with gold for 5 min, and observed with a Hitachi S4500 Field Emission scanning electron microscope (Surface Science Western). Images were recorded electronically.

Electrophoretic karyotyping. Karyotypes were obtained using contour-clamped homogeneous electric field electrophoresis (Bio-Rad CHEF DRII). Intact chromosomes were prepared according to the method of Gardner *et al.* (1993).

Table 1. Origin of the strains of *K. nitidulidarum* in sand dune ecosystems of Rio de Janeiro State, Brazil

The host plant was *P. arrabidaei*.

Locality	Substrate type	Mating type	Strain no. (UFMG)
Arraial do Cabo	Flower	h ⁺	96-353
		h ⁻	96-394 [†] , 96-352
Maricá	<i>Sigelgaita</i> sp. tunnel	h ⁻	96-340
	Flower	h ⁺	96-272 ^T , 96-328
		h ⁻	96-305, 96-329
	<i>Sigelgaita</i> sp. tunnel	h ⁻	96-319
Praia Seca	Flower	h ⁺	96-371

Table 2. Host plants and geographic origins of the strains of *Candida restingae* in sand dune ecosystems of Rio de Janeiro State, Brazil

Host plant	Locality	Substrate type	Strain no. (UFMG)
<i>Cereus pernanbucensis</i>	Arraial do Cabo	Flower	96-276 ^T
	Praia Seca	Flower	96-380
<i>Pilosocereus arrabidaei</i>	Arraial do Cabo	Flower	96-331
	Maricá	Flower	96-271
	Praia Seca	<i>Sigelgaita</i> sp. tunnel	96-308, 96-322
		<i>Sigelgaita</i> sp. tunnel	96-274

Electrophoresis was carried out in a 0.8% (w/v) agarose gel in 1 × TBE buffer under the following conditions: 110 V, 24 h with linear ramping of 100–300 s and 28 h with linear ramping of 400–700 s.

DNA sequence analysis. DNA was extracted as described previously (Lachance, 1990). The D1 and D2 variable domains of the large-subunit rDNA were amplified using primers NL1 (5' GCATATCAATAAGCGGAGGAAAG) and NL4 (5' GGTCCGTGTTTCAAGACGG; O'Donnell, 1993), purchased from Procyon Biopharma. The PCR was conducted following the instructions provided by the supplier of *Taq* polymerase (Boehringer Mannheim), in the presence of 1.5 mM MgCl₂, in a Perkin-Elmer System 2400 cyclor. After 2 min denaturation at 95 °C, the mixture was subjected to 35 cycles at 95 °C for 15 s, 53.7 °C for 25 s and 72 °C for 20 s, with a final extension for 5 min at 72 °C. The amplified DNA was concentrated and cleaned by ultrafiltration in MicroCon 100 concentrators (Amicon) and sequenced in an ABI sequencer at the John P. Robarts Research Institute, London, Ontario. The sequences were edited with the program DNAMAN, version 3.2 (Lynnon BioSoft). Existing sequences for other yeasts (Kurtzman & Robnett, 1997) were retrieved from GenBank. The CLUSTAL W (Thompson *et al.*, 1994) algorithm provided in the DNAMAN package was used to align the sequences and construct a neighbour-joining tree with 1000 bootstrap iterations.

RESULTS AND DISCUSSION

Latin diagnosis of *Kodamaea nitidulidarum* sp. nov.

