Note

Candida kunwiensis sp. nov., a yeast associated with flowers and bumblebeesSoon Gyu Hong,¹ Kyung Sook Bae,¹ Michel Herzberg,² Andreas Titze² and Marc-André Lachance³

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A novel asexual ascomycetous yeast, *Candida kunwiensis* (SG99-26^T = KCTC 17041^T = CBS 9067^T), was isolated from sweet potato (*Ipomoea batatas*) flowers in Korea and from the body surface of pollinating bumblebees in Germany. Comparative analysis of the D1/D2 domain of 26S rDNA of all available sequences for ascomycetous yeasts showed that the novel species was phylogenetically related to the genus *Metschnikowia*, but the sequence similarity was low. Morphologically and physiologically, *C. kunwiensis* in many ways resembles *Metschnikowia pulcherrima*, but can be distinguished from this species by its ability to assimilate lactic acid and its inability to produce pulcherrimin.

Certain yeast species with affinities to the genus *Metschnikowia* occur in flowers, fruits and associated vector insects (Miller & Phaff, 1998). *Metschnikowia hawaiiensis* (Lachance *et al.*, 1998b, 1990), *Metschnikowia continentalis* (Lachance *et al.*, 1998b), *Metschnikowia lochheadii*, *Metschnikowia drosophilae* (Lachance *et al.*, 2001a) and *Candida ipomoeae* (Lachance *et al.*, 1998a) occur on morning glories (*Ipomoea* spp.) and insects, including drosophilids, beetles and bees, found on these flowers (Lachance *et al.*, 2001b). Other yeasts isolated from flowers include *Metschnikowia gruessii*, *Metschnikowia lunata*, *Metschnikowia pulcherrima*, *Metschnikowia reukauffii* (Miller & Phaff, 1998) and *Metschnikowia koreensis* (Hong *et al.*, 2001).

In the course of an isolation programme of yeasts from various natural sources in Korea, a strain (SG99-26^T) of ascomycetous affinity was isolated from flowers of *Ipomoea batatas* Lam. Comparison of the sequence of the D1/D2 domain of the 26S rDNA with sequences from all currently recognized ascomycetous yeasts and comparison of physiological profiles indicated that strain SG99-26^T represented a novel yeast species. Independently, a study of yeasts associated with pollinating bumblebees in Germany yielded various strains of a yeast with the same D1/D2 sequences as that of strain SG99-26^T. Here, we describe the Korean strain and German strains as representing a novel yeast species, for

which the name *Candida kunwiensis* is proposed. This novel species has a close phylogenetic relationship with the genus *Metschnikowia*.

The origins of the novel strains discussed in this report are given in Table 1. Morphological and physiological characteristics were examined by the conventional techniques described by Yarrow (1998). For strain SG99-26^T, the assimilation of carbon sources was examined at 25 °C on a rotary shaker (120 r.p.m.) at 7-day intervals for 21 days. The utilization of nitrogen sources was examined by auxanography for 7 days. Urease activity was tested in Christensen's urea agar (Christensen, 1946). The determination of the coenzyme Q system was carried out as described by Yamada (1998) using an HPLC apparatus equipped with a Spherisorb S5 ODS2 column (Waters). The DNA base content (G+C mol%) was determined by the thermal denaturation method (T_m) using 0.1 × SSC solution. The G+C content was calculated using the equation $G+C \text{ mol\%} = 51.0 + 2.08 (T_{mx} - T_{mr})$, where a DNA preparation from *Escherichia coli* KCTC 2443 (K-12; G+C content, 51.0 mol%) was included as a reference (T_{mr}) (Owen & Pitcher, 1985). The German strains were characterized nutritionally by replica plating. All the novel strains are maintained in liquid nitrogen in the yeast collection of the Department of Plant Sciences, University of Western Ontario, Canada.

The GenBank accession number for the partial 26S rDNA sequence of *Candida kunwiensis* SG99-26^T is AF389527.

The D1/D2 domain of the nuclear 26S rDNA of strain SG99-26^T was amplified and sequenced using the primer

