Full Paper

Pichia koratensis sp. nov., a new ascomycetous yeast related to *Pichia acaciae* isolated from insect frass in Thailand

Takashi Nakase,^{1,2,*} Sasitorn Jindamorakot,¹ Kozaburo Mikata,² Shinya Ninomiya,² Hiroko Kawasaki,² Savitree Limtong,³ Wanchern Potacharoen,¹ and Morakot Tanticharoen¹

¹ Central Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC),
National Science and Technology Development Agency (NSTDA),
113 Thailand Science Park, Phaholyothin Road, Pathumthani 12120, Thailand

² NITE Biological Resource Center (NBRC), Department of Biotechnology,
National Institute of Technology and Evaluation, Kisarazu, Chiba 292–0818, Japan

³ Department of Microbiology, Faculty of Science, Kasetsart University,
Bangkok, 10900, Phaholyothin Road, Thailand

(Received August 22, 2007; Accepted October 17, 2007)

Two yeast strains (ST-235 and ST-237) isolated from insect frass collected in northeastern Thailand, were identified as *Pichia acaciae* and a hitherto undescribed species based on the sequence analysis of the D1/D2 domain of 26S rDNA, ITS regions, and DNA-DNA reassociation experiments. The latter strain, ST-237 (=BCC 11769=NBRC 103638=JCM 12576), was described as *Pichia koratensis* sp. nov. *Pichia koratensis* is closely related to *Pichia acaciae* in the D1/D2 domain sequence of 26S rDNA and phenotypical characteristics but differs by 5 nucleotides (0.9%) from the type strain of *P. acaciae* and is clearly distinguished by the assimilation and fermentation of sucrose.

Key Words—drum-shaped ascospores; new yeast from insect frass; new yeast from Thailand; *Pichia koratensis* sp. nov.

Introduction

In the course of a study of yeasts living in the tropical forests of Asia, two strains of ascomycetous yeasts were isolated from insect frass collected in northeastern Thailand. These strains, obtained from the same sample, produced ascospores and were phylogenetically close to *Pichia acaciae*, which was isolated from insect frass in South Africa (van der Walt, 1966). Strain ST-235, producing hat to helmet-shaped as-

cospores, was identified as *P. acaciae* based on sequence analysis of the D1/D2 domain and ITS regions, and DNA-DNA reassociation experiments with *P. acaciae*. The other strain ST-237, producing drum-shaped ascospores, is considered to represent a hitherto undescribed species based on the sequence analysis of the D1/D2 domain of 26S rDNA and ITS regions, and DNA-DNA relatedness. Phenotypic characteristics of this strain resemble *P. acaciae* as well but are clearly discriminated from it. It is described here as a new species of *Pichia*.

Materials and Methods

Yeast strains. The strains ST-235 and ST-237 were isolated from insect frass collected in Nong Kratone, Nakhonratchasima Province, northeastern Thai-

*Address reprint requests to: Dr. Takashi Nakase, NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, 2–5–8 Kazusakamatari, Kisarazu, Chiba 292–0818, Japan.

Tel: +81–438–20–5763 Fax: +81–438–20–5766

E-mail: nakase-takashi@nite.go.jp

land, in February 2001, by direct streaking on YM agar (Difco Lab., Detroit) plates supplemented with 100 ppm chloramphenicol. Names of the tree and infested insect could not be identified. Immediately after purification by conventional streaking technique, the cultures were suspended in YM broth supplemented with 10% (w/v) glycerol and maintained at -80°C .

Examination of morphological, physiological, and biochemical characteristics. Most of the morphological, physiological, and biochemical characteristics were examined by the methods described by Yarrow (1998). The assimilation of nitrogen compounds was investigated on solid media using starved inocula. Vitamin requirements were determined according to the method of Komagata and Nakase (1967). The maximum growth temperature was determined in YM broth using metal block baths.

Ubiquinone system. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml of YM broth on a rotary shaker at 150 rpm at 25°C and were harvested by centrifugation in early stationary growth phase. The cells were washed three times with distilled water. Ubiquinones were extracted, purified and identified according to Nakase and Suzuki (1986).

Isolation and purification of nuclear DNA. Nuclear DNA was isolated and purified according to Nakase and Suzuki (1985) or Holm et al. (1986).

DNA base composition. 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).

