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*Candida khmerensis* sp. nov., a novel cation-tolerant yeast isolated from dry salted shrimp and sewage in Cambodia

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Two cation-tolerant yeasts with powdered colonies, K28-3-2T and K26-1-4, were isolated from dry salted shrimp and sewage, respectively, in Siem Reap province, Cambodia. The D1/D2 sequences of the 26S rDNA data showed that the two isolates were conspecific and related to the *Pichia burtonii* and *Candida fennica*. Two isolates were examined by a polyphasic taxonomic approach, including molecular phylogenetic analysis, morphological, physiological and biochemical tests, DNA hybridization and MSP-PCR fingerprinting, in comparison with *P. burtonii* and *C. fennica*. The two isolates were found to grow by multilateral budding with true and pseudomycelium, to not produce ascospores, and to contain ubiquinone Q-8 similar to that of *P. burtonii* and *C. fennica*. The two isolates were not differentiated from the two closest species, *P. burtonii* and *C. fennica*, by the phenotypic character examined, except for the cation (Li⁺)-tolerance. From DNA-DNA reassociation studies, however, the two isolates showed low similarities to the closest two species. Based on D1/D2 sequences of 26S rDNA and DNA-DNA reassociation data, they were shown to be a new distinct species from *P. burtonii* and *C. fennica*. Therefore, a novel species is proposed, *Candida khmerensis* sp. nov., represented by strain K28-3-2T (=JCM 13262T=CBS 9784T). The novel species, *Candida khmerensis* sp. nov. can be clearly distinguished from *P. burtonii* and *C. fennica* by either the 26S rDNA D1/D2 or ITS region with 5.8S rDNA sequencing, or by the MSP-PCR fingerprinting pattern.

**Key Words**——*Candida fennica; Candida khmerensis* sp. nov.; cation-tolerance; halo-tolerance; MSP-PCR; osmo-tolerance; *Pichia burtonii*

Introduction

In the course of studying the taxonomic diversity of osmo-tolerant yeasts in Tropical Southeast Asia, many yeast strains have been isolated from traditional fermented food, fruits, and other natural habitats. Among our isolates several new species were predicted by 26S rDNA D1/D2 nucleotide sequence analysis and a cation-tolerance test using LiCl (Nagatsuka et al., 2002, 2005). *Citeromyces siamensis* sp. nov., which is isolated from dry salted squid and fermented soybeans in Thailand, has been previously reported as a second species of genus *Citeromyces* (Nagatsuka et al., 2002). *Pichia myanmarensis* sp. nov., which is isolated...
from palm sugar in Myanmar, has been reported (Nagatsuka et al., 2005). In this study, we determined the taxonomic characteristics of two powdery yeast isolates that were the closest to *P. burtonii* (97% similarity) and, secondarily to *C. fennica* (96% similarity), in the 26S rDNA D1/D2 domain nucleotides sequences, and we describe herein for those two isolates a new species, *Candida khmerensis* sp. nov.

Four other strains of *P. burtonii* with poor taxonomic data that were preliminarily identified as *P. burtonii* were preserved, in addition to *P. burtonii* IFO 10837T, in the Institute for Fermentation, Osaka (IFO), one of the Japanese culture collections. The taxonomic characteristics of these four other strains of *P. burtonii* were also examined. *P. burtonii* IFO 0844, IFO 1196, and IFO 1986 were isolated from tamari-koji in Japan, from sputum in The Netherlands, and from banana fruit, *Musa sapientum*, in Japan, respectively; IFO 6130 came from the CBS culture collection and its origin is unknown.

**Materials and Methods**

**Isolation of yeasts and reference strains.** Novel cation-tolerant yeasts, K28-3-2T and K26-1-4, were isolated, respectively, from dry salted shrimp that was sold in the market and from sewage at the factory where salted fish used in fish-sauce and paste (Pra-hoc) is produced, in Siem Reap province, Cambodia. The isolation was carried out by an enrichment culture using 20% glucose YM (yeast-extract and malt-extract) broth containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 0.005% chloramphenicol to inhibit bacterial growth. Cultivation was performed at field room temperature for a week or so. The purification of the two strains was done by employing the dilution method. Five strains of *P. burtonii*, IFO 10837T, IFO 0844, IFO 1196, IFO 1986 and IFO 6130, and the type strain of *C. fennica*, IFO 10276T, were used as reference strains. All strains were stocked on a YM (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) agar slant.

