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***Candida kanchanaburiensis* sp. nov., a new ascomycetous yeast species related to *Pichia nakazawae* isolated in Thailand**

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Three yeast strains, ST-633, ST-634 and ST-635, isolated from the fruit body of a mushroom, *Coprinus* sp., and rotted fruit of guava collected in the western region of Thailand, were found to represent a hitherto undescribed species. This yeast is related to *Pichia nakazawae* var. *akitaensis*, *P. nakazawae* var. *nakazawae* and *Pichia philogaea* in the D1/D2 domain of 26S rDNA but 12 (2.3%), 13 (2.5%) and 15 (2.8%) nucleotides are different from these taxa, respectively, suggesting the distinctness of the Thai strains at species level. Since ascospore formation was not detected, it is described as a new species of *Candida*, *Candida kanchanaburiensis*. This species is distinguished from *P. nakazawae* by the ability to assimilate 2-ketogluconic acid and L-lysine, and inability to assimilate soluble starch.

Key Words—*Candida kanchanaburiensis* sp. nov.; new anamorphic yeast; new yeast from Thailand

Introduction

The natural substrates of Thailand have long been considered to be rich in microbial biodiversity though this kind of study has not been carried out until recent years. Since the first report describing new yeast species, *Kockovaella thailandica* and *K. imperatae* from Thailand (Nakase et al., 1991), the reports describing new yeast taxa have increased year to year and 46 new yeast species are currently recognized based on Thai isolates, 24 of basidiomycetous yeasts (Fungsin et al., 2001, 2002a, b, 2003a, b, 2006; Nakase et al., 1991, 2006; Prillinger et al., 1997; Sugita et al., 2003;

Takashima and Nakase, 1998, 2000, 2001; Takashima et al., 1995) and 22 of ascomycetous yeasts (Jindamorakot et al., 2004, 2007; Limtong et al., 2004, 2005, 2007a, b, 2008a, b; Nagatsuka et al., 2002; Nakase et al., 2005, 2007a, b; Sugita et al., 2006; Sumpradit et al., 2005; Suzuki et al., 1994). These studies suggested that a vast number of unknown yeast species are living in the natural environment of Thailand.

In 2003, we isolated three yeast strains from fruit and a fruit body of a mushroom collected in western Thailand. These strains are related to *Pichia nakazawae*, especially *P. nakazawae* var. *akitaensis*, but differed from this variety by 12 nucleotides in the D1/D2 domain of 26S rDNA. Detailed taxonomic study revealed that they represented a hitherto undescribed species of anamorphic genus *Candida*. It is described as a new species, *Candida kanchanaburiensis*, in the present paper.

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Fig.1. Phylogenetic tree for *Candida kanchanaburiensis* sp. nov. constructed by the neighbor-joining method based on the D1/D2 domain of 26S rDNA sequences.

The numerals represent the percentages from 1,000 replicate bootstrap resamplings. Sequences were retrieved from the DDBJ databases under the accession numbers indicated.

Materials and Methods

Yeast strains. The strains ST-633^T (BCC 15316^T=NBRC 104475^T) and ST-634 (BCC 15317=NBRC 104476) were isolated from the fruit body of a mushroom, *Coprinus* sp., and ST-635 (BCC 15318=NBRC 104477) was isolated from rotted fruit of guava, *Psidium guajava* L. These samples were collected in Tong Pha Phum, Kanchanaburi Province, western Thailand, in Nov. 2003, by S. Jindamorakot by direct streaking on YM agar (Difco Lab., Detroit) plates supplemented with 100 ppm chloramphenicol. Immediately after purification by conventional streaking technique, the cultures were suspended in YM broth supplemented with 10% (w/v) glycerol and maintained by freezing at -80°C .

