Candida tumulicola sp. nov. and *Candida takamatsuzukensis* sp. nov., novel yeast species assignable to the *Candida membranifaciens* clade, isolated from the stone chamber of the Takamatsu-zuka tumulus

Yuka Nagatsuka,¹ Tomohiko Kiyuna,¹ Rika Kigawa,² Chie Sano,² Sadatoshi Miura² and Junta Sugiyama³

Correspondence

Junta Sugiyama

jsugiyam@tecsrg.co.jp

¹NCIMB Group, TechnoSuruga Laboratory Co., Ltd, 330 Nagasaki, Shimizu-ku, Shizuoka-shi, Shizuoka 424-0065, Japan

²Independent Administrative Institution, National Research Institute for Cultural Properties, Tokyo, 13-43 Ueno Park, Taito-ku, Tokyo 110-8713, Japan

³Tokyo Office, TechnoSuruga Laboratory Co., Ltd, Shinko Music Plaza Bldg 5F-N, 2-1 Kandaogawa-machi, Chiyoda-ku, Tokyo 101-0052, Japan

During a survey of the mycobiota in the stone chamber of the Takamatsu-zuka tumulus in the village of Asuka, Nara Prefecture, Japan, we isolated 19 yeast strains assigned to the genus *Candida* from various samples, taken mainly from mouldy spots where the colour of the murals had changed to black, white or another tone, and from viscous gels (biofilms) on plaster walls. The 26S rDNA D1/D2 domain sequence-based phylogeny clearly indicates two groups of isolates. Polyphasic characterization, including morphological, physiological and chemotaxonomic characteristics, and sequence analysis of the 26S rDNA D1/D2 domain and ITS regions suggest that each group is assignable to one of two novel species within the *Candida membranifaciens* clade. Proposed herein are the names *Candida tumulicola* sp. nov. (originally T6517-9-5^T; holotype JCM 15403^T; isotypes CBS 10917^T, NBRC 104392^T) and *Candida takamatsuzukensis* sp. nov. (originally T4922-1-1^T; holotype JCM 15410^T; isotypes CBS 10916^T, NBRC 104391^T). The 26S rDNA D1/D2 domain sequence divergence indicates that *C. tumulicola* differs from *Candida friedrichii* NBRC 10277^T, the type strain of the nearest species, in 15 nucleotides (3%), whereas *C. takamatsuzukensis* differs from *Candida insectorum* NBRC 10283^T and *Pichia mexicana* NBRC 10544^T, the type strains of the nearest species, in 20 nucleotides (4%). Both novel species are also clearly distinguishable from the species closest to them by various physiological characteristics.

After its excavation in 1972, the Takamatsu-zuka tumulus, located in Asuka-mura, Nara Prefecture, Japan, thought to have been built some time in the 7th or 8th century, was designated a special historical site in 1973. The colourful paintings on its plaster walls were designated National Treasures. The paintings are composed of star constella-

tions ('Seishuku') on the ceiling and nine murals, including a group of men and a group of women (named 'Asuka beauties', for a group of women on the west wall), on a plaster wall in a stone chamber (approx. 1.0 m wide, 1.1 m high and 2.7 m long). Following specialists' advice, the Agency for Cultural Affairs in Japan decided in 1973 to preserve these paintings within the stone chamber. Since the completion of a protective facility in 1976, the beauty and stability of the murals have been maintained under high humidity (about 100 % relative humidity) and within a temperature range of about 14–20 °C (Kigawa *et al.*, 2006). For an overview of the state of the paintings and the inside of the stone chamber from the time of discovery to the end of 2000, see Kigawa *et al.* (2004, 2006). According to official records, the mycobiota in the chamber was

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the combined ITS1 region, 5.8S rDNA, ITS2 region and 26S rDNA D1/D2 sequences determined in this study are AB365456–AB365477, as listed in Table 1.

A maximum-parsimony tree based on 26S rDNA D1/D2 domain sequences is available as supplementary material with the online version of this paper.

comparatively stable during periods with little perturbation. However, mould outbreaks occurred as a result of some stimuli, such as mural restoration work undertaken in the late 1970s and early 1980s or work to rehabilitate the space adjacent to the chamber in spring 2001. Fungal colonies appeared frequently and grew on the murals. Viscous gels (biofilms; mixtures of moulds, yeasts and bacteria), samples of which frequently contained mites, were also visible to the naked eye on the wall plaster after 2004.

At the Agency's request, in May 2004 we started to investigate changes in the chamber's mycobiota. In the course of our survey, we isolated yeasts from biofilms, mouldy spots and other samples collected in the stone chamber from September 2004 to December 2006. Here, we describe two novel species assignable to the *Candida membranifaciens* clade (Suh *et al.*, 2005) based on 19 isolates.

