***Bensingtonia thailandica* sp. nov., a novel basidiomycetous yeast species isolated from plant leaves in Thailand**

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Ten strains which were characterized by the formation of ballistoconidia, the absence of xylose in whole-cell hydrolysates, the presence of Q-9 as the major ubiquinone isoprenologue, the inability to ferment sugars and positive diazonium blue B and urease reactions were isolated from plant samples collected in Thailand. These isolates were closely related to *Bensingtonia phyllada* based on the analysis of 18S rDNA sequences. On the basis of the morphological, physiological and chemotaxonomic properties, the 10 isolates were assigned to the genus *Bensingtonia*. DNA complementarity showed that these isolates were genetically distinct from known species of the genus *Bensingtonia*. The isolates are described as *Bensingtonia thailandica* sp. nov. The type strain is strain TY-138^T (= JCM 10651^T).

Keywords: *Bensingtonia thailandica* sp. nov., ballistoconidium-forming yeasts, basidiomycetous yeasts, 18S rDNA

INTRODUCTION

The genus *Bensingtonia* was established by Ingold based on a single species, *Bensingtonia ciliata* (Ingold, 1986). Later, the description of this genus was emended by Nakase & Boekhout (1988). The genus is characterized by the formation of ballistoconidia, the presence of Q-9 as the major ubiquinone isoprenologue and the absence of xylose in whole-cell hydrolysates (Boekhout & Nakase, 1998). Ten species are currently known in this genus. The phylogenetic analysis based on 18S rDNA sequences revealed that *Bensingtonia* species were mainly divided into two groups, the Sporidiales group and the *Agaricostilbum*/*Bensingtonia* group, within the Urediniomycetes (Takashima *et al.*, 1995a; Sugita *et al.*, 1997; Hamamoto & Nakase, 2000).

In the course of a survey of yeasts living in the natural environment in Thailand, 10 strains of undescribed ballistoconidium-forming yeasts were isolated from plant leaves which were collected in a tropical rain forest in Thailand. The identification of these isolates

and the description of a new species in the genus *Bensingtonia*, *Bensingtonia thailandica* sp. nov., are reported here.

METHODS

Isolation. Plant samples were collected in a tropical rain forest of Sakearat Environmental Research Station in Nakhon Ratchasima Province, which is the north-eastern region of Thailand, in November 1996. The procedure used for yeast isolation and purification has been described previously (Nakase & Suzuki, 1985; Nakase & Takashima, 1993). Ten strains isolated from plant samples (Table 1) were investigated. *Bensingtonia ciliata* JCM 6865^T, *Bensingtonia miscanthi* JCM 5733^T, *Bensingtonia phyllada* JCM 7476^T, *Bensingtonia subrosea* JCM 5735^T and *Bensingtonia yuccicola* JCM 6251^T were used for the comparative study. All strains were grown at 17 or 25 °C in yeast extract/malt extract broth or on yeast extract/malt extract agar (Difco) for purification and cultivation.

Morphological, physiological and biochemical characteristics. The isolates were characterized morphologically, physiologically and biochemically by using the method currently used in yeast taxonomy (Yarrow, 1998). Assimilation of nitrogen compounds was examined on solid media with starved inoculum, as described by Nakase & Suzuki (1986). The vitamin-free medium reported by Komagata & Nakase (1967) was used for the vitamin requirement test. The medium consists of 1 % glucose, 0.5 % vitamin-free Cas-

The DDBJ accession numbers for the 18S rDNA sequences of *Bensingtonia thailandica* strains TY-138^T, TY-188, TY-191 and TY-223 are AB040114, AB040115, AB040116 and AB040117, respectively.