Examination of morphological, physiological, and biochemical characteristics. Most of these characteristics were examined by the methods described in *The Yeasts, a Taxonomic Study*, 4th ed. (Yarrow, 1998). Growth in vitamin-free medium was examined in a basal medium reported by Komagata and Nakase (1967). The maximum growth temperature was determined in YM broth at 1°C intervals using metal block baths.

Ubiquinone system. Ubiquinones were extracted from washed intact cells and purified and identified according to Mikata and Yamada (1999).

DNA base composition. Nuclear DNA was isolated and purified according to Holm et al. (1986). The DNA base composition was determined by HPLC after enzymatic digestion of DNA to deoxyribonucleosides as described by Tamaoka and Komagata (1984) using a DNA-GC Kit (Yamasa Shoyu Co., Ltd., Chiba).

Sequencing and phylogenetic analysis. Genomic DNA was prepared using Dr. GentLE for Yeast (TaKaRa) according to the manufacturer's protocol. The D1/D2 domains of 26S rDNA were amplified using primers NL-1 (5'-GCA TAT CAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTG AAG ACG G-3') (O'Donnell, 1993). The amplification was carried out in a reaction mixture containing TaKaRa Ex Taq (TaKaRa Bio) by a Gene Amp PCR System 9700 (Applied Biosystems).

PCR was performed according to the method previously described (Kurtzman and Robnett, 1998). Purification of PCR products and determination of the sequence of the D1/D2 domain of the 26S rDNA were performed as described by Imanishi et al. (2007). The

sequences determined in this study were deposited in the DDBJ database under the following accession numbers: ST-633^T (=BCC 15316^T=NBRC 104475^T), AB428757; ST-634 (=BCC 15317=NBRC 104476), AB428758; ST-635 (=BCC 15318=NBRC 104477), AB428759. Reference sequences used for the phylogenetic study, shown in Fig. 1, were obtained from the database. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using a CLUSTAL X software package, ver. 1.83 (Thompson et al., 1997). Evolutionary distances were calculated according to Kimura's two-parameter model (1980). Bootstrap values were calculated by computer software based on Felsenstein (1985) from 1,000 random trials.

Results and Discussion

In the phylogenetic tree based on the D1/D2 domain sequences of 26S rDNA, three examined strains, ST-633, ST-634 and ST-635, are located in a clade including several *Pichia* and *Candida* such as *Pichia philogaea* and *Pichia mexicana* reported by Kurtzman and Robnett (1998) and constitute a cluster which con-

nects with a cluster including *Pichia nakazawae* var. *nakazawae*, *Pichia nakazawae* var. *akitaensis* and *Pichia philogaea* though the bootstrap confidence level is not high (Fig. 1). These three strains had identical sequences in this domain and differed from *P. nakazawae* var. *akitaensis*, *P. nakazawae* var. *nakazawae* and *P. philogaea* in the same cluster by, without gaps, 12 (2.3%), 13 (2.5%) and 15 nucleotides (2.8%), respectively. According to a guideline of Kurtzman and Robnett (1998), this difference in the D1/D2 sequences indicates a difference at species level.

In the phenotypic characteristics, these three strains demonstrated essentially the same characteristics. The assimilation of L-sorbose, D-arabinose and D-ribose by these strains is not stable. They often started to grow on these sugars after 3 to 4 weeks and, sometimes, they did not grow even after 4 weeks. However, it is thought that they have an ability to assimilate these carbon compounds though it is much delayed. These strains showed similar physiological characteristics to *P. nakazawae* but clearly differentiated from the latter by the ability to assimilate 2-ketogluconic acid and L-lysine, and inability to assimilate soluble starch.

In liquid media, the strains examined produce sedi-

Fig. 2. Morphology of vegetative cells of *Candida kanchanaburiensis* sp. nov.