Samples for isolation were picked with a sterilized cotton swab from various spots in the stone chamber of the Takamatsu-zuka tumulus and maintained in a physiological salt solution until isolation. To isolate moulds and yeasts, the collected samples were directly streaked on potato-dextrose agar (PDA) plates supplemented with 5% chloramphenicol and 5% rose bengal. Single colonies were streaked on PDA plates with 5% chloramphenicol for purification of yeast isolates. All yeast isolates and reference strains *Candida friedrichii* NBRC 10277^T, *Candida insectorum* NBRC 10283^T and *Pichia mexicana* NBRC 10544^T were cultivated on yeast extract/malt extract (YM) agar at 25 °C. Voucher isolates including type strains have been deposited as L-dried cultures in the public culture collections listed in Table 1.

Procedures for DNA isolation and sequencing of the 26S rDNA D1/D2 domain and the internal transcribed spacer (ITS) region with 5.8S rDNA were given previously (Nagatsuka *et al.*, 2002, 2005; Yamada *et al.*, 1999). Sequences were aligned with the program CLUSTAL W (Thompson *et al.*, 1994) and phylogenetic trees were constructed based on an alignment of 497 nt of the 26S rDNA D1/D2 domain [609 nt determined of the 26S rDNA D1/D2 domain for the 14 isolates of group A (T5916-5-3, T5916-6-3, T6220-1-4, T6220-6-3, T6517-3-6, T6517-7-3, T6517-8-2, T6517-9-5^T, T61213-1-5, T61213-3-5, T61213-7-9, T61213-7-10, T61213-12-11 and T61213-14-8) and 594 nt determined for five isolates of group B (T4922-1-1^T, T5916-2-4, T61213-3-6, T61213-12-12 and T61213-13-9)] excluding gaps, uncertain positions within the sequence retrieved from the databases (designated N) and ambiguously aligned regions in considering the secondary structure of the 26S rRNA (Hwang & Kim, 2000); uncertain positions of alignment were positions 983–1002, 1049–1052 and 1062–1066 for group A, according to the sequence of strain T6517-9-5^T (GenBank accession no. AB365463), and positions 1030–1033, 1080–1083 and 1093–1096 for group B, according to the sequence of

strain T4922-1-1^T (AB365470). Phylogenetic trees were constructed by the neighbour-joining and maximum-parsimony programs of MEGA version 3.1 (Kumar *et al.*, 2004). Maximum-parsimony trees were obtained by 10 random-addition heuristic search replicates. Bootstrap values were calculated from 1000 replications (Felsenstein, 1985). The strains used in the phylogenetic analysis and their sequence GenBank accession numbers are listed in Fig. 1 and in Supplementary Fig. S1, available in IJSEM Online.

Physiological and biochemical tests were performed using the standard protocols described by Yarrow (1998). The ability of the strains to assimilate isopropyl alcohol, which had been used as a disinfectant in the chamber since September 2005 (Kigawa *et al.*, 2006), as their sole carbon source was tested. Assimilation testing of carbon compounds was done in liquid medium incubated at 25 °C for up to 4 weeks. The cation tolerance of the strains was determined from the ability to grow in YM broth (pH 6.2) supplemented with LiCl at various concentrations, as described previously (Nagatsuka *et al.*, 2002). Cell morphology was observed under differential interference contrast optics on a light microscope (Olympus BX51) after cultivation on YM agar at 25 °C. Quinone systems were determined by the HPLC method (Yamada *et al.*, 1973; Kuraishi *et al.*, 1985) using the Waters 996 PDA HPLC System. Nuclear DNA for determination of DNA base compositions was extracted and purified from freeze-dried cells by a modified version of the method of Marmur (1961). A Shimadzu SCL-10A vp HPLC system was used to determine DNA base compositions (Tamaoka & Komagata, 1984).

Forty-four yeast strains were isolated from samples collected in the stone chamber of the Takamatsu-zuka tumulus between September 2004 and December 2006. All of the isolates were grouped based on 26S rDNA D1/D2 sequence divergence. A BLAST similarity search with 26S rDNA D1/D2 sequences for the respective groups revealed groups A and B (Table 1), distinct from any known species, in the *C. membranifaciens* clade (Suh *et al.*, 2005). Fourteen isolates of group A differed in their 26S rDNA D1/D2 domain sequences from the type strain of the closest species, *C. friedrichii* NRRL Y-17653^T (GenBank accession no. U45781), by 15 nucleotides (12 substitutions and 3 gaps). On the other hand, five isolates of group B differed in their 26S rDNA D1/D2 domain sequences from the type strains of the closest species *C. insectorum* NRRL Y-7787^T (U45791) by 20 substitutions and *P. mexicana* NRRL Y-11818^T (U45797) by 19 substitutions and 1 gap. In view of the number of nucleotide substitutions in the 26S rDNA D1/D2 domain (Kurtzman & Robnett, 1998), the isolates within each of groups A and B are considered to represent a single species; both of these species are distinct not only from the other but also from any previously described species. 26S rDNA D1/D2 sequences identical to that of strain T4922-1-1^T have already been deposited in GenBank, under accession numbers AY559041 (*Candida* sp. CBS