In medio liquido post dies tres cellulae singulae, binae, aut in catenis brevis, ovoidae (1–3 × 3–6 µm). Cellulae cum extuberatione formari possunt. Post unum mensem sedimentum formatur. Cultura in agar malti post dies 14 (17 °C), tumulosa, aliquando cum lineis radialibus, glabra aut rugosa, candida, butyrosa aut firma. In agar farinae Zea mays post dies 14 mycelium verum formatur. Post dies unum, cellulae stirpum interfertiliu mixtarum in agar glucosi et extracti levidinis tubi junctionis formantur. Post dies tres, asci conjugati possunt videri. Asci stabiles sunt. Ascosporae petasosae. Glucosum (lente) fermentatur. Sucrosum, raffinsum, galactosum, maltosum, melezitsum, methyl α-D-glucosidum, cellobiosum (exigue et lente), salicinum (lente), L-sorbosum, D-arabiosum (variabile et exigue), ethanolum (lente), glycerolum (variabile et exigue), ribitolum (lente), manitolum, glucitolum, acidum succinicum (exigue), acidum citricum, 2-ketogluconatum, glucosaminum (exigue), N-acetylglucosaminum et hexadecanum (exigue) assimilantur at non inulinum, melibiosum, lactosum, trehalosum, amyllum solubile, L-rhamnosum, D-xylosum, L-arabiosum, D-ribosum, methanolum, 1-propanolum, 2-propanolum, 1-butanolum, erythritolum, xylitolum (aliquando exigue), galactitolum, meso-inositolum, acidum lacticum, acidum malicum, acidum gluconicum, glucono-δ-lactonum (aliquando exigue), acetolum, nec ethyl acetat. Ethylaminum (lente), lysinum et cadaverinum assimilantur at non natrium nitricum nec natrium nitrosum. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 37 °C. Habitatio in floribus Pilosocereus arrabidae et in scarabeis

junctis Nitidulidae in Brasil. Typus UFMG96-272^T (h⁺). Isotypus UFMG96-394¹ (h⁻). In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Bata-vorum, sub no. CBS 8491^T [= UFMG96-272^T] et CBS 8492¹ [= UFMG96-394¹] depositae sunt.

Description of *Kodamaea nitidulidarum* sp. nov.

Kodamaea nitidulidarum (ni.ti.du.li.da'rum. L. gen. plur. masc. n. *nitidulidarum* of *nitidulidae*, referring to the family of beetles that vector this yeast species).

In yeast extract (0.5%)/glucose (2%) broth after 3 d at 25 °C, the cells are ovoid to ellipsoidal, occur singly, in parent–bud pairs, or occasionally in short chains, and measure 1–3 × 3–6 µm. Buds may be formed on a short protuberance. A sediment is formed after a month. On malt agar after 2 weeks at 17 °C, colonies are umbonate, sometimes fringed, glabrous, smooth or rugose, white, and butyrous to tough due to filamentous growth. In Dalmau plate cultures on cornmeal agar after 2 weeks true hyphae are formed. After 1 d on glucose (1%)/yeast extract (0.01%), mixed strains of complementary mating types give rise to short conjugation tubes. Conjugated pairs and zygotes are also present. Conjugation often occurs between an elongate cell and a more globose cell (Fig. 1d) that develops into the ascus. After 3 d, mature asci are visible. Two to four hat-shaped ascospores are formed. Evanescence is not observed even after prolonged incubation. Fermentation: in glucose, gas production begins after 3–5 d and a full tube of gas develops after 6–10 d. Assimilation of carbon compounds: sucrose, raffinose, galactose, maltose, melezitose, methyl α-D-glucoside, cellobiose (slow and weak), salicin (slow), L-sorbose, D-arabinose (variable and weak), ethanol (slow), glycerol (weak and slow), ribitol (slow), manitol, glucitol, succinic acid (weak), citric acid, 2-ketogluconic acid, D-glucosamine (weak), N-acetylglucosamine and hexadecane (weak) are assimilated; no growth occurs on inulin, melibiose, lactose, trehalose, starch, L-rhamnose, D-xylose, L-arabinose, D-ribose, methanol, 2-propanol, 1-butanol, erythritol, xylitol (rarely weak), galactitol, meso-inositol, lactic acid, malic acid, D-gluconic acid, glucono-δ-lactone (rarely weak), acetone or ethyl acetate. Assimilation of nitrogen compounds: ethylamine (slow), lysine and cadaverine positive; sodium nitrate and sodium nitrite negative. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Gelatin liquefaction and casein hydrolysis are negative. Lipolytic activity on Tween 80 agar is positive. Acid formation on chalk agar is negative. Growth on YM agar with 10% sodium chloride is positive; with 15% sodium chloride it is negative. Growth on 50% glucose/yeast extract agar is slow. Growth in the presence of 10 µg cycloheximide ml⁻¹ is negative. Growth in the presence of 8 µg digitonin ml⁻¹ at 25 °C is negative. Production of starch-like compounds is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The

Table 3. Isolation sources of *K. anthophila*

The numbers of isolates of each mating type (h⁺/h⁻) are indicated. UWO(PS) strain numbers are given for reference strains only.