**Sequencing and phylogenetic analyses of the 26S rDNA D1/D2 domain, 18S rDNA, and the ITS region with 5.8S rDNA.** The procedure for the determination and analyses of the rDNA sequence has been previously described (Nagatsuka et al., 2002; Yamada et al., 1999). Primers NL-1 and NL-4 (O'Donnell, 1993) were used to synthesize and sequence the 26S rDNA D1/D2 domain fragment. Primers P1 and P2 were used to synthesize the 18S rDNA fragment; and primers P1, P2, P3, P4, P5, P6, P7, and P8 (Yamada et al., 1999), were used to sequence the 18S rDNA. For amplification and sequencing of the ITS region with 5.8S rDNA, the primers ITS-1 and ITS-4 were used (White et al., 1990). For sequencing of the ITS region with 5.8S rDNA, the primers ITS-2 and ITS-3 were also used (White et al., 1990). The sequences of the 26S rDNA D1/D2 domain, 18S rDNA, and the ITS region with 5.8S rDNA determined in this study were deposited in the DDBJ database under the following accession numbers: the D1/D2 sequences of 26S rDNA of isolates, K28-3-2T and K26-1-4, are AB158648 and AB158649; the ITS region with 5.8S rDNA and the 18S rDNA of isolates, K28-3-2T and K26-1-4, *P. burtonii* IFO 10837T and *C. fennica* IFO 10276T are AB158650–AB158657. Considering the secondary structure of 26S rRNA (Hwang and Kim, 2000), the sequence regions for which the alignment was questionable (nucleotide positions 62–78, 97–114, 172–194 and 379–384, according to the *C. khmerensis* numbering system (AB158648)) were not used in the construction of the phylogenetic tree based on the 26S rDNA D1/D2 sequences. Gaps and uncertain positions in the database (submitted as “n”) were excluded when the tree was drawn using CLUSTAL W ver 1.6 (Thompson et al., 1994). The phylogenetic tree was constructed based on 399 nucleotides of the 26S rDNA D1/D2 domain sequences out of a total of 525 nucleotides determined, excluding uncertain aligned regions. The species and accession numbers in the nucleotide sequence cited from the database are listed in Fig. 1.

**Physiological, biochemical, and morphological characteristics and the ubiquinone system.** The physiological and biochemical tests were performed using the standard methods described in *The Yeasts, A Taxonomic Study* (Yarrow, 1998). The effects of osmotic pressure were tested in terms of the ability to grow at high concentrations of sugar, 50% and 60% (w/w) glucose (Yarrow, 1998). The cation-tolerance of the yeast strains has been evaluated according to their growth ability in YM broth containing the Li cation (LiCl), which is the analogue of the sodium cation with higher toxicity than sodium. After incubation for 2 weeks at 28°C in YM broth supplemented with LiCl at various concentrations, the growth was scored (Nagatsuka et al., 2002). Cell morphology was determined using light mi-
Scanning electron microscopy after cultivation on YM agar at 28°C for 3 days. The quinone systems were determined by high-performance liquid chromatography (Hitachi La Chrom L-7400) with the column (COSMOSIL 5C18-MS) according to the method of Yamada and Kondô (1973) and Kuraishi et al. (1985).

**DNA base composition and DNA-DNA relatedness.** The yeast DNA for the DNA base composition analysis and DNA-DNA hybridization test was extracted and purified by a modified version of Marmur’s method (1961) from freeze-dried cells mashed in ceramic bowls and purified again by ultra-centrifugation (Hamamoto and Nakase 1995; Nagatsuka et al., 2002). The DNA base composition was determined by high-performance liquid chromatography (Hitachi La Chrom L-7400) according to the method of Tamaoka and Komagata (1984). DNA-DNA hybridization was carried out according to the photobiotin-microplate method of Ezaki et al. (1989). Hybridization was performed at 39°C in a 2× SSC buffer containing 50% (v/v) formamide on an immuno plate (Nunc). The detection after hybridization has been described (Nagatsuka et al., 2002).