A: Vegetative cells in sediment of ST-633^T grown in YM broth for 3 days at 25°C. B: Vegetative cells in pellicle of ST-633^T grown in YM broth for 3 days at 25°C. C: Pseudomycelia produced by ST-633^T on slide culture with cornmeal agar after 4 days at 25°C. D: Pseudomycelia produced by ST-634 on slide culture with cornmeal agar after 4 days at 25°C. E: Pseudomycelia produced by ST-635 on slide culture with cornmeal agar after 4 days at 25°C. Scale bars=10 μm.

ment and pellicle. Cells of the sediment are more or less short bodies and those of the pellicle are more or less elongate bodies showing a tendency to form pseudomycelia (Fig. 2A and 2B). The three strains produce well developed pseudomycelia and bear blastoconidia on media commonly used for yeast cultivation. On slide cultures with cornmeal agar, various types of pseudomycelia are produced, some with a few blastoconidia (Fig. 2C) and others with many of them (Fig. 2D, 2E). Blastoconidia are arranged in chains, often stalagmoidal (Fig. 2D), or in clusters especially inside of the agar media (Fig. 2E).

The three strains did not produce ascospores in respective single cultures on YM agar, cornmeal agar, potato dextrose agar, glutathione-acetate agar or ME sporulation medium of Wickerham or mixed cultures of possible pairs of the three strains.

Based on the results mentioned above, we concluded that the three strains examined represent a new species of the genus *Candida*. It is described as *Candida kanchanaburiensis* sp. nov.

Candida kanchanaburiensis Nakase, Jindamorakot, Ninomiya, Imanishi, Kawasaki & Potacharoen, sp. nov.

In liquido "YM," post dies 3 ad 25°C, cellulae ex sedimento globosae, subovoideae vel ovoideae, 2.3–5.6 × 3.7–7.9 µm, singulae, binae aut catenatae (Fig. 2A), cellulae ex pelliculo ovoideae, longi-ovoideae, ellipsoideae, longi-ellipsoideae, cylindratae vel allantoideae, 2.1–3.7 × 3.7–9.3 µm, singulae, binae aut catenatae (Fig. 2B). Annulus, pellicula incompleta et sedimentum formantur. In agaro "YM," post unum mensem ad 15°C, cultura subflavi-cremea, glabra, non-nitida, mollis, margine erosa. Pseudomycelium formatur (Fig. 2C–2E). Ascosporae non formantur.

Glucosum et galactosum (fortasse exguum) fermentantur at non sucrosum, maltosum, trehalosum, xylosum, methyl- α -D-glucosidum nec cellobiosum. Glucosum, galactosum, L-sorbose (lente vel lente & exiguum), sucrosum, maltosum, cellobiosum, trehalosum, melezitotum, D-xylosum, L-arabinosum, D-arabinosum (lente vel nullum), D-ribosum (lente vel nullum), L-rhamnosum (fortasse lente), D-glucosaminum (tardum), N-acetyl-D-glucosaminum, ethanolum, glycerolum, erythritolum, ribitolum, D-mannitolum, D-glucitolum, xylitolum, L-arabinitolum, methyl- α -D-glucosidum, salicinum, arbutinum, acidum D-gluconicum, acidum succinicum, acidum citricum et propanum-1,2-diolum (lente) assimilantur at

non lactosum, melibiosum, raffinose, inulinum, amylo-solubile, methanolum, galactitolum, glucono- δ -lactonum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum, acidum D-glucuronicum, acidum D-galacturonicum, inositolum, butanum-2,3-diolum nec hexadecanum. Kalium nitricum non assimilatur. Maxima temperatura crescentiae: 35–37°C. Ad crescentiam biotinae et thiaminae necessarium sunt. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 34.2–35.0 mol% (per HPLC). Ubiquinonum majus: Q-9.

Holotypus. BCC 15316 in statu lyophilo ex stirps ST-633, cultura viva ex *Coprinus* sp., Tong Pha Phum, Kanchanaburi, Thailandia isolata et in collectione culturarum in 'BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC),' Pathumthani, Thailandia conservata. Isotypus ut NBRC 104475 in statu lyophilo in collectione culturarum in 'NITE Biological Resource Center (NBRC),' Kisarazu, Chiba, Japonia conservatus.