Locality	Host species	Substrate type		
		Flower*	Beetle	<i>Drosophila</i>
Hapuna Beach, Hawaii Island	<i>Ipomoea pes-caprae</i>	1/0 91-893.2 ¹ (h ⁻)		
Queensland				
Conondale	<i>Ipomoea</i> sp.	2/2 95-602.1 ^T (h ⁺)	1/1	
Charlie Moreland State Forest	<i>Hibiscus heterophyllus</i> (white)	6/7	2/3	3/0
Bell Bird Creek	<i>Hibiscus heterophyllus</i> (yellow)	0/3	1/1	0/1
Noosa River Ferry	<i>Hibiscus diversifolius</i>	2/2	0/1	
	<i>Ipomoea</i> sp.	1/2		
Pile Gully State Forest	<i>Hibiscus heterophyllus</i> (yellow)	5/6	2/2	1/0
Rainbow Beach	<i>Ipomoea pes-caprae</i>	1/2	1/0	
Boreen Point	<i>Hibiscus diversifolius</i>	1/1	0/1	
Coolool National Park	<i>Hibiscus heterophyllus</i> (yellow)	5/6	2/1	0/1
Kirks Road	<i>Hibiscus divaricatus</i>	2/1		
Goodnight Scrub State Forest	<i>Hibiscus splendens</i>	2/1 95-725.1 ^T (h ⁻)	1/1	1/1
Burnett Creek	<i>Hibiscus heterophyllus</i> (yellow)	2/1		
Cedars Creek Crossing	<i>Hibiscus heterophyllus</i> (yellow)	1/2	1/0	
	<i>Hibiscus heterophyllus</i> × <i>Hibiscus splendens</i> hybrid	0/1		
	<i>Hibiscus splendens</i>	1/1	1/0	
Moore Park Beach	<i>Ipomoea</i> sp.	1/2		
	<i>Ipomoea pes-caprae</i>	0/1		
Manoo	<i>Hibiscus splendens</i>	1/2		
Mount Larcom	<i>Hibiscus heterophyllus</i> (yellow)	0/2		0/1
Rockhampton	<i>Hibiscus tiliaceus</i>	2/2		
Byfield State Forest	<i>Hibiscus heterophyllus</i> (yellow)	0/1		0/1
Byfield National Park	<i>Hibiscus heterophyllus</i> (yellow)	1/0		
Yeppoon	<i>Hibiscus tiliaceus</i>	0/1		
	<i>Ipomoea</i> sp.	1/1		
The Caves	<i>Hibiscus heterophyllus</i> (yellow)	0/1	0/1	1/0
Toolombah Creek	<i>Hibiscus heterophyllus</i> (yellow)	1/0	0/1	
Eungella National Park	<i>Hibiscus heterophyllus</i> (yellow)	0/1		
Mount Ossa Road	<i>Hibiscus heterophyllus</i> (yellow)			1/0
Beatrice Creek	<i>Hibiscus splendens</i>	1/0	1/1	
	<i>Hibiscus heterophyllus</i> (yellow)	2/0	1/0	
Coppabella	<i>Hibiscus divaricatus</i>	1/0		1/1
Carnarvon National Park	<i>Hibiscus heterophyllus</i> (yellow)	1/0		
	<i>Hibiscus splendens</i>	1/1	2/2	0/1
	<i>Hibiscus divaricatus</i>	0/1		
Esk turnoff	<i>Hibiscus tiliaceus</i>	0/1		
Mount Crosby	<i>Hibiscus heterophyllus</i> (white)	1/1	0/1	1/1
Goodna	<i>Hibiscus splendens</i>	1/0	0/1	
	<i>Ipomoea</i> sp.	1/2		
New South Wales				
Tyagarah Road	<i>Hibiscus diversifolius</i>	1/1	1/0	0/1
Giro	<i>Hibiscus heterophyllus</i> (white)	3/2	1/0	1/1
Martins Creek	<i>Hibiscus heterophyllus</i> (white)	1/0	0/1	
Ourimbah	<i>Ipomoea</i> sp.	0/1	0/1	

Table 3 (cont.)