DNA-DNA homology. DNA-DNA homology was examined by the reassociation method using the photobiotin microplate-hybridization technique according to Kaneko and Banno (1991). Hybridization was performed at 40°C in $2\times$ SSC buffer containing 50% (v/v) formamide on a microplate (Immunoplate, Nunc).

Sequencing and phylogenetic analysis. The nucleotide sequences of the D1/D2 domain of 26S rDNA were directly determined according to the method reported by Kurtzman and Robnett (1998), as described in a previous paper (Fungsin et al., 2002). The sequences determined in this study were deposited in the DDBJ database under the following accession numbers: ST-235=BCC 11767=NBRC 103639, D1/D2 domain of 26S rDNA (AB354210), ITS regions including 5.8S rDNA (AB354211); ST-237=BCC 11769=NBRC 103638 =JCM 12576, D1/D2 domain of 26S

rDNA (DQ404484), ITS regions including 5.8S rDNA (AB354209); *Pichia acaciae* NBRC 1681^T, D1/D2 domain of 26S rDNA (AB354212), ITS regions including 5.8S rDNA (AB354213). The sequences were aligned with related species by CLUSTAL X ver. 1.8 (Thompson et al., 1997).

The phylogenetic tree was constructed from the evolutionary distance data of Kimura (1980) using the neighbor-joining method (Saitou and Nei, 1987). Sites where any gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were done based on 1,000 random resamplings.

Examination of ascospore morphology by scanning electron microscope. After confirmation of sufficient production of ascospores using a light microscope, the ascosporeogenous cells were treated with an enzyme mixture of 1 mg of Zymolyase 100T (Seikagaku Kogyo Co.) for 2 h at 37°C to digest ascus walls, and washed twice with 0.1 M phosphate buffer (pH 7.2) by centrifugation. The free ascospores were then fixed with 2% glutaraldehyde in phosphate buffer for 2 h at 4°C and 1% osmic acid in phosphate buffer. The fixed samples were dehydrated by passing through a graded acetone concentration series of 30, 50, 60, 70, 80, 90, 95, and 100% at 15-min intervals, and then were transferred to isoamylacetate for more than 2 h. The dehydrated samples of ascospores were mounted on a small disk of cover glass and subjected to critical point drying in an HCP-2 (Hitachi Ltd.). The glass disk with the dried ascospores was coated with platinum at 10 mA for 3.5 min in vacuo (4 Pa) using ion sputtering apparatus JUS-5000 (JEOL Ltd.). The preparations were examined under a scanning electron microscope JSM-5400 (JEOL Ltd.) at a voltage of 15 kV.

Results and Discussion

In the phylogenetic tree based on the D1/D2 domain sequences of 26S rDNA, strains ST-235 and ST-237 constituted a cluster with *Pichia* (*Yamadazyma*) *acaciae* with high bootstrap values, and connected with a cluster comprising *Pichia* (*Yamadazyma*) *farinosa* and *Candida cacaui* (Fig. 1).

The strain ST-235 differed by 1 nucleotide from *P. acaciae*. However, ST-237 differed by 5 nucleotides (0.9%) from *P. acaciae*, the nearest species in D1/D2 sequences, and by 6 nucleotides (1.1%) from ST-235 and was presumed to represent a species distinct from *P. acaciae* according to the guidelines of Kurtzman

Fig. 1. Phylogenetic tree for *Pichia koratensis* ST-237^T 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.

Table 1. DNA relatedness among ST-235, ST-237 and *Pichia acaciae* NBRC 1681^T.

Source of unlabeled DNA		Mol% G+C	Relative DNA binding of labeled DNA from (%)		
			ST-237	ST-235	NBRC 1681 ^T
<i>Pichia</i> sp.	ST-237	40.7	100	51	45
<i>Pichia</i> sp.	ST-235	39.6	42	100	91
<i>P. acaciae</i>	NBRC 1681 ^T	41.2	43	109	100
<i>S. cerevisiae</i>	NBRC 10217 ^T	37.8	16	19	26