Table 1. Origin and DNA base composition of the strains examined

Strains of groups A and B were isolated from samples collected in the stone chamber of the Takamatsu-zuka tumulus in Nara Prefecture, Japan. Dates are given in the form D/M/Y. Accession numbers relate to sequences of the combined ITS1 region, 5.8S rDNA, ITS2 region and 26S rDNA D1/D2 sequences. ND, Not determined.

Strain	Other strain designation(s)	Sampling date	Isolation source	Sampling location	GenBank accession no.	G + C content (mol%)
<i>Candida tumulicola</i> sp. nov. (group A)						
T5916-5-3	JCM 15396	16/9/2005	Viscous gel (biofilm)	Lower section near the rightmost of the four women on the east wall	AB365456	ND
T5916-6-3	JCM 15397	16/9/2005	Viscous gel (biofilm)	Lower section near the four women on the east wall	AB365457	ND
T6220-1-4	JCM 15398	20/2/2006	Brownish viscous gel (biofilm)	Left part near the four women on the west wall	AB365458	ND
T6220-6-3	JCM 15399	20/2/2006	Brownish viscous gel (biofilm)	Near the hind legs of the blue dragon on the east wall	AB365459	42.5
T6517-3-6	JCM 15400	17/5/2006	Part of the mural that had turned black	Back part of the head of the leftmost of the four women on the west wall	AB365460	ND
T6517-7-3	JCM 15401	17/5/2006	As above	Upper part near the head of the white tiger on the west wall	AB365461	ND
T6517-8-2	JCM 15402	17/5/2006	Part of the mural that had turned white	Above the rightmost of the four women on the east wall	AB365462	ND
T6517-9-5 ^T	JCM 15403 ^T , CBS 10917 ^T , NBRC 104392 ^T	17/5/2006	Viscous gel (biofilm)	Lower part near the feet of the rightmost of the four women on the east wall	AB365463	43.1
T61213-1-5	JCM 15404	13/12/2006	Black mould, viscous gel (biofilm)	Foreheads of the group of women on the west wall	AB365464	ND
T61213-3-5	JCM 15405	13/12/2006	Part of the mural that had turned black	Back part of the head of the leftmost of the four women on the west wall	AB365465	ND
T61213-7-9	JCM 15406	13/12/2006	Part of the mural that had turned white	Upper part near the rightmost of the four women on the east wall	AB365466	ND
T61213-7-10	JCM 15407	13/12/2006	As above	Upper part near the rightmost of the four women on the east wall	AB365467	ND
T61213-12-11	JCM 15408	13/12/2006	Viscous gel (biofilm)	Right part, near the ceiling on the north wall	AB365468	ND
T61213-14-8	JCM 15409	13/12/2006	Springtail (Collembola)	The floor in the middle west	AB365469	ND
<i>Candida takamatsuzukensis</i> sp. nov. (group B)						
T4922-1-1 ^T	JCM 15410 ^T , CBS 10916 ^T , NBRC 104391 ^T	22/9/2004	Air	In the stone chamber	AB365470	36.7
T5916-2-4	JCM 15411	16/9/2005	Viscous gel (biofilm)	Lower part near the hind legs of the white tiger on the west wall	AB365471	36.8
T61213-3-6	JCM 15412	13/12/2006	Part of the mural that had turned black	Back part of the head of the leftmost of the four women on the west wall	AB365472	ND
T61213-12-12	JCM 15413	13/12/2006	Viscous gel (biofilm)	Right part, near the ceiling on the north wall	AB365473	ND
T61213-13-9	JCM 15414	13/12/2006	Part of the mural that had turned black	Back part of the heads of the leftmost of the four women on the west wall	AB365474	ND
<i>Candida friedrichii</i> NBRC 10277 ^T			Sorbitol solution	Germany	AB365475	ND
<i>Candida insectorum</i> NBRC 10283 ^T			Insect tunnels, <i>Euclea natalensis</i>	South Africa	AB365476	ND
<i>Pichia mexicana</i> NBRC 10544 ^T			Soft-rot pocket of agria cactus, <i>Stenocereus gummosis</i>	Mexico	AB365477	ND

Fig. 1. Phylogenetic positions of strains T6517-9-5^T (*Candida tumulicola* sp. nov.; group A) and T4922-1-1^T (*Candida takamatsuzukensis* sp. nov.; group B) in the *C. membranifaciens* clade, excluding subclade C (Suh *et al.*, 2005), in a neighbour-joining analysis of the 26S rDNA D1/D2 domain sequences (497 nt). Numbers indicate percentages of bootstrap sampling, derived from 1000 replications. Bar, 5 changes per 1000 nucleotide positions. *Candida temnochilae* NRRL Y-27763^T, which is assignable to subclade C in the *C. membranifaciens* clade, was used as an outgroup.