Locality	Host species	Substrate type		
		Flower*	Beetle	<i>Drosophila</i>
Narrabeen Lagoon	<i>Hibiscus diversifolius</i>	2/3	1/0	
	<i>Ipomoea</i> sp.	1/3	1/0	
Tarrawanna School	<i>Hibiscus splendens</i>	1/2		0/1
	<i>Hibiscus splendens</i>		0/2	
Balgownie	<i>Hibiscus heterophyllus</i> (white)	2/2	0/1	0/1
Hewitt Creek	<i>Hibiscus diversifolius</i>		1/0	0/1
	<i>Ipomoea</i> sp.		0/1	
Bungwahl Creek	<i>Hibiscus heterophyllus</i> (white)	0/1		1/1
Middle Brother State Forest	<i>Hibiscus splendens</i>	1/0		
Port Macquarrie	<i>Ipomoea</i> sp.	2/2	1/1	
Lake Innes Drive	<i>Hibiscus heterophyllus</i> (white)	4/2	1/0	1/0
Bellingen	<i>Hibiscus heterophyllus</i> (white)	0/2	1/1	1/1
Bellingen (Thora, 1990)†	<i>Hibiscus heterophyllus</i> (white)	3/4		

* T, Holotype; I, designated isotypes.

† Thora was the name formerly used to designate the Bellingen site. A preliminary collection was made there in 1990, whereas the bulk of the work was done in 1995.

habitat is in flowers of *Pilosocereus arrabidae* and associated nitidulid beetles in sand dune ecosystems in Brazil. The type strain is UFMG96-272^T. Its mating type was designated arbitrarily h⁺. The designated isotype, strain UFMG96-394^I, has the mating type h⁻. They were isolated from flowers of *Pilosocereus arrabidae* in Rio de Janeiro, Brazil. They have been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, the Netherlands, as strains CBS 8491^T and CBS 8492^I, respectively.

Latin diagnosis of *Candida restingae* sp. nov.

In medio liquido post dies tres cellulae singulae aut binae, ovoidae (2–4 × 4–6 µm). Cellulae cum extuberatione formari possunt. Post unum mensem sedimentum formatur. Cultura in agar malti post dies 14 (17 °C) infimo-convexa, lucida, candida et butyrosa. In agar farinae Zea mays post dies 14 hyphae veres formantur. Glucosum (lente) fermentatur. Sucrosum, raffinose, galactosum, trehalosum (lente), maltosum, melezitosum, methyl α-D-glucosidum, cellobiosum (lente), salicinum (lente), L-sorbose, D-xylosum (lente), ethanolum (lente), glycerolum (lente), ribitolum, xylitolum, mannitolum, glucitolum, acidum succinicum, acidum citricum, acidum malicum (exiguum), glucono-δ-lactonum (lente), acidum gluconicum (exiguum), 2-ketogluconatum, glucosaminum, N-acetylglucosaminum, et hexadecanum (exiguum) assimilantur at non inulinum, melibiosum, lactosum, amyllum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum, methanolum, 2-propanolum, 1-butanolum, erythritolum, galactitolum, meso-inositolum, acidum lacticum, acetolum, nec ethyl acetum. Ethylaminum, lysinum et cadaverinum assimilantur at non natrium nitricum nec natrium nitrosum. Ad

crescentiam vitaminarum externarum necessariae sunt. Augmentum in 37 °C. Typus stirps UFMG96-276^T. Habitatio in floribus cactorum in Brasil. In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum, sub no. CBS 8493^T [= UFMG96-276^T] deposita est.

Description of *Candida restingae* sp. nov.

Candida restingae (res.tin'gae. L. gen. fem. n. *restingae* of the restinga, referring to the coastal sand dune ecosystem where this yeast species was isolated).

