Candida thaimueangensis sp. nov., an anamorphic yeast species from estuarine water in a mangrove forest in Thailand

Savitree Limtong,¹ Wichien Yongmanitchai,¹ Hiroko Kawasaki² and Tatsuji Seki²

Correspondence
Savitree Limtong
fscistl@ku.ac.th

¹Department of Microbiology, Faculty of Science, Kasetsart University, 50 Paholyothin Rd, Bangkok 10900, Thailand

²The International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita-City, Osaka 565-0871, Japan

Four yeast strains (TM1-01^T, TM1-07, TM3-47 and TM3-49) were isolated by membrane filtration from estuarine water collected from a mangrove forest in Phang-Nga province, southern Thailand. Analysis of the D1/D2 domains of the large-subunit rDNA sequence revealed that the sequences of the four strains were identical. The closest recognized species in terms of pairwise sequence similarity was *Pichia deserticola*, but the level of nucleotide substitution (4.8 %) was sufficient to justify the description of a separate species. Phylogenetic analysis demonstrated that the four strains occupy a basal position with respect to *Pichia membranifaciens* and close relatives. The four strains showed identical phenotypic characteristics, including proliferation by multilateral budding, absence of ascospores, arthrospores and ballistospores and negative reactions for Diazonium blue B and urease. The major ubiquinone was Q-7. On the basis of the above findings, these four strains were assigned to a single novel species of the genus *Candida*, for which the name *Candida thaimueangensis* sp. nov. is proposed. The type strain is TM1-01^T (= CBS 10360^T = NBRC 101967^T = BCC 21229^T).

Yeasts of the genus *Candida*, one of the largest genera in terms of numbers of species, are widely distributed in nature. Species of this genus have been isolated from various sources in terrestrial and aquatic habitats (Butinar *et al.*, 2005; de Almeida, 2005; Pimentel *et al.*, 2005; Rhishipal & Philip, 1998; Ruivo *et al.*, 2004; Soares *et al.*, 1997; Suh *et al.*, 2004). In Thailand, both basidiomycetous and ascomycetous yeasts are rich in species diversity (Nakase, 2000; Nakase *et al.*, 2001; Jindamorakot *et al.*, 2004). At the time of writing, 10 novel ascomycetous yeast species have been described from Thailand (Nagatsuka *et al.*, 2002; Nakase *et al.*, 2005; Jindamorakot *et al.*, 2004; Limtong *et al.*, 2004, 2005; Sumpradit *et al.*, 2005; Suzuki *et al.*, 1994). Among these, six species belong to the genus *Candida*: *Candida stellimalicola*, isolated from star apple (Suzuki *et al.*, 1994), *C. easanensis*, *C. pattaniensis* and *C. nakhonratchasimensis*, isolated from insect frass (Jindamorakot *et al.*, 2004), and *C. krabiensis* and *C. sithepensis*, isolated from soil (Limtong *et al.*, 2004). Until now there has been no description of novel yeast species isolated from water in a natural ecosystem owing to the lack of studies of yeast diversity in aquatic environments in Thailand.

In the course of an investigation of yeasts in aquatic habitats in Thailand, including estuarine water in mangrove forests and seawater, various strains of yeasts were isolated by membrane filtration. In this study we describe four strains obtained from estuarine water in a mangrove forest as representing a novel species of the genus *Candida*.

Four yeast strains, TM1-01^T, TM1-07, TM3-47 and TM3-49, were isolated by membrane filtration of water samples collected from a mangrove forest in Khao Lumpee-Haad Thaimueang National Park, Amphoe Thaimueang, Phang-Nga province, Thailand. Fifty to two hundred millilitres of water was filtered through 0.8-µm pore size membrane filters, which were placed on acidified yeast extract/malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose, adjusted to pH 3.7–3.8 with 1 M HCl) supplemented with 0.025 % sodium propionate and 200 mg chloramphenicol l⁻¹, and incubated for 2–3 days at room temperature. Yeast colonies were picked and purified by cross streaking on YM agar.