Table 1. List of the isolates used in this study

Isolate	Accession no.*		Source†
	JCM	TISTR	
TY-138 ^T	10651 ^T	5733 ^T	<i>Cratoxylum maingayi</i>
TY-173	10652	5734	<i>Vitis vinifera</i>
TY-180	10653	5735	<i>Xylia xylocarpa</i>
TY-181	10654	5736	<i>Xylia xylocarpa</i>
TY-188	10655	5737	<i>Seteria pallide-fusca</i>
TY-190	10656	5738	<i>Seteria pallide-fusca</i>
TY-191	10657	5739	<i>Seteria pallide-fusca</i>
TY-223	10658	5740	<i>Dendrolobium triangulare</i>
TY-262	10659	5741	<i>Boesenbergia pandurata</i>
TY-361	10660	5742	<i>Pennisetum polystachyon</i>

* JCM, Japan Collection of Microorganisms, RIKEN, Saitama, Japan; TISTR, Thailand Institute of Scientific and Technological Research, Chatuchak, Bangkok, Thailand.

† All strains were isolated from a dead leaf of the species shown at Nakhon Ratchasima, Thailand.

amino acids (Difco), 0.1 % KH_2PO_4 , 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 % NaCl and 0.01 % $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, adjusted to pH 5.5.

Chemotaxonomy. The presence of xylose in whole-cell hydrolysates was analysed by HPLC as described previously (Suzuki & Nakase, 1988). Ubiquinones were extracted and purified by the method of Yamada & Kondo (1973) with slight modifications and determined by HPLC as described previously (Hamamoto & Nakase, 1995).

Determination of DNA base composition and DNA relatedness. DNA isolation, the determination of G + C content by HPLC and DNA–DNA reassociation experiments using the membrane-filter method were carried out as described previously (Hamamoto & Nakase, 1995).

Nucleotide sequence analyses of 18S rDNA. DNA extraction for PCR and the sequencing of 18S rDNA of the representative four isolates (strains TY-138^T, TY-188, TY-191 and TY-223) were performed as described by Hamamoto & Nakase (2000) with slight modifications. The primers used for amplification and sequencing of 18S rDNA, 5.8S rDNA and the internal transcribed spacer (ITS) region were those described by White *et al.* (1990). The sequences were aligned using CLUSTAL W 1.75 (Thompson *et al.*, 1994) with the selected sequences (Fig. 1) retrieved from the GenBank and DDBJ libraries and manually adjusted. Evolutionary distances were calculated using the PHYLIP 3.57c program DNADIST (Felsenstein, 1995) with Kimura's two-parameter model and a tree was constructed in NEIGHBOR by the neighbour-joining method (Saitou & Nei, 1987). The confidence values of branches were determined by performing a bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

RESULTS AND DISCUSSION

All of our isolates were characterized by the formation of ballistoconidia, whose shapes were ovoid to ellipsoid, the inability to assimilate inositol and to ferment sugars, positive diazonium blue B (DBB) and urease reactions, the absence of xylose in whole-cell hydrolysates and the presence of Q-9 as the major

ubiquinone isoprenologue. On the basis of these results, these 10 isolates were assigned to the genus *Bensingtonia*. They were divided into four groups based on the ability to assimilate galactose, galactitol, D-glucitol and 2-ketogluconic acid: TY-138^T; TY-173, TY-180, TY-181, TY-190, TY-191, TY-262 and TY-361; TY-188; and TY-223. Representative strains of each group, strains TY-138^T, TY-188, TY-191 and TY-223, were used for the further investigations.

Long insertions in 18S rDNA were found in strains TY-138^T (401 nt), TY-188 (377 nt), TY-191 (389 nt) and TY-223 (397 nt) and these were presumed to be introns. The inserted regions of the four strains were considered to be group I introns, because they had conserved sequence elements P, Q, R and S (Cech, 1988). The sequence of 1713–1805 nt of 18S rDNA was determined for strains TY-138^T, TY-188, TY-191 and TY-223. The levels of 18S rDNA sequence similarity among these four strains ranged from 99.8 to 99.9 %. The sequence data for these yeasts were aligned with 28 sets of published data. A total of 1424 nt present in all species between positions 99 and 1683 (in *Saccharomyces cerevisiae*), excluding introns, were used for analysis. Our four isolates formed a cluster together with *B. miscanthi*, *B. phyllada*, *B. subrosea*, *B. yuccicola* and *Kondoa malvinella* in the *Agaricostilbum/Bensingtonia* lineage (Hamamoto & Nakase, 2000), statistically well supported (bootstrap = 100 %), and closer to *B. phyllada* than to the other species in this cluster (Fig. 1).