In yeast extract (0.5 %)/glucose (2 %) broth after 3 d at 25 °C, the cells are ovoid to ellipsoidal, occur singly or in parent-bud pairs, and measure 2–4 × 4–6 µm. Buds may be formed on a short protuberance. A sediment is formed after a month. On malt agar after 2 weeks at 17 °C, colonies are small, low convex, glossy, smooth, white and butyrous. In Dalmau plate cultures on cornmeal agar after 2 weeks, some true hyphae are formed. Asci and ascospores are not formed. Fermentation: in glucose, gas production begins after 2–3 d and a full tube of gas develops after 4–5 d. Assimilation of carbon compounds: sucrose, raffinose, galactose, trehalose (slow), maltose, melezitose, methyl α-D-glucoside, cellobiose (slow), salicin (slow), L-sorbose, D-xylose (slow), ethanol (slow), glycerol (slow), ribitol, xylitol, mannitol, glucitol, succinic acid, citric acid, malic acid (weak), glucono-δ-lactone (slow), D-gluconic acid (weak), 2-ketogluconic acid, N-acetylglucosamine and hexadecane (weak) are assimilated; no growth occurs on inulin, melibiose, lactose, starch, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, 2-propanol, 1-butanol, erythritol, galactitol, meso-inositol, lactic acid, D-gluconic acid,

acetone or ethyl acetate. Assimilation of nitrogen compounds: ethylamine, lysine and cadaverine positive; sodium nitrate and sodium nitrite negative. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Gelatin liquefaction and casein hydrolysis are negative. Lipolytic activity on Tween 80 agar is positive. Acid formation on chalk agar is negative. Growth on YM agar with 10% sodium chloride is slow; with 15% sodium chloride it is negative. Growth on 50% glucose/yeast extract agar is negative. Growth in the presence of 10 µg cycloheximide ml⁻¹ is weak; with 100 µg ml⁻¹ it is negative. Growth in the presence of 8 µg digitonin ml⁻¹ at 25 °C is negative. Production of starch-like compounds is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is in flowers of columnar cacti in sand dune ecosystems in Brazil. The type strain is UFMG96-276^T. It was isolated from a flower of the cactus *Cereus pernanbucensis* in Brazil. This strain has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, the Netherlands, as strain CBS 8493^T.

Latin diagnosis of *Kodamaea anthophila* sp. nov.

In medio liquido post dies tres cellulae singulae aut binae, ovoidae (2–4 × 4–6 µm). Post unum mensem sedimentum et velum rugosum formantur. Cultura in agar multi post dies 14 (17 °C), tumulosa, hebes aut rugosa, candida et butyrosa aut firma. Lineis radialibus possunt formari. In agar farinae Zea mays post dies 14 mycelium verum formatur. Post dies unum, cellulae stirpum interfertiliū mixtarum in agar carbonis fundamento plures tubi junctionis formantur. Post dies tres, asci conjugati possunt videri. Ascosporae petasosae, liberatae. Glucosum, (lente) fermentatur. Sacrosum, raffinsum, galactosum, trehalosum, maltosum, mel-ezitosum, methyl α-D-glucosidum, cellobiosum, salicinum, L-sorboseum, D-xylosum (lente et variabile), D-arabinosum (exiguitate et variabile), ethanolum (exiguitate), glycerolum (lente), ribitolum (lente), xylitolum (lente), mannitolum, glucitolum, acidum succinicum, acidum citricum, acidum malicum (exiguitate), glucono-δ-lactonum, 2-ketogluconatum et N-acetylglucosaminum assimulantur at non inulinum (raro exiguitate), melibiosum, lactosum, amyllum solubile, L-rhamnosum, L-arabinosum, D-ribosum methanolum, 1-propanolum, 2-propanolum, 1-butanolum, erythritolum, galactitolum, meso-inositolum, acidum lacticum, acidum gluconicum, glucosaminum, acetonum, ethyl acetum nec hexadecanum. Ethylaminum, lysinum et cadaverinum assimulantur at non natrium nitricum nec natrium nitrosum. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 37 °C. Habitatio in floribus et insectibus junctis Ipomoea et Hibiscus spp. in Australia et Hawaii. Typus stirpis UWO(PS)95-602.1^T (h⁺). Isotypus stirpis UWO(PS)91-893.2¹ et UWO(PS)95-725.1¹ (h⁻). In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum, sub no. CBS 8494^T [= UWO(PS)95-602.1^T], CBS 8495¹ [= UWO(PS)91-

893.2¹] et CBS 8496¹ [= UWO(PS)95-725.1¹] depositae sunt.