Table 1. Origins of strains of *Candida kunwiensis*

Strain no.	Substrate	Host plant	Isolated in
SG99-26 ^T	Flower	<i>Ipomoea batatas</i>	Kunwi, Korea
MH266, MH277, MH402	<i>Bombus terrestris</i>	<i>Helleborus foetidus</i>	Marburg, Germany
MH275	<i>Bombus cryptarum</i>	<i>Helleborus foetidus</i>	Marburg, Germany
MH304	<i>Bombus hortorum</i>	<i>Helleborus foetidus</i>	Marburg, Germany
MH394	<i>Bombus lapidarius</i>	<i>Helleborus foetidus</i>	Marburg, Germany
MH356	Flower	<i>Helleborus foetidus</i>	Marburg, Germany
MH397	<i>Bombus pascuorum</i>	<i>Helleborus foetidus</i>	Marburg, Germany

pair No.4 (ACCCG CTGAA YTAA GCATA T) and No.11 (CTCCT TGGTC CGTGT TTCAA GACGG) (Van der Auwera *et al.*, 1994). The DNA sequence has been deposited in GenBank under accession no. AF389527. The 26S rDNA D1/D2 sequence of the strain was aligned with other 26S rDNA sequences from species of the genus *Metschnikowia* and related species on the basis of similarity in the primary and secondary structures using the PHYDIT program, version 3.1 (Chun, 1995), which enables manual and semi-computerized alignment of nucleotide sequences using secondary structure information (available at <http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were reconstructed using Kimura's two-parameter model (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987) using the PHYLIP package (version 3.57c; Felsenstein, 1995) with or without the D2 domain sequences that showed large deletions in some taxa. *Saccharomyces cerevisiae* NRRL Y-12632^{NT} (U44806) was used as an outgroup. The relative robustness of individual branches was estimated by bootstrapping (Felsenstein, 1985), in which 1000 bootstrapped trees were generated from the resampled data. The neighbour-joining tree was compared with those constructed by parsimony methods. The parsimony tree was reconstructed by using the heuristic search option of PAUP 4.0 beta version (Swofford, 1998) with 100 replicates of random sequence addition. The D1/D2 sequence of strain MH266 was determined as described by Lachance *et al.* (1999).

Latin diagnosis of *Candida kunwiensis* Hong, Bae, Herzberg, Titze et Lachance sp. nov.

Cultura in aqua extracto malti post dies 3 ad 25 °C cellulae globosae ad subglobosae (4–7 × 4–7 µm), singulae. Post unum mensem sedimentum formatur. Cultura in agaro malti post dies 3 ad 25 °C albida crenea et butyrosa. In agaro farinae Zea mays post dies 10 pseudomycelium nullum vel rudimentarium. Chlamydosporae formantur. Glucosum (aliquando exigue) fermentatur, at non galactosum, saccarum, maltosum, lactosum, raffinose nec inulinum. Glucosum, galatosum, L-sorbose, saccharum, maltosum, cellobiosum, trehalosum, melezitosum, D-xylosum (aliquando lente), D-glucosaminum (aliquando exigue), N-acetylglucosaminum, ethanolum, glycerolum, ribitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum D-gluconicum, glucono-δ-lactonum, acidum DL-lacticum (aliquando exigue), acidum succinicum et hexadecanum (lente et variabile) assimilantur

at non lactosum, melibiosum, raffinose, inulinum, amyllum solubile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, methanolum, erythritolum, galactitolum, acidum citricum, inositolum, hexadecanum, nec acidum D-glucuronicum. Assimilatio ethylamini, cadaverini et L-lysini ad non kallii nitratis, sodii nitrosi, D-glucosaminum, creatini nec creatinini. Vitaminae externae ad crescentiam necessariae sunt. Maxima temperatura crescentiae: 30 °C. Materia amyloidea non formantur. Ureum non finditur. Proportio molaris guanini plus cytosini in acido deoxyribonucleico: 41.1 ± 1.3

Fig. 1. (a) Differential interference contrast (DIC) micrograph of vegetative cells of *C. kunwiensis* SG99-26^T. Bar, 10 µm. (b, c) Phase-contrast micrographs of (b) cell with tubular bud (YM agar) and (c) chlamydospores (dilute V8 agar) of *C. kunwiensis* MH266.

mol%. *Typus*: SG99-26^T, *ex flora Ipomoea batatas isolatus est*. In collectione *zymotica* Korean Collection for Type Cultures (KCTC 17041^T = CBS 9067^T) *preservatus*.

Description of *Candida kunwiensis* Hong, Bae, Herzberg, Titze & Lachance sp. nov.

Candida kunwiensis (kun.wi.en'sis. N.L. nom. fem. adj. *kunwiensis* of Kunwi, referring to the town where the type strain was isolated).