7880) and AY731727 (*Candida* sp. Mary 148) (Gadanhó & Sampaio, 2005). There is a high possibility that *Candida* sp. CBS 7880 and *Candida* sp. Mary 148 are conspecific with T4922-1-1^T. *Candida* sp. CBS 7880 (<http://www.cbs.knaw.nl/yeast/>) and *Candida* sp. Mary 148 (Gadanhó & Sampaio, 2005) were respectively isolated from liquid sweetener in the Netherlands and from seawater in the Mid-Atlantic Ridge hydrothermal fields near the Azores.

Phylogenetic trees were generated from 26S rDNA D1/D2 sequence comparisons using the neighbour-joining (Fig. 1) and maximum-parsimony (Supplementary Fig. S1) methods. The topologies generated in the neighbour-joining and maximum-parsimony phylogenetic trees are basically identical. Group A has no clearly identifiable sister species and occupies the basal position in the *C. membranifaciens* clade defined by Suh *et al.* (2005). No reliable connection of group B with any known species was recognized, although weak links with *Candida atlantica* and *Candida atmosphaerica* were found.

In the ITS region with the 5.8S rDNA sequence, all the isolates were identical within each group. Group A and *C. friedrichii* NBRC 10277^T, to which group A showed the highest similarity in the 26S rDNA D1/D2 sequence, showed 23.8 % difference (51 substitutions and 42 gaps) in

the ITS1 and 2 regions. Group B and *C. insectorum* NBRC 10283^T or *P. mexicana* NBRC 10544^T, to both of which group B showed the highest similarities in the 26S rDNA D1/D2 sequence, showed differences of 19.1 % (54 substitutions and 23 gaps) and 16.6 % (43 substitutions and 24 gaps), respectively, in the ITS1 and 2 regions. Sugita *et al.* (1999) reported that conspecific strains based on DNA–DNA relatedness have overall nucleotide differences of less than 1 % in both the ITS1 and 2 regions. Based on the nucleotide differences in the ITS1 and 2 regions, all the isolates in each group are considered to represent a single species, and each group forms a species distinct from the most closely related species, based on only the 26S rDNA D1/D2 sequence similarity.

The phenotypic characteristics of group A were compared with those of *C. friedrichii* (Table 2) and those of group B were compared with those of *C. insectorum* and *P. mexicana* (Table 3). Within each group, all of the examined isolates had similar features. Group A differed from *C. friedrichii* in the ability to assimilate arbutin, melibiose, lactose, raffinose, galactitol, ethanol and hexadecane, in the ability to grow at 30, 35 and 37 °C and in tolerance to cation toxicity (0.5 and 0.8 M Li⁺), osmotic pressure (50 % glucose) and cycloheximide (0.01 or 0.1 %) (Table 2). Group B differed from *C. insectorum* in the ability to

Table 2. Differential phenotypic characteristics between *C. tumulicola* sp. nov. (group A) and *C. friedrichii*

Group A strains included were T6517-9-5^T, T5916-5-3, T5916-6-3, T6220-1-4, T6220-6-3, T6517-3-6 and T6517-7-3. Maltose, sucrose, lactose and raffinose were not fermented by any of the strains. Glucose, galactose, D-xylose, L-arabinose, glycerol, erythritol, ribitol, L-arabinitol, D-glucitol, D-mannitol, N-acetyl-D-glucosamine, ethylamine and cadaverine were assimilated by all strains. Soluble starch, inositol, L-rhamnose, D-glucuronate, D-galacturonic acid, methanol, butane-2,3-diol, isopropyl alcohol, nitrate, nitrite and creatinine were not assimilated by any of the strains. All strains grew at 28 °C, but none grew at 40 °C. Growth in vitamin-free medium was negative. Growth in 16 % (w/v) NaCl/5 % (w/v) glucose and 1.0 M LiCl/YM broth was negative, but growth in 0.3 M LiCl/YM broth was positive in all cases. Production of amyloid compounds was negative in all cases. Diazonium blue B and urease reactions were negative in all cases. ND, No data; +, positive; −, negative; L, delayed positive (latent); S, slow; W, weak; V, variable. Data for *C. friedrichii* NBRC 10277^T were obtained in this study.