**Microsatellite PCR fingerprinting.** The yeast DNA for Microsatellite PCR (MSP-PCR) was prepared using the GenTieKun™ kit (TaKaRa Co.). PCR amplification using the microsatellite primers (GTG)$_5$ and (GAC)$_5$ was performed according to the modified and combined versions of Baleiras Couto et al. (1996) and Gadanho et al. (2002), as follows. Amplification reactions were performed in a volume of 25 μl containing 200 μM dNTPs, 0.2 μM primer, 0.15–0.2 ng of genomic template DNA, 2.5 units of TaKaRa Ex Taq™ DNA polymerase, and 2.5 μl of 10× Ex Taq™ Buffer attached by the company (TaKaRa Co.). The GeneAmp® PCR System 9700 (Perkin Elmer) was programmed for 40 cycles of 15 s at 94°C, 45 s at 55°C for primer (GTG)$_5$, or 45°C for primer (GAC)$_5$, and 90 s at 72°C, and a final extension of 4 min at 72°C. The PCR products amplified with primer (GTG)$_5$ and (GAC)$_5$ were analyzed by electrophoresis in 2.0% agarose gel at 50 V for 4 h and 1.8% at 50 V for 3 h, respectively. The gels were stained with ethidium bromide and photographed in UV light by the Gel Doc 1000 (Bio Rad). Similarities among the isolates were estimated using the Dice coefficient (Dice, 1945), and cluster analysis was performed using the UPGMA (unweighted pair-group method using arithmetic averages) algorithm (Sneath and Sokal, 1973).

**Results and Discussion**

**Phylogenetic analyses based on the 26S rDNA D1/D2 domain and 18S rDNA sequences**

Five hundred and twenty five nucleotides in the 26S rDNA D1/D2 domain of two isolates, K28-3-2 and K26-1-4, were determined. The 26S rDNA D1/D2 sequences of isolates, K28-3-2 and K26-1-4, were found to be identical. The 26S rDNA D1/D2 sequences for isolates, K28-3-2 and K26-1-4, were the closest to those of *P. burtonii* NRRL Y-1933T (U45712) with 97% similarity (10 substitutions and 1 gap), and were the second closest to *C. fennica* NRRL Y-7505T (U45715) with 95% similarity (17 substitutions and 6 gaps). The 26S rDNA D1/D2 sequences of the *P. burtonii* NRRL Y-1933T differed by 16 substitutions and 7 gaps (95% similarity) from that of *C. fennica* NRRL Y-7505T.
four other strains of *P. burtonii*, IFO 0844, IFO 1196, IFO 1986 and IFO 6130, were identical to that of *P. burtonii* NRRL Y-1933\(^T\). Isolates, K28-3-2\(^T\) and K26-1-4, clustered together with *P. burtonii* and *C. fennica* by a robust node with a bootstrap value of 100% in the phylogenetic tree based on the 26S rDNA D1/D2 sequence (Fig. 1). From the phylogenetic analysis based on the 26S rDNA D1/D2 nucleotide sequences, it appeared that two isolates, K28-3-2\(^T\) and K26-1-4, should be assigned as new species (Kurtzman and Robnett, 1998). A total of 1,733 nucleotides in the 18S rDNA of two isolates, K28-3-2\(^T\) and K26-1-4, and *P. burtonii* IFO 10837\(^T\) and 1,732 nucleotides in the 18S rDNA of *C. fennica* IFO 10276\(^T\) were determined. The 18S rDNA sequence of the two isolates, K28-3-2\(^T\) and K26-1-4, were identical, and differed by only 1 substitution from that of the *P. burtonii* IFO 10837\(^T\) and by 6 substitutions and 1 gap from *C. fennica* IFO 10276\(^T\). Those of *P. burtonii* IFO 10837\(^T\) and *C. fennica* IFO 10276\(^T\) differed from each other by 7 substitutions and 1 gap.