In 5 % malt extract broth after 3 days incubation at 25 °C, cells are globose to subglobose (4–7 × 4–7 µm), occurring (mainly) singly. Vegetative reproduction is proceeded by multilateral budding (Fig. 1a). Cells may produce a tubular bud equivalent to three or more cell lengths (Fig. 1b). Sediment is formed after 4 weeks incubation. A pellicle is absent. On 10 % malt extract agar after 3 days incubation at 25 °C, colonies are white and butyrous; colonies have dull or smooth surfaces, and smooth and entire margins. In Dalmau plate cultures on cornmeal agar after 10 days incubation at 25 °C, no growth under the coverslip is observed, except in the margin where air is transmitted through the media. No pseudomycelia or hyphae are observed. Does not sporulate on oatmeal agar, potato glucose agar, water agar, YM agar, cornmeal agar, 5 % malt extract agar, 10 % malt extract agar, 1 % acetic acid agar, V8 agar or diluted V8 agar at various temperatures (Yarrow, 1998). However, after one week on dilute (20 %) V8 agar, chlamydospores of the type found in *Metschnikowia pulcherrima* are formed in abundance (Fig. 1c). Ferments D-glucose (sometimes weakly); D-galactose, sucrose, maltose, lactose, raffinose and trehalose are not fermented.

Assimilates the carbon compounds D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose (sometimes slow), D-glucosamine (sometimes weak), *N*-acetyl-D-glucosamine, ethanol, glycerol, ribitol, D-mannitol, D-glucitol, methyl α -D-glucoside, salicin, D-gluconate, glucono- δ -lactone, DL-lactate (sometimes weak) and succinate. Does not assimilate lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, methanol, erythritol, galactitol, citrate, inositol or glucuronate. Assimilation of hexadecane is slow and variable. Assimilates the nitrogen compounds ethylamine, cadaverine and L-lysine; does not assimilate nitrate, nitrite, D-glucosamine, creatine or creatinine. Grows in the presence of 10 % NaCl/5 % glucose, 16 % NaCl/5 % glucose and 50 % glucose. Does not grow in the presence of 0.01 % cycloheximide, 0.1 % cycloheximide or 1 % acetic acid. Does not grow in vitamin-free medium. Does not form starch. Urease-negative. Diazonium Blue B stain-negative. Grows at 30 °C, but not at 35 °C. Major ubiquinone system of the type strain (SG99-26^T = KCTC 17041^T = CBS 9067^T) is Q9; Q8 is also present in a significant amount (Q9:Q8 = 4:1). DNA G+C content is 41.1 ± 1.3 mol% (mean value of three determinations). The closest relative of *Candida kunwiensis*, based on D1/D2 domain sequences of 26S rDNA, is *Metschnikowia gruessii* NRRL Y-17809^T (85 % sequence similarity; 72 bases different per 488 sites; Fig. 2).

Phylogenetic placement. The 26S rDNA sequence of strain MH266 was identical to that of strain SG99-26^T, verifying their conspecificity. Unambiguous alignment of variable regions was very difficult because multiple insertion or deletion events have occurred in the 26S rDNA

Fig. 2. Phylogeny of *Metschnikowia* and related *Candida* spp. based on the D1 domain of the 26S rDNA sequences (*S. cerevisiae* numbering 45–405). The tree was constructed by the neighbour-joining algorithm using a distance matrix calculated using Kimura's two-parameter model. The numbers at the nodes indicate the bootstrap values for 1000 iterations, expressed as percentages. Internal branches that were conserved both in the distance tree and in six equally parsimonious trees are represented by thick lines.

M. hibisci

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A
A
GGUGCAGCAACCAAACUAACACCCA
|||||
CCACUUCGUUGGU GGUUGUUAA
A
C

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M. hawaiiensis

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A
G
GGGAGGGAAGAUGUUAAGGACUCCUUACU
|||||
CUCUCCUUUCUGAGAACCCCUAGGAUGC
G
C

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M. lunata

```

A
G
GGCUUGACCGGCUGGGGAGGU CCGGGAAAAAGAGGCCAAACAGCAAAGGAAUGUA ACUCU
|||||
CUGAACCGGCCUACCCCCUCUAAGGGCCCCUUUCACUCCCACACCGCUCCUAUAUUAUGAGC
G
C