and Robnett (1998). In the ITS regions including 5.8S rDNA, ST-235 showed an identical sequence with the type strain of *P. acaciae* while ST-237 differed by 20 nucleotides (3.1%) in this region. The sequence analyses mentioned above strongly presumed the conspecificity of ST-235 with *P. acaciae* and distinctness of ST-237 from this species. In order to confirm these presumptions, we carried out DNA-DNA reassociation experiments with the type strain of *P. acaciae* and ST-235 and ST-237 (Table 1). High DNA similarity values of 91–109% found between *P. acaciae* NBRC 1681^T and ST-235 clearly indicated the conspecificity of these strains as suggested by the sequence analyses mentioned above, and the low similarity values of 43–45% and 42–51% found between ST-237 and *P. acaciae* NBRC 1681^T, and between ST-237 and ST-235, respectively, clearly indicated the difference between ST-237 and *P. acaciae* at the species level. It is concluded that strain ST-237 represents a new species closely related to *P. acaciae*, and that ST-235 is a strain of *P. acaciae*.

In the phenotypic characteristics, ST-237 resembles

P. acaciae as well but was clearly differentiated from this species by the assimilation and fermentation of sucrose and the growth in the vitamin-deficient medium.

In various growth media commonly employed for yeasts, such as YM agar, vegetative cells of ST-237 were transformed to the asci after heterogamic conjugation. The ascus contains 1–4, usually 2–4, ascospores which are not liberated from the ascus at maturation. Under the light microscope, these ascospores look spheroidal or oblate spheroidal, and sometimes have unclear brims (Fig. 3A–E). Some ascospores look drum-shaped (Fig. 3A, arrows). Under the scanning electron microscope, the ascospores are apparently drum-shaped (Fig. 3F). *Pichia acaciae*, the nearest species, produced ascospores usually after heterogamic conjugation as well as ST-237 but the shape of the ascospores is hat-shaped with short brims, and the mature ascospores are rapidly released from the ascus and tend to agglutinate (van der Walt, 1966). ST-237 possessed Q-9 as the major component of ubiquinones, like *P. acaciae*. Billon-Grand (1989) divided species of the genus *Pichia* into three

Fig. 2. Morphology of vegetative cells of *Pichia koratensis* ST-237^T.

A, B: Vegetative cells grown in YM broth for 3 days at 25°C. C: Pseudomycelia produced on corn meal agar after 4 days at 25°C. Scale bars=10 µm.

groups based on the type of coenzyme Q (ubiquinone) and transferred all Q-9 having species producing hat-shaped ascospores to the newly proposed genus *Yamadazyma*. However, Kurtzman and Robnett (1998) found that species assigned to *Yamadazyma* were placed in several clades, all of which are characterized by coenzyme Q-9. Based on this finding, Kurtzman (1998) did not accept the genus *Yamadazyma* and retained all of the species of this genus in the genus *Pichia*. We think that the ubiquinone type is one of the important taxonomic criteria to define the genus, and that species within a genus should contain the same major ubiquinone. Therefore, Q-9-possessing species should be excluded from the genus *Pichia* in the future because *Pichia membranifaciens*, type species of the genus *Pichia*, have Q-7. At present, however, the genus *Yamadazyma* is not well-defined, as pointed out by Kurtzman (1998). Therefore, we decided to place ST-237 in the genus *Pichia* and describe it as *Pichia koratensis*.

Pichia koratensis Nakase, Jindamorakot, Mikata, Ni-nomiya, Kawasaki & Limtong, sp. nov.

In liquido "YM," post dies 3 ad 25°C, cellulae globosae, subovoideae vel ovoideae, 2.5–5×2.5–8 µm, singulae, binae aut catenatae (Fig. 2A). Pseudomycelium formatur. Cellulae in pseudomycelium allantoideum et elongatae (Fig. 2B). Annulus, pellicula in-