Characteristic	Group A	<i>C. friedrichii</i>	
		NBRC 10277 ^T	Meyer <i>et al.</i> (1998a)
Fermentation of:			
Glucose	L	L	+
Galactose	L	L	S/−
Trehalose	−	−	S
Melibiose	−	−	ND
Assimilation of:			
L-Sorbose	L	+	+
D-Glucosamine	S	L	−/L
D-Ribose	L/S	L	+
D-Arabinose	+	S	V
Sucrose	+/L	+	+
Maltose	+/S	L	+
Trehalose	L/S	L	+
Methyl α-D-glucoside	+/L	L	+
Cellobiose	+	L	+
Salicin	+	L	+
Arbutin	+	−	+
Melibiose	−	S	+
Lactose	+	−	−
Raffinose	−	L	+
Melezitose	+/−	L	+
Xylitol	L/+	S	+
Galactitol	−	L	+
D-Glucono-1,5-lactone	L	S	ND
2-Keto-D-gluconate	L	+	+
5-Keto-D-gluconate	−	−	ND
D-Gluconate	S/L	S	+
DL-Lactate	W/−	L	V
Succinate	+	S	+
Citrate	L/+	+	+
Ethanol	L	−	+
Isopropyl alcohol	−	−	ND
Propane-1,2-diol	S	L	−
Hexadecane	−	+	L

Table 2. cont.

Characteristic	Group A	<i>C. friedrichii</i>	
		NBRC 10277 ^T	Meyer <i>et al.</i> (1998a)
Saccharate	−	−	ND
L-Lysine	+	S	+
Growth at/with:			
30 °C	+	W	ND
35 °C	+	−	W/−
37 °C	+	−	−
0.01 % Cycloheximide	+	−	−
0.1 % Cycloheximide	+	−	ND
50 % (w/v) D-Glucose	−	+	+
10 % NaCl/5 % glucose	S	+	+
0.5 M LiCl	+	−	ND
0.8 M LiCl	+	−	ND

assimilate methyl α-D-glucoside, raffinose, melezitose and 2-keto-D-gluconate and in the ability to grow at 35 °C; group B also differed from *P. mexicana* in the ability to assimilate L-sorbose, methyl α-D-glucoside, lactose, raffinose, melezitose and D-glucono-1,5-lactone, in the ability to grow at 35 °C and in its tolerance to osmotic pressure [10 % NaCl/5 % glucose (w/v)] (Table 3). The growth responses of group B (Table 3) and *Candida* sp. CBS 7880 (<http://www.cbs.knaw.nl/yeast/>) were very similar except for fermentation of glucose (CBS 7880, positive), assimilation of DL-lactate (CBS 7880, delayed positive) and growth in 50 % glucose (CBS 7880, positive). The G+C contents of the DNA of strains of group A (T6517-9-5^T and T6220-6-3) and group B (T4922-1-1^T and T5916-2-4) were 42.5–43.1 and 36.7–36.8 mol%, respectively. In groups A (T6517-9-5^T and T6220-6-3) and B (T4922-1-1^T and T5916-2-4), the major ubiquinone was Q-9, the same as in all members of the *C. membranifaciens* clade (Meyer *et al.*, 1998c).

In groups A and B, asexual reproduction occurred by multilateral budding (Figs 2 and 3), and pseudohyphae and septate hyphae were formed. No sexual state that included the formation of ascospores was observed in any of the crosses within groups A or B on YM agar, 2 % malt agar or cornmeal agar after 6 weeks. Therefore, in light of the phenotypic, phylogenetic and morphological evidence, we conclude that groups A and B should each be classified as a novel species of the anamorphic genus *Candida* in the *C. membranifaciens* clade, and we propose the names *Candida tumulicola* sp. nov. for group A strains and *Candida takamatsuzukensis* sp. nov. for group B strains.

Latin diagnosis of *Candida tumulicola* Nagatsuka, Kiyuna, Kigawa & Sugiyama sp. nov. (Fig. 2)

In medio liquido YM *post dies* 3 *ad* 25 °C, *cellulae ellipsoideae* (2.5–3.5 × 3.8–6.5 μm), *singulae aut binae*, *per*

Table 3. Differential phenotypic characteristics between *C. takamatsuzukensis* sp. nov. (group B), *C. insectorum* and *P. mexicana*

Group B strains included were T4922-1-1^T and T5916-2-4. Maltose, lactose and raffinose were not fermented by any of the strains. Glucose, galactose, D-xylose, maltose, cellobiose, arbutin, erythritol, ribitol, L-arabinitol, D-glucitol, D-mannitol, N-acetyl-D-glucosamine, ethylamine, L-lysine and cadaverine were assimilated by all strains. Soluble starch, inositol, D-glucuronate, D-galacturonic acid, DL-lactate, methanol, butane-2,3-diol, saccharate, nitrate, nitrite and creatine were not assimilated by any strain. All strains grew at 28 and 30 °C. Growth in vitamin-free medium was negative. Growth in medium containing 16 % NaCl/5 % glucose (w/v) or 0.8 M or 1.0 M LiCl/YM broth was negative, but growth in 0.3 M and 0.5 M LiCl/YM broth was positive in all cases. Production of amyloid compounds was negative in all cases. Diazonium blue B and urease reactions were negative in all cases. ND, No data; +, positive; –, negative; L, delayed positive (latent); s, slow; w, weak; v, variable. Data for *C. insectorum* NBRC 10283^T and *P. mexicana* NBRC 10544^T were obtained in this study.