Morphological and physiological characteristics

The morphological and growth characteristics of the two isolates were tested in comparison with five strains of *P. burtonii* and the type strain of *C. fennica*. The results of the physiological test regarding *P. burtonii* IFO 10837\(^T\), IFO 0844, IFO 1196, IFO 1986 and IFO 6130, and *C. fennica* IFO 10276\(^T\) in this study are consistent with the assertion in *The Yeasts, A Taxonomic Study* (Kurtzman, 1998), that is to say *P. burtonii* and *C. fennica* closely resemble one another in their phenotypic characteristics. Two isolates, K28-3-2\(^T\) and K26-1-4, were also found to resemble *P. burtonii* and *C. fennica* in their phenotypic characteristics, except for the cation (Li\(^+\))-tolerance property. The growth of two isolates, K28-3-2\(^T\) and K26-1-4, could be detected in YM broth containing 0.5 M LiCl, but not 0.8 M LiCl, in contrast with *P. burtonii* (≤0.8 or 1.0 M) and *C. fennica* (≤0.3 M) (Table 1). Ascospores of isolates, K28-3-2\(^T\) and K26-1-4, were not observed under either condition, either singly cultured or mixed and cultured on 5% malt extract, PDA, or YM agar. All of the morphological and phenotypic characteristics of the two isolates that were studied are listed in the description of *Candida khmerensis* sp. nov.

Ubiquinone system, DNA base composition, and DNA-DNA relatedness

The two isolates and *C. fennica* IFO 10276\(^T\) were found to have Q-8 as the major ubiquinone, the same as *P. burtonii* (Kurtzman, 1998). The G+C contents of K28-3-2\(^T\) and K26-1-4 were 29.9–30.5 mol% and 30.2–30.6 mol%, respectively, which are slightly lower than that of the type strain of *P. burtonii* (32.2–32.7 mol%) and almost the same as that of the type strain of *C. fennica* (30.1–30.6 mol%). The DNA relatedness values are summarized in Table 2. These values indicate that two isolates, K28-3-2\(^T\) and K26-1-4, belong to a single species and can be clearly distinguished from *P. burtonii* and *C. fennica*. Moreover the DNA-DNA hybridization data in this study show that *P. burtonii* and *C. fennica* are clearly distinct from each based on DNA relatedness. Accordingly, we propose that the K28-3-2\(^T\) and K26-1-4 isolates should be considered a new species of the genus *Candida* as *Candida khmerensis* sp. nov.

<table>
<thead>
<tr>
<th>C. khmerensis</th>
<th>LiCl (m)</th>
<th>NaCl (m)</th>
<th>10% NaCl+5% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>K28-3-2(^T)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>K26-1-4</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>P. burtonii</td>
<td>IFO 10837(^T)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IFO 0844</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IFO 1196</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IFO 1986</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IFO 6130</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. fennica</td>
<td>IFO 10276(^T)</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

* Results are scored as: +, with growth; –, no growth.
dida khmerensis sp. nov.