```

M. pulcherrima

```

A
G
GGCUUGCAAGCAGACACUUACUGGGCCAGCAUUCGGGGCGGGGAGCAAACACCGGGGAAUGUACCUUU
|||||
CUGAACGUUGGUAGGAGCCCCACCACCCCUCAUUCCCUGGCCCCAUAUGGAGC
G
C

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C. kunwiensis

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A
G
GGCUUGCAAGUAUUGGGCGCAUUCGGGGGAGAGGAAACCAAAUAGCCCCGGGAAUGUAACUUU
|||||
CUGAACGUUCAUGUAGGAGCCCCCUCUCCUUGGUAGAUCGGCCCUAUAUUGAGC
G
C

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M. guessii

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A
G
GGCUUGCAAGCAGACACAACUUUUUGUGGCAGCAUUCGGGGAGUAGGAGGAAAAGGAGUAGAAAUGUAGCUU
|||||
CUGAACGUUGGUUAGUCCGUAGGUAGCCCUCCACCUCCAUAUCUGGUCUGUAUAUUGGC
G
C

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M. bicuspidata

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A
G
GGCUUGCAAGCAGACACAACUUUUGUUGGGCCAGCAUUCGGGGCGAGGGGAAGAAAGAAAAAGGAAUGUAACUCU
|||||
CUGAACGUUGGUUAGGAAGCCCUACUCUACCUUCAAACUUCUCCAGUAUAUGAGC
G
C

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Fig. 3. Model of the secondary structure of the D2 domain for selected *Metschnikowia* spp. and *C. kunwiensis*.

sequences of *M. continentalis* var. *continentalis*, *M. continentalis* var. *borealis*, *M. hawaiiensis*, *M. hibisci*, *M. lochheadii* and *Candida ipomoeae*. The D2 domains of these species ranged from 48 bp for *M. hibisci* to 63 or 64 bp for the other five species, in comparison to 139 bp for *M. lunata*, the sister taxon to the group containing the above-mentioned six species. The other *Metschnikowia* species also have short D2 domains (154–184 bp) compared to *S. cerevisiae* (210 bp). The secondary structures of the D2 domains (Fig. 3) of *M. hibisci* and *M. hawaiiensis* are clearly different from those of other *Metschnikowia* species. By contrast, the D2 domains of *M. hawaiiensis*, *C. ipomoeae*, *M. continentalis* and *M. continentalis* var. *borealis* differ only by minor changes in loop and bulge motifs. *M. hibisci* also has short stem-loop structures with small bulges in the D2 domain, but the sequence is completely different from those of the aforementioned species. The D2 domain of *M. lunata* has a similar secondary structure and sequence motifs to that of *M. bicuspidata*, but several deletions were also observed. The secondary structure of the D2 domain of *C. kunwiensis* SG99-26^T was similar to those of *M. pulcherrima*, *M. gruessii* and *M. bicuspidata* with some minor differences. Based on the secondary structure models, it is proposed that *C. kunwiensis* is most closely related to the core group of *Metschnikowia* species, which includes *M. bicuspidata*, the type species of the genus, and more distantly related to the species that share shorter D2 domains, such as *M. hibisci* and *M. hawaiiensis*.

In view of their differences in secondary structure, one might even question whether the D2 domains of all *Metschnikowia* species are in fact homologous. Their inclusion in data used for phylogenetic reconstructions can be a source of serious distortion. For this reason, the tree presented in Fig. 2 is based only on that portion of the D1/D2 sequence that can be aligned unambiguously, and excludes most of the variable D2 region. A tree based on the entire D1/D2 sequence (not shown) suggested different relationships amongst *M. continentalis* var. *continentalis*, *M. continentalis* var. *borealis*, *M. lochheadii* and *C. ipomoeae*. The tree also suggested different phylogenetic positions of *M. pulcherrima*, *M. hibisci*, *M. lunata*, *C. torresii*, *M. drosophilae* and *M. agaves*. In the phylogeny based on partial sequences (Fig. 2), *C. kunwiensis* and *M. gruessii* appeared as sister species related to the core group, with a fairly high bootstrap value.

Identification. The physiological characteristics of *C. kunwiensis* are typical of those of most *Metschnikowia* species and nearly identical to those of *M. pulcherrima*. Lack of pulcherrimin production and growth on lactic acid by *C. kunwiensis* probably represent the only useful features to distinguish the two species. Unfortunately, growth on lactic acid exhibited some variation (positive or weak) amongst our strains, and is reported as negative or weak in *M. pulcherrima* (Miller & Phaff, 1998). Pulcherrimin production is also reported as variable in *M. pulcherrima*. This opens the possibility that some of

the strains identified in the past as *M. pulcherrima* might in fact be isolates of *C. kunwiensis*.

Ecology. In the German study, *C. kunwiensis* was recovered almost exclusively from the body surface of five different species of bumblebees, suggesting an association with pollen (Table 1). *Candida bombi* was also frequent in these samples. In contrast, flowers visited by the bumblebees, the nectar of these flowers and the probosces of the bumblebees normally had a different yeast community, composed predominantly of *M. reukaufii*, with lesser proportions of *M. gruessii* and *C. bombi*. It will therefore be of great ecological interest to identify the principal habitat of *C. kunwiensis* in its Korean environment, to see if an association with pollen exists there too.

Acknowledgements

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