completa et fragile, et sedimentum formantur. In agaro "YM," post unum mensem ad 20°C, brunneola vel brunneo-grisea pallida, rugulosa, impolita, mollis vel butyracea, margine erosa. Pseudomycelium formatur (Fig. 2C). Asci formantur post conjugationem heterogametam. Ascosporae tympani-formes, 1–4 in asco, 1.2–3.1×1.8–3.1 µm (Fig. 3). Glucosum, sucrosum et maltosum fermentantur at non galactosum nec lactosum. Glucosum, galactosum, L-sorbosum (fortasse lente), sucrosum, maltosum, cellobiosum, trehalosum, lactosum (lente), amyllum solubile, D-xylosum, D-ribosum, D-glucosaminum, N-acetyl-D-glucosaminum, ethanolum, glycerolum, erythritolum, ribitolum, D-mannitolum, D-glucitolum, xylitolum, L-arabinitolum, α-methyl-D-glucosidum, salicinum, glucono-δ-lactonum (lente), acidum D-gluconicum, acidum succinicum, acidum citricum, propanum-1,2-diolum (lente), butanum-2,3-diolum et hexadecanum (lente et exiguum) assimilantur at non melibiosum, raffinolum, melezitolum, inulinum, L-arabinosum, D-arabinosum, L-rhamnosum, methanolum, galactitolum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum, acidum saccharicum, acidum D-glucuronicum, acidum D-galacturonicum nec inositolum. Kalium nitricum non assimilatur. Maxima temperatura crescentiae: 43–44°C. Ad crescentiam vitaminum non necessarium est. Proportio molaris guanini+cytosini in acido deoxyribonucleico: 40.7 mol% (per HPLC). Ubiquinonum

Fig. 3. Morphology of ascospores of *Pichia koratensis* ST-237^T.

A–E: Light microscopic pictures of ascospores produced on YM agar after 8 days at 25°C. F: Scanning electron microscopical picture of ascospores. Scale bars=5 μm.

majus: Q-9.

Holotypus: BCC 11769 in statu lyophilo ex stirpis ST-237, cultura viva ex ligno pulvereo ab insecto efferenti, Nong Kratone, Nakhonratchasima, 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 103638 in statu lyophilo in collectione culturarum in 'NITE Biological Resource Center (NBRC),' Kisarazu, Chiba, Japonia, et ut JCM 12576 in statu lyophilo in collectione culturarum in 'Japan Collection of Microorganisms (JCM),' RIKEN, Wako, Saitama, Japonia conservatus.

Growth in YM broth: After 3 days at 25°C, cells are spheroidal, short ovoidal, ovoidal or long ovoidal, single, in pairs, in chains or in clusters, 2.5–5×2.5–8 μm (Fig. 2A). Pseudomycelium is often observed. Pseudomycelial cells are sausage-shaped or cylindrical, often elongated (Fig. 2B). A ring, sediment and an incomplete fragile pellicle, which easily falls down to the bottom, are produced. After 1 month at 20°C, a ring, incomplete pellicle and heavy sediment are present.

Growth on YM agar: After 1 month at 20°C, the

streak culture is pale brown to pale brownish-grey, finely wrinkled especially near the bottom of the slant, dull, soft to butyrous and has an erose margin.

Slide culture on corn meal agar: After 4 days at 25°C, the pseudomycelium is abundantly produced. They are well-developed (Fig. 2C).

Ascospore formation: The ascus is produced after heterogamic conjugation. Under the light microscope, ascospores look spheroidal or oblate spheroidal, and sometimes have unclear brims, some ascospores look drum-shaped, 1–4, usually 2–4 per ascus and measure 1.2–3.1×1.8–3.1 μm (Fig. 3A–E). Under scanning electron microscope, ascospores are proved to be drum-shaped (Fig. 3F).

Fermentation: Glucose, sucrose and maltose are fermented. Galactose and lactose are not fermented.

Assimilation of carbon compounds:

Glucose	+
Galactose	+
L-Sorbose	+ (may be latent)
Sucrose	+
Maltose	+
Cellobiose	+
Trehalose	+

Lactose	+ (latent)
Melibiose	—
Raffinose	—
Melezitose	—
Inulin	—
Soluble starch	+
D-Xylose	+
L-Arabinose	—
D-Arabinose	—
D-Ribose	+
L-Rhamnose	—
D-Glucosamine	+
N-Acetyl-D-glucosamine	+
Methanol	—
Ethanol	+
Glycerol	+
Erythritol	+
Ribitol	+
Galactitol	—
D-Mannitol	+
D-Glucitol	+
Xylitol	+
L-Arabinitol	+
α-Methyl-D-glucoside	+
Salicin	+
Glucono-δ-lactone	+ (latent)
D-Gluconic acid	+
2-Ketogluconic acid	—
5-Ketogluconic acid	—
DL-Lactic acid	—
Succinic acid	+
Citric acid	+
Saccharic acid	—
D-Glucuronic acid	—
D-Galacturonic acid	—
Inositol	—
Propane-1,2-diol	+ (latent)
Butane-2,3-diol	+
Hexadecane	+ (latent and weak)
Assimilation of nitrogen compounds:	
Potassium nitrate	—
Sodium nitrite	—
Ethylamine	+
L-Lysine	+
Cadaverine	+
Vitamins required: None.	
Production of starch-like substances: Negative.	
Growth in 10% NaCl+5% glucose: Positive.	
0.1% Cycloheximide resistance: Negative.	