Methods for DNA isolation, amplification of the D1/D2 domains of the large-subunit (LSU) and small-subunit (SSU) rDNA by PCR and sequencing with the ABI BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) by using an ABI PRISM 3100 automated DNA

The GenBank/EMBL/DDBJ accession number for the D1/D2 domain of the LSU rDNA sequence of TM1-01^T is AB264009.

sequencer (Applied Biosystems) were as described previously (Limtong *et al.*, 2007). The sequences were compared pairwise by BLAST searches (Altschul *et al.*, 1990) and were aligned with the sequences of related species retrieved from GenBank by using the multiple alignment program CLUSTAL X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the phylogenetic tree was estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). The new strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula following the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation on yeast extract/peptone/dextrose (YPD) agar (1 % yeast extract, 2 % peptone, 2 % glucose and 2 % agar). Ubiquinones were extracted from intact packed cells cultivated in YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). The isoprenologues were identified by HPLC by using a Cosmosil (Waters 5C18) 4.6 × 250-mm column and methanol/2-propanol (2:1) at 1 ml min⁻¹ as the elution system with spectrophotometric detection (wavelength 275 nm).

The sequences of the D1/D2 domains of the LSU rDNA of the four strains were identical. The closest species to the four strains in terms of pairwise sequence similarity was *Pichia deserticola*, but with 4.8 % nucleotide substitutions (27 nucleotide substitutions in 558 nt). According to Kurtzman & Robnett (1998), yeast strains showing nucleotide substitutions of greater than 1 % in the D1/D2 domain of the LSU rDNA are usually representative of different species. The phylogenetic tree based on the sequences of the D1/D2 domains of the LSU rDNA further demonstrated that the four strains are placed at a position distinct from

P. deserticola and other related species in the *Pichia membranifaciens* clade (Fig. 1). The SSU rDNA sequences of the four new strains were also identical (data not shown). Accordingly, the four new strains are considered to represent a single, novel, phylogenetically distinct species.

The four strains proliferated by multilateral budding (Fig. 2), were negative for Diazonium blue B and urease reactions and had Q-7 as the major ubiquinone, as do other members of the *P. membranifaciens* clade. They did not produce ascospores on YM agar, 5 % malt extract agar, Fowell's acetate agar or Gorodkova agar after 5 weeks at 28 °C, and on this basis are assigned to the genus *Candida*. The name *Candida thaimueangensis* sp. nov. is proposed to accommodate these new strains.

In practice, *Candida thaimueangensis* can be distinguished from *P. deserticola*, its closest phylogenetic relative, only on the basis of sequence comparisons, as differences in phenotypic characteristics are minor (Table 1).

Latin diagnosis of *Candida thaimueangensis* Limtong, Yongmanitchai, Kawasaki et Seki sp. nov.

In agaro YM post dies 3 ad 28 °C cellulae globosae, ellipsoideae aut elongatae (2.2–5.2 × 2.6–6.5 µm), singulae aut binae, per gemmationem multipolarem reproducentes. Cultura butyrosa, cremea, seminitidae, glabra, margine glabra. In agaro farinae Zea mays post dies 7 ad 28 °C et agaro YM post dies 21 ad 25 °C pseudohyphae formantur, hyphae non formantur. Ascosporae non formantur. In medio liquido YM post dies 3 ad 28 °C, repens pellicula formatur. Fermentatio nulla. Glucosum, D-xylosum (lente), glycerolum, acidum DL-lacticum, acidum succinicum et ethanolum assimilantur at non D-galactosum, L-sorbosum, D-ribosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosus, maltosum, trehalosum, cellobiosum, melibiosum, lactosum, raffinose, melezitose, inulinum, erythritolum, D-mannitolum, galactitolum,

Fig. 1. Phylogenetic tree based on the D1/D2 domains of the LSU rDNA, showing positions of the four new strains with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), by using the neighbour-joining method. Numbers indicate percentages of bootstrap support, derived from 1000 samples.