Rapid diagnostic separation of C. khmerensis, P. burtonii, and C. fennica

To provide a rapid diagnostic method for separation of the three close species, C. khmerensis sp. nov., P. burtonii, and C. fennica, MSP-PCR fingerprinting with micro-satellite oligonucleotide primers (GTG)$_5$ and (GAC)$_5$, which have been demonstrated to be appropriate for inter- and intra-species differentiation for ascomycetous yeasts (Baleiras Couto et al., 1996; Capece et al., 2003; Rodrigues and Fonseca, 2003), was carried out. The PCR fingerprints are depicted in Fig. 2, and their respective dendrograms resulting from the numerical analysis of the PCR profiles obtained with the two primers are shown in Fig. 3. The banding profiles of C. khmerensis sp. nov., K28-3-2$^T$ and K26-1-4, are almost identical to each other and are clearly different from those of P. burtonii and C. fennica (Fig. 2 and Fig. 3). The banding profile of P. burtonii showed intra-heterogeneity (Fig. 2), but all of the strains of each single species had similar banding patterns and clustered together in the dendrogram (Fig. 3). The ITS regions with 5.8S rDNA were examined with regard to their usefulness as molecular markers to distinguish the three close species, C. khmerensis, P. burtonii, and C. fennica, from each other. The nucleotide sequences of 5.8S rDNA in C. khmerensis K28-3-2$^T$ and K26-1-4, and C. fennica IFO 10276$^T$ were identical. The nucleotide sequences of the ITS region with 5.8S rDNA in C. khmerensis K28-3-2$^T$ and K26-1-4, were identical but differed by 28 substitutions and 18 gaps.
from that of the P. burtonii IFO 10837T and by 46 substitutions and 25 gaps from C. fennica IFO 10276T. The ITS regions of P. burtonii IFO 0844, and IFO 6130, were found to be nearly identical, differing by 3 substitutions and 3 gaps from P. burtonii IFO 10837T. P. burtonii IFO 1196 and IFO 1986 had several kinds of ITS regions (data not shown). The heterogeneity of P. burtonii was predicted from the nucleotide sequence data for the ITS regions, MSP-PCR fingerprinting, and cation (Li⁺/H₁¹₀₀₁)·tolerant properties. However, the variability of those characteristics was somewhat lower than the differences among those of the three species, C. khmerensis, P. burtonii, and C. fennica. Consequently, C. khmerensis, P. burtonii, and C. fennica can be easily identified by using a primer, (GTG)₅ or (GAC)₅, or by either the 26S rDNA D1/D2 or ITS region with 5.8S rDNA nucleotide sequencing.

**Description**

Latin diagnosis of *Candida khmerensis* sp. nov. Nagatsuka, Kawasaki, Mikata & Seki


Glucosum, sucrosum, maltosum et trehalosum fermentantur, sed non galactosum (infirme aut non) lactosum et raffinosum. Glucosum, galactosum, L-sorbosum (tarde aut occultus), sucrosam, maltosum, cellulobiosum, trehalosum, raffinosum, amyllum solubile, d-xylosum, d-ribosum, N-acetyl-d-glucosaminum, ethanolum, glycerolum, erythritolum, ribitolum, d-mannitolum, d-glucitolum, methyl α-d-glucosidum, salicinum, acidum d-gluconicum, acidum succinicum, et acidum citricum, sed non lactosum, melibiosum, melitzosum (infirme aut non), inulimum, L-arabinosinum, D-arabinosinum, L-rhamnosinum, D-glucosaminum, methanolum, galactitolum, acidum DL-lacticum assimilantur nec inositolum. Kalium nitricum, ethylaminum, L-lysinum (tardus) et cadaverinum, sed non kalium nitracum (infirme) assimilantur. Augmentum in 37°C. Crescens in 10% natrio chlorido/5% glucoso, in 50% glucoso et 0.5M litio chlorido/liquido YM. Non crescens in glucoso nec 0.8 M litio chlorido/liquido YM. Non crescens in medio sine vitamino. Non crescens in 0.01 et 0.1% cycloheximido. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Amyllum non formatur. Systema coenzymatis Q-8 adest. Guaninum et cytosinum acidi deoxyribonucleati 29.9–30.6 mol% (per HPLC).

Typus stirpis K28-3-2² (=JCM 13262²=CBS 9784²) isolatus ex scilla salina desiccata, in Siem Reap Preuf., Cambodia, conservatur in collectionibus culturaris, National Institute of Technology and Evaluation, Japan Collection of Microorganisms (JCM), et The Centraalbureau voor Schimmelcultures (CBS) deposita est.

**Description of Candida khmerensis** Nagatsuka, Kawasaki, Mikata & Seki sp. nov.

*Candida khmerensis* (kh.me.r'en'sis. L. adj.
**Candida khmerensis**, pertaining to Khmer (Cambodia), where the yeast was originally isolated.