Characteristic	Group B	C. insectorum		P. mexicana	
		NBRC 10283 ^T	Meyer <i>et al.</i> (1998b)	NBRC 10544 ^T	Kurtzman (1998)
Fermentation of:					
Glucose	W	L	+	L	+
Galactose	W	W	S	S	+ /W
Sucrose	—	—	S/—	—	—
Trehalose	W	—	S	—	+
Melibiose	—	—	ND	—	ND
Assimilation of:					
L-Sorbose	L	—	L	—	—
D-Glucosamine	L	W	—/L	S	+
D-Ribose	+ /L	L	+	+	+
L-Arabinose	+ /L	S	+	+	+
D-Arabinose	L	S	+ /L	S	V
L-Rhamnose	L	S	+	+	+
Sucrose	L	+	+	+	+
Trehalose	+ /L	+	+	+	+
Methyl α-D-glucoside	—	S	+	+	+
Salicin	+ /L	L	+	+	+
Melibiose	—	L	—/L	S	W/—
Lactose	—	S	—/L	+	+
Raffinose	—	L	L	L	+
Melezitose	—	+	+	+	+
Glycerol	L	S	+	+	+
Xylitol	L	+	+	+	ND
Galactitol	—	—	—	L	V
D-Glucono-1,5-lactone	—	—	ND	S	ND
2-Keto-D-gluconate	+	—	—	S	S
5-Keto-D-gluconate	—	—	ND	—	—
D-Gluconate	+	S	+	+	+
Succinate	+	S	+	+	+
Citrate	+	S	+	+	+
Ethanol	L	+	+	+	+
Isopropyl alcohol	—	—	ND	—	ND
Propane-1,2-diol	—	—	—	L	ND
Hexadecane	—	+	+	+	+
Growth at/with:					
35 °C	—	+	ND	+	ND
0.01 % Cycloheximide	—	—	V	—	ND
0.1 % Cycloheximide	—	—	V	—	ND
50 % (w/v) D-Glucose	—	W	—	L	ND
10 % NaCl/5 % glucose	W	W	ND	+	+

Fig. 2. Micrographs of *C. tumulicola* T6517-9-5^T. (a) Vegetative cells in YM broth for 3 days at 25 °C. Bar, 10 µm. (b) Dalmau plate culture on YM agar for 6 days at 25 °C. Bar, 20 µm. (c) Dalmau plate culture on 2% malt agar for 5 days at 25 °C. Bar, 10 µm.

gemmationem multipolarem reproducentes. In medio liquido fermentatione sedimentum et annulus tenuis formantur. In agarō YM post hebmas 3 ad 25 °C, cultura linearis albida vel crenea et butyracea cum margine filamentoso et elevatione pulvinato cum radiatim stria. In agarō YM post dies 6 ad 25 °C, pseudohyphae et hyphae septatae formantur. Teleomorphosis ignota. Characteres physiologici in tabula secunda demonstrantur. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Amylum non formatur. Systema coenzymatis Q-9 adest. Guaninum et cytosinum acidi deoxyribonucleati 43.1 mol% (per HPLC). Typus: holotypus JCM 15403^T (isotypi, CBS 10917^T, NBRC 104392^T; originaliter ut T6517-9-5^T), lyophilus in collectionibus culturarum quas Japan Collection of Microorganisms (JCM), RIKEN, Wako, Saitama, Japonia sustentat.

Description of *Candida tumulicola* Nagatsuka, Kiyuna, Kigawa & Sugiyama sp. nov.

Candida tumulicola (tu.mu.li'co.la. N.L. nom. fem. n. *tumulicola* referring to a tumulus dweller, where the yeast was originally isolated).

Growth in YM broth: after 3 days at 25 °C, cells are ellipsoidal (2.5–3.5 × 3.8–6.5 µm) and appear singly or in pairs. Budding is multipolar (Fig. 2a). Sediment and a thin ring are formed on the surface of the fermentation medium. Aerobic growth on YM agar: after incubation for 3 weeks at 25 °C, the streak culture is white to creamy in colour, smooth and butyrous; it has a filamentous margin and pulvinate elevation with radial striations. Dalmau plate culture on YM agar or 2% malt agar: after incubation for 5–6 days at 25 °C, pseudohyphae of

branched chains of cylindrical cells (Fig. 2b) and septate hyphae (Fig. 2c) are present. The sexual state is unknown after 1 month at 25 °C from individual strains or from strains crossed in all combinations on YM, 2% malt or cornmeal agars. For details of physiological and biochemical characteristics, see Table 2. The major ubiquinone system is Q-9. The G+C content of the nuclear DNA of the type strain is 43.1 mol%, as determined by HPLC.