Fig. 2. Cells of strain TM1-01^T. (a) Vegetative cells grown on YM agar after 3 days at 28 °C and (b) pseudohyphae produced on cornmeal agar after 7 days at 28 °C. Bars, 10 µm.

inositolum, glucono-δ-lactonum, acidum 2-ketogluconicum, acidum D-gluconicum, acidum D-glucuronicum, acidum D-galacturonicum, acidum citricum nec methanolum. Ethylaminum, L-lysinum et cadaverinum assimilatur at non nitrium nitricum nec nitrium nitrosum. Ad crescentiam vitamina externa non necessaria. Crescit in temperatura 37 °C at non in 40 °C. Crescit (exigue) in 50 % glucosum. Non crescit in 0.01 % cycloheximido, 60 % glucosum nec 10 % natrii chloridum/5 % glucosum. Amylum non formatur. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Ubiquinonum majus: Q-7.

Typus stirpis TM1-01^T (= CBS 10360^T = NBRC 101967^T = BCC 21229^T) *isolatus aqua*, Phang-Nga Provincia, Thailandia, conservatur in collectionibus culturarum quas Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), NITE Biological Resource Center (Chiba, Japan) et BIOTEC Culture Collection, National Central for Genetic Engineering and Biotechnology, Thailand (Pathumthani, Thailand) *deposita est*.

Description of *Candida thaimueangensis* Limtong, Yongmanitchai, Kawasaki & Seki sp. nov.

Candida thaimueangensis (thai.mueang.en'sis. N.L. fem. adj. *thaimueangensis* referring to Thaimueang, Thailand, where the four strains were isolated).

After growth on YM agar for 3 days at 28 °C, cells are spheroidal, ellipsoidal to elongate (2.2–5.2 × 2.6–6.5 µm) and occur singly, in pairs or in short chains (Fig. 2). Colonies are butyrous, cream-coloured, semi-glistening and raised, with a smooth surface and have an entire margin. Budding is multilateral. After 7 days in Dalmau plate cultures on cornmeal agar at 28 °C and YM agar at 25 °C, pseudohyphae are formed but true hyphae are not formed. No arthrospores are produced. No ascospores are produced from individual strains on YM agar, 5 % malt extract agar, Fowell's acetate agar or Gorodkova agar after 5 weeks at

28 °C. In YM broth, after 3 days at 28 °C, creeping pellicles and floating islets are present. A pellicle is not present during growth on the surface of assimilation medium. The major ubiquinone is Q-7. Phenotypic characteristics of the species are given in Table 1.

Table 1. Differential phenotypic characteristics between strain TM1-01^T and *Pichia deserticola*

Strains TM1-01^T, TM1-07, TM3-47 and TM3-49 gave identical results. All strains were negative for fermentation. D-Glucose, glycerol, ethanol, ethylamine hydrochloride, L-lysine hydrochloride and cadaverine were assimilated by all strains, but D-galactose, L-sorbose, L-rhamnose, D-ribose, L-arabinose, D-arabinose, sucrose, maltose, trehalose, cellobiose, melibiose, lactose, raffinose, melezitose, inulin, erythritol, mannitol, galactitol, *myo*-inositol, 2-keto-gluconic acid, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, citric acid, methanol, potassium nitrate and sodium nitrite were not. All strains grew at 25, 30, 35 and 37 °C. Growth in vitamin-free medium was negative. Growth on medium containing 0.01 % cycloheximide, 60 % glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose was negative in all cases. Production of amyloid compound was negative in all cases. Diazonium blue B and urease reactions were negative in all cases. Symbols: +, positive; – negative; D, delayed positive; S, slow; W, weak.

Characteristic	Strain TM1-01 ^T	<i>P. deserticola</i>
Ascospore formation	–	+
Assimilation of:		
D-Xylose	S	–
D-Glucono-1,5-lactone	–	D, –
DL-Lactate	+	+, W
Succinic acid	+	W, –
Growth:		
At 40 °C	–	+, –
With 50 % glucose	W	+, D

The type strain, TM1-01^T (=CBS 10360^T=NBRC 101967^T=BCC 21229^T), was isolated from water in a mangrove forest in Kho Lumpee-Haad Thaimueang National Park, Phang-Nga province, Thailand.

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