The G + C content of the DNA of the representative strains, TY-138^T, TY-188, TY-191 and TY-223, was 47.1, 47.7, 47.3 and 47.9 mol %, respectively. They showed high levels of DNA complementarity (76–100 %) to each other, but strain TY-138^T showed low levels (5–22 %) of complementarity with the type strains of *B. ciliata*, *B. miscanthi*, *B. phyllada*, *B. subrosea* and *B. yuccicola* whose G + C contents are similar to those of our isolates. These results indicate that our isolates represent a new species of the genus *Bensingtonia*. The name *Bensingtonia thailandica* has been proposed for this new species. Table 2 shows the phenotypic characteristics of 11 species of the genus *Bensingtonia*.

Latin diagnosis of *Bensingtonia thailandica* sp. nov. Fungsin, Hamamoto & Nakase

In medio liquido post dies 3 cellulae vegetativae ovoideae vel ellipsoideae, 2.0–3.0 × 5.0–8.0 µm, singulae aut binae. Post unum mensem pellicula imperfecta et sedimentum formatur. Cultura in agar YM, albo-cremea, non-nitida, mollis et margine glabra. Hyphae et pseudohyphae non formantur. Ballistospores reniformes aut ellipsoideae, 3.0–4.0 × 7.0–8.0 µm. Fermentatio nulla. Glucosum, galactosum (varium), saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinose, melezitose, amyllum solubile, glycerolum (varium), galactitolum (varium), D-mannitolum (varium), D-glucitolum (varium), acidum 2-ketogluconicum (varium),

Fig. 1. Phylogenetic positions of four *Bensingtonia* isolates based on 18S rDNA or 18S rRNA sequence comparison. The branching pattern was generated by the neighbour-joining method as described in the text. The numbers on the tree indicate bootstrap values greater than 50 %.

Table 2. Characteristics differentiating *B. thailandica* from known species

+, Positive; —, negative.

Characteristic	<i>B. thailandica</i>	<i>B. ciliata</i>	<i>B. ingoldii</i>	<i>B. miscanthi</i>	<i>B. musae</i>	<i>B. naganoensis</i>	<i>B. phyllada</i>	<i>B. sakaguchii</i>	<i>B. subrosea</i>	<i>B. yamatoana</i>	<i>B. yuccicola</i>
Assimilation of:											
L-Sorbose	—	+	—	+	+	—	+	—	+	—	+
Sucrose	+	—	+	+	—	+	+	—	+	+	—
Cellobiose	+	—	+	+	—	+	+	+	+	—	+
Trehalose	+	+	+	+	+	—	+	+	+	+	+
Lactose	—	—	+	+	—	—	+	+	+	—	—
Melibiose	+	—	+	—	—	+	—	—	—	—	—
Raffinose	+	—	+	+	—	+	—	—	+	—	—
DNA G+C content (mol %)	47.1–47.9	46.1*	56.0*	48.5*	52.3*	53.7*	45.9*	52.6–52.8†	45.5*	51.4*	44.5*

* Data from Takashima *et al.* (1995b).

† Data from Sugita *et al.* (1997).

acidum 5-ketogluconicum (varium), *acidum succinicum*, *acidum DL-lacticum* (varium) et *acidum citricum* assimulantur, at non *L-sorbosum*, *lactosum*, *inulinum*, *D-xylosum*, *L-arabinosum*, *D-arabinosum*, *D-ribosum*, *L-*

rhamnosum, *ethanolum*, *erythritolum*, *ribitolum*, *methyl α-D-glucosidum*, *salicinum*, *glucono-δ-lactonum*, *inositolum*, *acidum D-glucuronicum* nec *acidum D-galacturonicum*. *Kalium nitricum* et *natrium nitricum* assimi-

Fig. 2. Photomicrographs of *B. thailandica* TY-138^T (= JCM 10651^T). (a) Vegetative cells grown on YM agar after 5 d at 25 °C. (b) Ballistoconidia produced on corn meal agar after 7 d at 17 °C. Bars, 10 µm.

lantur at non ethylaminum, L-lysinum nec cadaverinum. Ad crescentiam thiaminum necessarium est. G + C acidi deoxyribonucleati 47.1–47.9 mol % (per HPLC). Ubiquinonum majus Q-9. Typus stirps TY-138^T ex folio Cratoxylum maingayi, Nakhon Ratchasima Province, Thailand, isolata est. In collectionibus culturarum quas Japan Collection of Microorganisms, Wako, Saitama, sustentant, no. JCM 10651 deposita et in collectionibus culturarum quas Thailand Institute of Scientific and Technological Research, Chatuchak, Bangkok, sustentant, no. TISTR 5733 sunt.