Description of *Kodamaea anthophila* sp. nov.

Kodamaea anthophila (an.tho'phi.la. L. nom. fem. adj. *anthophila* flower-loving, referring to the flowers from which these yeasts were isolated).

In yeast extract (0.5%)/glucose (2%) broth after 3 d at 25 °C, the cells are ovoid to ellipsoidal, occur singly or in parent-bud pairs, and measure 2–4 × 4–6 µm. A fragile matted pellicle is formed after 1–3 d. On malt agar after 2 weeks at 17 °C, colonies are white to cream, smooth to rugose, often stellate or umbonate, glabrous to hairy, and butyrous to tough due to filamentous growth. Considerable variation occurs in colony morphology even amongst isolates from a single sample. In Dalmau plate cultures on cornmeal agar after 2 weeks, true septate hyphae are formed. After 1 d on glucose (1%)/yeast extract (0.01%) agar, mixed strains of complementary mating types give rise to one or more conjugation tubes. Conjugated pairs and zygotes are also present. Conjugation is often between an elongate cell and a more globose cell that becomes the ascus. After 2–3 d, mature asci are visible. Two to four hat-shaped ascospores are formed. Evanesence is frequently observed after 4 d. Fermentation: in glucose, gas production begins after 3–5 d and a full tube of gas develops in 5–9 d. Assimilation of carbon compounds: sucrose, raffinose, galactose, trehalose, maltose, methyl α-D-glucoside, cellobiose, salicin, L-sorbose, D-xylose (slow and variable), D-arabinose (weak and variable), ethanol (weak), glycerol (slow), ribitol (slow), xylitol (slow), mannitol, glucitol, succinic acid, citric acid, malic acid (weak), glucono-δ-lactone, 2-ketogluconic acid and N-acetylglucosamine are assimilated; no growth occurs on inulin (rarely weak), melibiose, melezitose, lactose, starch, L-rhamnose, L-arabinose, methanol, ethanol, 2-propanol, 1-butanol, erythritol, galactitol, meso-inositol, lactic acid, gluconic acid, D-glucosamine, acetone, ethyl acetate or hexadecane. Assimilation of nitrogen compounds: ethylamine, lysine and cadaverine positive; sodium nitrate and sodium nitrite negative. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Gelatin liquefaction and casein hydrolysis are negative. Lipolytic activity on Tween 80 agar is negative. Acid formation on chalk agar is negative. Growth on YM agar with 10% sodium chloride is positive; with 15% sodium chloride it is negative. Growth on 50% glucose/yeast extract agar is slow. Growth in the presence of 10 µg cycloheximide ml⁻¹ is positive; with 100 µg ml⁻¹ it is negative. Growth in the presence of 8 µg digitonin ml⁻¹ at 25 °C is negative. Production of starch-like compounds is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is flowers of morning glories and *Hibiscus* species as well as associated beetles and *Drosophila* species. The type strain is UWO(PS)95-602.1^T. Its mating type was designated arbitrarily as

Fig. 1. Scanning electron micrographs of the sexual cycle of *K. nitidulidarum*. (a) Budding vegetative cells. (b, c) Detail of bud scars. (d) Ascus. (e–g) Ascospores liberated after Mureinase treatment. Bars, 1 µm.

h^+ . The designated isotypes, strains UWO(PS)91-893.2^I and UWO(PS)95-725.1^I, have mating type h^- . They were isolated from flowers of beach morning glory in Hawaii, and flowers of morning glory and *Hibiscus splendens* in Australia, respectively. These strains have been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmeltcultures, Delft, the Netherlands, as strains CBS 8494^T, CBS 8495^I and CBS 8496^I, respectively.