The holotype, JCM 15403^T (=CBS 10917^T =NBRC 104392^T; originally T6517-9-5^T), was isolated on 17 May 2006 by one of the authors (T.K.) from a sample of a viscous gel collected near the feet of the rightmost of the four women depicted on the east plaster wall in the stone chamber of the Takamatsu-zuka tumulus in the village of Asuka, Nara Prefecture, Japan. This holotype is deposited as a lyophilized preparation in the culture collection of the Japan Collection of Microorganisms, RIKEN, Wako, Saitama Prefecture, Japan.

Latin diagnosis of *Candida takamatsuzukensis* Nagatsuka, Kiyuna, Kigawa & Sugiyama sp. nov. (Fig. 3)

In medio liquido YM post dies 3 ad 25 °C, cellulae sphaeroidae vel ovoideae (1.9–4.1 × 2.1–6.1 µm), singulae aut binae, per gemmationem multipolarem reproducentes. In medio liquido fermentatione sedimentum et pellicula crassa formantur. In agarō YM post hebmas 3 ad 25 °C, cultura linearis albida vel crenea et siccata cum margine lobato et elevatione pulvinato. In agarō YM post dies 6 ad 25 °C, pseudohyphae et hyphae septatae formantur. Teleomorphosis ignota. Characteres physiologici in tabula tertia demonst-

Fig. 3. Micrographs of *C. takamatsuzukensis* T4922-1-1^T. (a) Vegetative cells and pseudohyphae in YM broth for 3 days at 25 °C. Bar, 10 µm. (b) Dalmat plate culture on YM agar for 3 days at 25 °C. Bar, 10 µm.

tur. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Amylum non formatur. Systema coenzymatis Q-9 adest. Guaninum et cytosinum acidi deoxyribonucleati 36.7 mol% (per HPLC). Typus: holotypus JCM 15410^T (isotypi, CBS 10916^T, NBRC 104391^T; originaliter ut T4922-1-1^T), lyophilus in collectionibus culturarum quas Japan Collection of Microorganisms (JCM), RIKEN, Wako, Saitama, Japonia sustentat.

Description of *Candida takamatsuzukensis* Nagatsuka, Kiyuna, Kigawa & Sugiyama sp. nov.

Candida takamatsuzukensis (ta'ka.mat.su.zuk.en'sis. N.L. fem. adj. *takamatsuzukensis* referring to the Takamatsu-zuka tumulus, in the village of Asuka, Nara Prefecture, Japan, the collection site of the type strain).

Growth in YM broth: after 3 days at 25 °C, cells are spherical to ovoid (1.9–4.1 × 2.1–6.1 µm) and appear singly or in pairs. Budding is multipolar (Fig. 3a). Sediment and thick pellicles form on the surface of the fermentation medium. Growth on YM agar: after incubation for 3 days at 25 °C, the streak culture is white to creamy in colour, wrinkled and dry; it has a lobate margin and pulvinate elevation. Dalmat plate culture on YM agar: after incubation for 6 days at 25 °C, simple to elaborate pseudohyphae and septate hyphae are present (Fig. 3b). The sexual state is unknown after 1 month at 25 °C from individual strains or strains crossed in all combinations on YM, 2 % malt or cornmeal agar. For details of physiological and biochemical characteristics, see Table 3. The major ubiquinone system is Q-9. The G+C content of the nuclear DNA of the type strain is 36.7 mol%, as determined by HPLC.

The holotype JCM 15410^T (=CBS 10916^T =NBRC 104391^T; originally T4922-1-1^T) was isolated on 22 September 2004 by one of the authors (T.K.) from the air of the stone chamber at the Takamatsu-zuka tumulus in

Asuka, Nara Prefecture, Japan. This holotype is deposited as a lyophilized preparation in the culture collection of the Japan Collection of Microorganisms, RIKEN, Wako, Saitama Prefecture, Japan.