Growth in YM medium: after 3 days at 28°C, the cells are spherical to ovoid (2.5–4.4 μm), single or in pairs. Budding is multipolar (Fig. 5-A). Sediment and pericle are formed on the surface of the fermentation media. Growth on YM agar medium: after 3 days at 28°C, the streak culture is white to off-white in color, powdering with a clear mycelial border. Dalmatian plate culture on YM and MA agar: after 3 days at 28°C, true and pseudo-hyphae are formed (Fig. 5-B). Blastoconidia are formed on denticles (Fig. 5-B). Formation of ascospores was not detected.

Glucose, sucrose, maltose, and trehalose are fermented, but galactose (weak or negative), lactose and raffinose are not. Glucose, galactose, L-sorbose (slow or latent), sucrrose, maltose, cellobiose, trehalose, raffinose, soluble starch, D-xylose, D-ribose, N-acetyl-D-glucosamine, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, D-gluconate, succinate, and citrate are assimilated as carbon compounds, but lactose, melibiose, melezitose (weak or negative), inulin, L-arabinose, D-arabinose, L-rhamnose, D-glucosamine, methanol, galactitol, DL-lactate, and inositol are not.

Potassium nitrite, ethylamine, L-lysine (slow), and cadaverine are assimilated as sole nitrogen sources, but potassium nitrate is not. Growth at 37 and 40°C is negative. Growth in 10% NaCl plus 5% glucose in a yeast nitrogen base is positive. Growth on 50% (w/w) glucose-yeast extract agar and in 0.5 M LiCl YM broth is positive. Growth on 60% (w/w) glucose-yeast extract agar and in 0.5 M LiCl YM broth is positive. Growth on 60% (w/w) glucose-yeast extract agar and in 0.5 M LiCl YM broth is positive.

Fig. 4. Variable nucleotide positions within the ITS 1-5.8S-ITS 2 region of *P. burtonii* IFO 10837T, and *C. fennica* IFO 10276T compared to the corresponding sequence of *C. khmerensis* sp. nov., K28-3-2T and K26-1-4 (DDBJ accession no. AB 158650).

Hyphens indicate deletions.
agar or in 0.8 M LiCl YM broth is negative. Growth in vitamin-free medium is positive. No growth occurs in the presence of 0.01 or 0.1% cycloheximide. Urease activity is negative. Diazonium blue B reaction is negative. Production of starch-like substances is negative. The major ubiquinone system is Q-8. The G+C content of the nuclear DNA is 29.9–30.6 mol%, as determined by HPLC. The closest relatives of \textit{C. khmerensis}, based on D1/D2 domain sequences of 26S rDNA, are \textit{P. burtonii} NRRL Y-1933\textsuperscript{T} (U45712) (97% sequence similarity; 11 bases (including caps) different per 525 sites) and \textit{C. fennica} NRRL Y-7505\textsuperscript{T} (U45715) (95% sequence similarity; 23 bases (including gaps) different per 525 sites). The closest relatives of \textit{C. khmerensis}, based on the ITS regions sequences, are \textit{P. burtonii} IFO 10837\textsuperscript{T} (77% sequence similarity; 46 bases different per 201 sites; Fig. 5) and \textit{C. fennica} IFO 10276\textsuperscript{T} (67% sequence similarity; 63 bases different per 191 sites). The MSP-PCR fingerprinting pattern using (GTG)\textsubscript{5} or (GAC)\textsubscript{5} primers of \textit{C. khmerensis} can be distinguished from those of \textit{P. burtonii} or \textit{C. fennica}. The type strain, K28-3-2\textsuperscript{T} (=JCM 13262\textsuperscript{T}=CBS 9784\textsuperscript{T}) and K26-1-4 (=JCM 13263=CBS 9785), were respectively isolated from dry salted shrimp and sewage in a factory where salted-fish used in fish sauce and paste (Prahoc) is produced in Siem Reap province, Cambodia in February of 2002.

Fig. 5. Microscopic illustration of \textit{C. khmerensis} K28-3-2\textsuperscript{T}.  
(A) Vegetative cells in YM broth for 3 days at 28°C. Scale bars, 10 μm. (B) Pseudohyphae and branched short chains of blastoconidia under the coverglass on YM agar for 3 days at 28°C. Scale bars, 20 μm.

Acknowledgments

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