Ecology and habitat specificity

The localities and host plants of the three new yeast species are listed in Tables 1, 2 and 3. *K. nitidulidarum* and *Candida restingae* were isolated from the columnar cacti *P. arrabida*e and *Cereus pernanbucensis* in sand dune ecosystems in Brazil. Beetles that used the cactus flowers as breeding and feeding sites vector these yeast species (Rosa *et al.*, 1994, 1995). *K. nitidulidarum* was isolated only from flowers and tunnels of the moth *Sigelgaita* sp. larvae in *P. arrabida*e. Larvae of *Sigelgaita* sp. penetrate cactus stems, sometimes entering through flowers. This could explain the occurrence of *K. nitidulidarum* in association with the moth tunnels as well as the flowers (Rosa *et al.*, 1992). An unidentified nitidulid beetle was the principal vector of this species among the flowers (Rosa *et al.*, 1994). *Candida restingae* was isolated from tunnels of *Sigel-*

Fig. 2. Scanning electron micrographs of the vegetative cells and the sexual cycle of *K. anthophila*. (a) Budding vegetative cells. (b) Conjugating cells and vegetative cells. (c) Mature ascus in the process of deliquescing. (d) Hat-shaped ascospores liberated from asci. Bars, 2 µm.

gaita sp. and decaying flowers of *C. pernanbucensis*. Two beetle species were predominant in flowers of this cactus, namely *Cyclocephala laminata* (Scarabeidae) and the same unidentified species present in flowers of *P. arrabida*e (Rosa *et al.*, 1995). Presumably, these beetles are the vectors of *Candida restingae* among flowers of the two cactus species.

K. anthophila was the dominant yeast species in flowers of Australian morning glories and *Hibiscus* as well as in *Drosophila hibisci* and a nitidulid beetle associated with them. It occurred in practically every one of over 400 samples examined. A single strain was isolated in beach morning glory (*I. pes-caprae*) in Hawaii, where over 100 morning glories (*I. pes-caprae* and *Ipomoea acuminata*) were examined (Lachance *et al.*, 1989; and

Fig. 3. Scanning electron micrographs of *Candida restingae*. (a) Budding cell. (b) Cell with bud scars. (c) A pedunculate bud. Bars, 2 μ m.

later unpublished data). The ecology of yeast communities associated with these plants will be described elsewhere.

Fig. 4. Electrokaryotypes of selected strains of *K. ohmeri*, *K. nitidulidarum*, *K. anthophila* and *Candida restingae*. *S. cerevisiae* is the Bio-Rad standard. The mating types (h^+ and h^-) are indicated.

Life cycles

The life cycles of *K. nitidulidarum* and *K. anthophila* are nearly identical. The buds are formed multilaterally, but are generally concentrated near the apical region of the cells (Figs 1 and 2). Multiple scars are formed in both sides of the parent cells. Buds are normally elongate with a narrow base. Asci are formed after conjugation of two cells of compatible mating types. The asci of *K. nitidulidarum* are persistent and ascospores germinate inside the ascus. In *K. anthophila* the asci usually deliquesce (Fig. 2c) and the ascospores are liberated (Fig. 2d). The mating types of both species are distributed more or less equally, and both mating types were frequently isolated in the same sample. This suggests that these species are sexually active. Self-sporulating cultures have not been seen, however, which indicates that the vegetative phase is haploid in the natural habitat. Sexual reproduction was not observed in *Candida restingae* (Fig. 3). Budding is also multilateral and the buds tend to be ovoid to cylindrical. An elongate tube is sometimes observed between the parent cell and the bud (Fig. 3c).

Electrophoretic karyotypes

The electrophoretic karyotypes of *Kodamaea* species and *Candida restingae* were very distinct (Fig. 4). Strains of *K. nitidulidarum* produced a low-molecular-size band that may represent a plasmid. Most strains of

Fig. 5. Phylogeny of *Kodamaea* and related yeast species based on the D1/D2 variable domains of the large rDNA subunit. The tree was constructed by neighbour-joining analysis of aligned sequences. The numbers at the nodes indicate the percentage bootstrap values for 1000 iterations. The scale corresponds to 2% substitution.

this species exhibited killer activity against *Candida glabrata* Y-55 under standard conditions (Lachance *et al.*, 1988). Whereas this might have suggested a link between killer ability and the putative plasmid, *Candida restingae* and *K. anthophila* also could exhibit the killer phenotype. *K. ohmeri* and *Candida restingae* have at least four chromosomes whereas *K. anthophila* and *K. nitidulidarum* have at least five. The electrokaryotypes are useful in distinguishing species from one another but strains of the same species had similar profiles.