Significance of the novel strains in the tumulus environment

To eliminate micro-organisms found in the stone chamber at the Takamatsu-zuka tumulus, fumigation with paraformaldehyde was employed (Kigawa *et al.*, 2006). Ethanol was also applied to eliminate micro-organisms on the plaster paintings, as it was thought to be the mildest treatment for the pigments used in the paintings. It was tentatively concluded that ethanol seemed to be effective against most of the micro-organisms. However, in the stone chamber at the Takamatsu-zuka tumulus, the humidity was always kept very high, almost 100 %, and thus the ethanol may have been diluted with a good deal of moisture and may have been used as a carbon source by the small population of micro-organisms that had survived the treatments. Beginning in September 2005, isopropyl alcohol was used as a disinfectant instead of ethanol (Kigawa *et al.*, 2006). An assimilation test of all the strains of *C. tumulicola* and *C. takamatsuzukensis* revealed that these strains assimilated ethanol latently but did not assimilate isopropyl alcohol at all (Tables 2 and 3). Thus, isopropyl alcohol would be more effective than ethanol for removing strains of *C. tumulicola* and *C. takamatsuzukensis*.

Suh *et al.* (2005) showed that many invertebrate-associated yeast species were accommodated in the *C. membranifaciens* clade. *C. tumulicola* T61213-14-8 was isolated from a springtail (Collembola). On the other hand, many strains of *C. tumulicola* and *C. takamatsuzukensis* were isolated in this study from biofilm samples. There is no record of the isolation of species in the *C. membranifaciens* clade from biofilms or plaster walls. Information on the origin of

known species in the *C. membranifaciens* clade maybe provide some clue as to the culprit behind the biodeterioration that is occurring in the Takamatsu-zuka tumulus.

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References

- Felsenstein, J. (1985).** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gadanhó, M. & Sampaio, J. P. (2005).** Occurrence and diversity of yeasts in the mid-Atlantic ridge hydrothermal fields near the Azores Archipelago. *Microb Ecol* **50**, 408–417.
- Hwang, S.-K. & Kim, J.-G. (2000).** Secondary structural and phylogenetic implications of nuclear large subunit ribosomal RNA in the ectomycorrhizal fungus *Tricholoma matsutake*. *Curr Microbiol* **40**, 250–256.
- Kigawa, R., Sano, C. & Miura, S. (2004).** Past and present situation of microorganisms in Takamatsuzuka tumulus. *Sci Conserv* **43**, 79–85.
- Kigawa, R., Sano, C., Ishizaki, T. & Miura, S. (2006).** Concept and measures of the conservation of Takamatsuzuka tumulus for thirty years and the present situation of biodeterioration. *Sci Conserv* **45**, 33–58.
- Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kuraishi, H., Katayama-Fujimura, Y., Sugiyama, J. & Yokoyama, T. (1985).** Ubiquinone systems in fungi. I. Distribution of ubiquinones in the major families of ascomycetes, basidiomycetes, and deuteromycetes, and their taxonomic implications. *Trans Mycol Soc Jpn* **26**, 383–395.
- Kurtzman, C. P. (1998).** *Pichia mexicana* Miranda, Holzschu, Phaff & Starmer. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 322–323. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. & Robnett, C. J. (1998).** Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.
- Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Meyer, S. A., Payne, R. W. & Yarrow, D. (1998a).** *Candida friedrichii* van Uden & Windisch. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 506–507. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Meyer, S. A., Payne, R. W. & Yarrow, D. (1998b).** *Candida insectorum* D. S. Scott, van der Walt & van der Klift (van der Walt et al.) Windisch. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 515–516. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Meyer, S. A., Payne, R. W. & Yarrow, D. (1998c).** *Candida Berkhout*. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 454–573. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Nagatsuka, Y., Kawasaki, H., Limtong, S., Mikata, K. & Seki, T. (2002).** *Citeromyces siamensis* sp. nov., a novel halotolerant yeast isolated in Thailand. *Int J Syst Evol Microbiol* **52**, 2315–2319.
- Nagatsuka, Y., Kawasaki, H., Mikata, K. & Seki, T. (2005).** *Candida khmerensis* sp. nov., a novel cation-tolerant yeast isolated from dry salted shrimp and sewage in Cambodia. *J Gen Appl Microbiol* **51**, 235–243.
- Sugita, T., Nishikawa, A., Ikeda, R. & Shinoda, T. (1999).** Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *J Clin Microbiol* **37**, 1985–1993.
- Suh, S. O., Nguyen, N. H. & Blackwell, M. (2005).** Nine new *Candida* species near *C. membranifaciens* isolated from insects. *Mycol Res* **109**, 1045–1056.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Yamada, Y., Okada, T., Ueshima, O. & Kondō, K. (1973).** Coenzyme Q system in the classification of the ascosporogenous yeast genera *Hansenula* and *Pichia*. *J Gen Appl Microbiol* **19**, 189–208.
- Yamada, Y., Kawasaki, H., Nagatsuka, Y., Mikata, K. & Seki, T. (1999).** The phylogeny of the cactophilic yeasts based on the 18S ribosomal RNA gene sequences: the proposals of *Phaffomyces antillensis* and *Starmera caribaea*, new combinations. *Biosci Biotechnol Biochem* **63**, 827–832.
- Yarrow, D. (1998).** Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.