Description of *Bensingtonia thailandica* sp. nov.
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Bensingtonia thailandica (thai'lan.di.ca. M.L. adj. *thailandica* referring to Thailand where the 10 strains were isolated).

After 3 d at 25 °C in YM broth (Difco), the cells are ovoidal to ellipsoidal (2.0–3.0 × 5.0–8.0 µm), single or in pairs (Fig. 2a). A sediment and an incomplete ring are formed after 1 month. After 1 month at 17 °C on YM agar, streak cultures are pale yellowish/cream, dull to shiny and smooth. The margin is entire. True

hyphae or pseudohyphae are not formed in Dalmau plate cultures on cornmeal agar (Difco). Ballistoconidia are produced abundantly on cornmeal agar and YM agar. They are reniform, allantoid or ellipsoidal, (3.0–4.0 × 7.0–8.0 µm) (Fig. 2b). Does not ferment D-glucose. Assimilate D-glucose, galactose (variable), sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch, glycerol (variable), galactitol (variable), D-mannitol (variable), D-glucitol (variable), 2-ketogluconic acid (variable), 5-ketogluconic acid (variable), succinic acid, DL-lactic acid (variable) and citric acid. Do not assimilate the carbon of L-sorbose, lactose, inulin, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, erythritol, ribitol, methyl α-D-glucoside, salicin, glucono-δ-lactone, inositol, D-glucuronic acid and D-galacturonic acid. Does not assimilate the following nitrogen sources: ethylamine, L-lysine and cadaverine. Assimilation of nitrate and nitrite is positive. Thiamine is required for growth. Growth does not occur on 50 % (w/w) glucose-yeast extract agar. No starch-like substance is produced. Does not liquefy gelatin. Urease activity is positive. The diazonium blue B reaction is positive. The major ubiquinone is Q-9. The G + C content of the nuclear DNA is 47.1–47.9 mol %, as determined by HPLC. Type strain TY-138^T was isolated from a dead leaf of *Cratoxylum maingayi* in a tropical rain forest of Sakearat Environmental Research Station in Nakhon Ratchasima Province, Thailand. This strain has been deposited in the Japan Collection of Microorganisms (JCM), Riken, Wako, Saitama, Japan, as strain JCM 10651^T and also deposited in the Thailand Institute of Scientific and Technological Research (TISTR), Chatuchak, Bangkok, Thailand, as strain 5733^T. The other 9 strains examined have also been deposited in the JCM and the TISTR as JCM 10652/TISTR 5734 (TY-173), JCM 10653/TISTR 5735 (TY-180), JCM 10654/TISTR 5736 (TY-181), JCM 10655/TISTR 5737 (TY-188), JCM 10656/TISTR 5738 (TY-190), JCM 10657/TISTR 5739 (TY-191), JCM 10658/TISTR 5740 (TY-223), JCM 10659/TISTR 5741 (TY-262) and JCM 10660/TISTR 5742 (TY-361).

B. thailandica can be distinguished from the other four known species in the same cluster of the 18S rDNA-based tree, *B. miscanthi*, *B. phyllada*, *B. subrosea* and *B. yuccicola*, and from the type species of the genus *Bensingtonia*, *B. ciliata*, on the basis of the assimilation of melibiose and the lack of assimilation of L-sorbose (Table 2). The differences in the G + C content between *B. thailandica* and the other five known species in the genus, *Bensingtonia ingoldii*, *Bensingtonia musae*, *Bensingtonia naganoensis*, *Bensingtonia sakaguchii* and *Bensingtonia yamatoana*, is significant enough to discriminate it from these species (Table 2).

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