Evolutionary relatedness

Analysis of the sequences of the D1/D2 regions of the large rDNA subunit showed that the *Kodamaea* species and *Candida restingae* form a homogeneous assemblage (Fig. 5). The two strains of *K. nitidulidarum* studied differed by five substitutions, which is at the high end of the range usually observed for members of the same species (Kurtzman & Robnett, 1997). The cactus flower species were the most closely related. A close kinship between these species was expected as they share the same microhabitat in the Brazilian sand dunes and have not been found in any other plants in that ecosystem (Rosa *et al.*, 1995). The strains of *K. anthophila* (three examined), although isolated in geographically distant locations, differed only by a single substitution.

The *Kodamaea* clade appears relatively recent, and *K. ohmeri*, by virtue of its less specialized ecology and its position in the rDNA phylogeny, may represent an ancestral form. This is consistent with the isolation of *K. ohmeri* from a number of different substrates, including food and clinical specimens (Kreger-van Rij, 1970), in contrast to the specificity of its sister taxa. However, a clear physiological specialization is not observed.

Species identification and genus circumscription

The morphology and physiological properties of *Kodamaea* species and *Candida restingae* exhibit a small amount of variation, and the identification based on such characters is tenuous, although not impossible. Mating reactions among the sexual species and rDNA sequencing provide good diagnoses. Below is an identification key based on the assimilation of a few carbon sources.

Key to *Kodamaea* species and *Candida restingae*

1. Melezitose assimilation rapid 2
Melezitose assimilation negative or delayed 3
2. Trehalose and xylose assimilated
→ *Candida restingae*
Trehalose and xylose not assimilated
→ *K. nitidulidarum*
3. Xylitol assimilated
→ *K. anthophila*
Xylitol not assimilated
→ *K. ohmeri*

Comparison of the species of the *Kodamaea* clade with other known yeasts indicated that identification strictly on the basis of physiology is not satisfactory. Specifically, separation from *Candida guilliermondii*, *Candida melibiosica* and *Debaryomyces* (syn. *Schwanniomyces*) *occidentalis* is difficult based on the nutritional tests normally administered in our laboratories, even though these species are unrelated to *Kodamaea* (Kurtzman & Robnett, 1997). Other similar yeasts can be resolved on the basis of glucose fermentation and raffinose assimilation (positive in *Kodamaea* species and *Candida restingae*) and assimilation of lactose (negative in *Kodamaea* species and *Candida restingae*).

Expansion of the genus *Kodamaea* to three teleomorphic species warrants a modification of the generic definition given by Yamada *et al.* (1995b). The genus is now defined as follows. The genus belongs to the Saccharomycetaceae. Colonies are butyroid, membranous to tough, and may be furry or have a fringed border. Cells are globose, ellipsoid, ovoid to cylindrical. Budding is multilateral and may involve formation of a short protuberance. Pseudomycelium or true mycelium may be formed. Species in the genus are heterothallic, usually haploid, and occasionally diploid. Asci arise by transformation of diploid cells or by conjugation of independent cells. They may be evanescent or persistent, containing one to four ascospores. Ascospores are pileiform or globose and glabrous. Fermentation is present. Sucrose, raffinose, maltose, L-sorbose, mannitol, glucitol, citric acid, 2-ketogluconate, L-lysine and cadaverine are assimilated. Growth occurs at 37 °C.

ACKNOWLEDGEMENTS

We acknowledge financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brazil-CNPq (Postdoctoral Fellowship to C.A.R.

201125/96-0), the Natural Science and Engineering Research Council of Canada (M.-A.L.), the National Science Foundation International Programs (W.T.S.) and the Australian Research Council (J.S.F.B.). Permission to collect was obtained from the Queensland National Parks and Wildlife Service and the Queensland Forest Service. We thank Dr C. P. Kurtzman for sending type cultures.

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