Growth in YM broth: After 3 days at 25°C, a fragile pellicle and sediment are formed. Cells in the sediment are spheroidal, short ovoidal to ovoidal, ellipsoidal to long ellipsoidal, 2.3–5.6 × 3.7–7.9 µm, occur singly, in pairs or in chains (Fig. 2A). Cells in the pellicle are ovoidal to long ovoidal, ellipsoidal to long ellipsoidal, cylindrical or sausage-shaped, 2.1–3.7 × 3.7–9.3 µm, occur singly, in pairs or in chains (Fig. 2B). Pseudomycelium is often observed. After 1 month at 15°C, a ring, incomplete pellicle and heavy sediment are present.

Growth on YM agar: After 1 month at 15°C, the streak culture is pale yellowish grey, smooth, dull, soft and has an erose margin.

Slide culture: Pseudomycelium is abundantly produced on media commonly used for the cultivation of yeasts. They developed very well. Blastoconidia occur singly or in short chains (Fig. 2C), or in fairly long chains and stalagmoidal (Fig. 2D) or in clusters (Fig. 2E).

Fermentation: Glucose and galactose (may be weak) are fermented. Sucrose, maltose, trehalose, xylose, methyl- α -D-glucoside and cellobiose are not fermented.

Assimilation of carbon compounds:

Glucose	+
Galactose	+
L-Sorbose	+
Sucrose	(latent or latent & weak)

Maltose	+
Cellobiose	+
Trehalose	+
Lactose	–
Melibiose	–
Raffinose	–
Melezitose	+
Inulin	–
Soluble starch	–
D-Xylose	+
L-Arabinose	+
D-Arabinose	+
	(latent) or –
D-Ribose	+
	(latent) or –
L-Rhamnose	+
	(may be latent)
D-Glucosamine	+
	(slow)
N-Acetyl-D-glucosamine	+
Methanol	–
Ethanol	+
Glycerol	+
Erythritol	+
Ribitol	+
Galactitol	–
D-Mannitol	+
D-Glucitol	+
Xylitol	+
L-Arabinitol	+
Methyl- α -D-glucoside	+
Salicin	+
Arbutin	+
Glucono- δ -lactone	–
D-Gluconic acid	+
2-Ketogluconic acid	–
5-Ketogluconic acid	–
DL-Lactic acid	–
Succinic acid	+
Citric acid	+
D-Glucuronic acid	–
D-Galacturonic acid	–
Inositol	–
Propane-1,2-diol	+
	(latent)
Butane-2,3-diol	–
Hexadecane	–
Assimilation of nitrogen compounds:	
Potassium nitrate	–

Sodium nitrite –

L-Lysine +

(latent or weak)

Ethylamine +

Cadaverine +

Vitamins required: Biotin and thiamine.

Production of starch-like substances: Negative.

Growth in 10% NaCl+5% glucose: Variable.

0.1% cycloheximide resistance: Negative.

Maximum growth temperature: 35–37°C.

Liquefaction of gelatin: Negative.

Acid production on chalk agar: Negative.

G+C content of nuclear DNA: 34.2–35.0 (3 strains by HPLC).

Major ubiquinone: Q-9.

Type strain: ST-633, isolated from fruit body of *Coprinus* sp. collected in Tong Pha Phum, Kanchanaburi Pref., western Thailand, in Nov. 2003, is the holotype strain of this species. It was deposited at BIOTEC Culture Collection (BCC), National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand, as BCC 15316. This strain was also deposited at NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kisarazu, Chiba 292-0818, Japan, as NBRC 104475. The strain is maintained by freezing and lyophilization in these culture collections.

Etymology: The specific epithet was derived from “Kanchanaburi” in western Thailand, where samples for isolation of this species were collected.

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