Maximum growth temperature: 43–44°C.

Liquefaction of gelatin: Positive (latent and weak).

Acid production on chalk agar: Positive (weak).

Diazonium blue B color reaction: Negative.

Urease: Negative.

G+C content of nuclear DNA: 40.7 mol% (by HPLC).

Major ubiquinone: Q-9.

Type strain: ST-237, isolated from insect frass collected in Nong Kratone, Nakhonratchasima, Thailand, in Feb. 2001, 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 11769. This strain was also deposited at NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kisarazu, Chiba 292–0818, Japan, as NBRC 103638 and at Japan Collection of Microorganisms (JCM), RIKEN, Wako, Saitama 351–0198, Japan, as JCM 12576. The strain is maintained by freezing and/or lyophilization in these culture collections.

Etymology: The specific epithet was derived from “Korat,” a common name of Nakhonratchasima, where a sample of insect frass containing *Pichia koratensis* was collected.

The strain *P. acaciae* ST-235 is maintained in BCC and NBRC as BCC 11767 and NBRC 103639, respectively.

Acknowledgments

We thank Dr. C. P. Kurtzman, Agricultural Research Service, US Department of Agriculture, for his suggestion on the terminology of the shape of ascospores and for reviewing the manuscript, and Ms. Hathairat Jan-ngam and Ms. Somjit Am-in, BIOTEC, for their help on several experiments.

References

- Billon-Grand, G. (1989) A new ascosporogenous yeast genus: *Yamadazyma* gen. nov. *Mycotaxon*, **35**, 201–204.
- Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.
- Fungsin, B., Takashima, M., Artjariyasripong, S., Arunpairojana, V., Hamamoto, M., and Nakase, T. (2002) *Bullera arundinariae* sp. nov., a new species of ballistoconidium-forming yeast, isolated from a plant in Thailand. *Microbiol. Cult. Coll.*, **18**, 83–90.
- Holm, C., Meeks-Wagner, D. W., Fangman, W. L., and Botstein, D. (1986) A rapid, efficient method for isolating DNA from

- yeast. *Gene*, **42**, 169–173.
- Kaneko, Y. and Banno, I. (1991) Reexamination of *Saccharomyces bayanus* strains by DNA-DNA hybridization and electrophoretic karyotyping. *IFO Res. Commun.*, **15**, 30–41.
- Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**, 111–120.
- Komagata, K. and Nakase, T. (1967) Microbiological studies on frozen foods. V. General properties of yeasts isolated from frozen foods. *J. Food Hyg. Soc. Jpn.*, **8**, 53–57.
- Kurtzman, C. P. (1998) *Pichia* E. C. Hansen emend. Kurtzman. In *The Yeasts, A Taxonomic Study*, 4th ed., ed. by Kurtzman, C. P. and Fell, J. W., Elsevier, Amsterdam, pp. 273–352.
- Kurtzman, C. P. and 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.
- Nakase, T. and Suzuki, M. (1985) Taxonomic studies on *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij and related species. I. Chemotaxonomic investigations. *J. Gen. Appl. Microbiol.*, **31**, 49–69.
- Nakase, T. and Suzuki, M. (1986) The ubiquinone system in strains of species in the ballistospore-forming yeast genera *Sporidiobolus*, *Sporobolomyces* and *Bullera*. *J. Gen. Appl. Microbiol.*, **32**, 251–258.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
- Tamaoka, M. and Komagata, K. (1984) Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Lett.*, **25**, 125–128.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **24**, 4876–4882.
- van der Walt, J. P. (1966) *Pichia acaciae* sp. n. *Antonie van Leeuwenhoek*, **32**, 159–161.
- Yarrow, D. (1998) Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, A Taxonomic Study*, 4th ed., ed. by Kurtzman, C. P. and Fell, J. W., Elsevier, Amsterdam, pp. 77–100.