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Bullera panici sp. nov. and *Bullera siamensis* sp. nov., two new yeasts in the *Bullera variabilis* cluster isolated in Thailand

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Four strains of ballistoconidiogenous yeasts that contain xylose and have Q-10 ubiquinone were isolated from leaves collected in a protected rain forest in Thailand and were found to represent two new species. The taxonomic properties of both coincide with the genus *Bullera* so they are described as *Bullera panici* sp. nov. and *Bullera siamensis* sp. nov. In phylogenetic trees based on the sequence of small subunit and D1/D2 domain of large subunit ribosomal DNAs, these two species are located in a cluster that includes *Bullera variabilis* and *Bullera psedovariabilis*, and that is well separated from clusters including the remaining hymenomycetous ballistoconidiogenous yeasts and related taxa.

Key words : *Bullera panici* sp. nov.; *Bullera siamensis* sp. nov.; new ballistoconidiogenous yeasts from Thailand ; new yeasts from leaves

INTRODUCTION

Nakase and Suzuki (10) described *Bullera variabilis*, based on the isolates from leaves in Japan and Canada. They stated that the electrophoretic pattern of enzymes of this species differs from strain to strain, which suggests that these yeasts constitute several different species. However, they described these strains as a single species because they could not distinguish any distinct groups based on enzyme patterns or other taxonomic characteristics. Bai et al. (1) studied 20 strains of *B. variabilis* and retained five strains in *B. variabilis*, reassigned 6 strains in *Bullera mrakii*, and proposed three novel species for eight of the nine remaining strains, namely *Bullera pseudohuiaensis*, *Bullera pseudoschimicola* and *Bullera komagatae*. In the phylogenetic tree based on ribosomal DNAs, *B. variabilis* is located at a position distant from other yeast species in Hymenomycetes (4, 9, 14). Recently, Bai et al. (2) described a new species, *Bullera pseudovariabilis,*

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based on a strain isolated from a leaf in Yunnan, China. This species is closely related to *B. variabilis* and constitutes a cluster with the latter species in the phylogenetic tree based on the sequence of small subunit (SSU) rDNA.

During a survey of ballistoconidiogenous yeasts in the tropical phyllosphere of Thailand, four strains of hymenomycetous yeasts were isolated from leaves collected at a protected rain forest in Northeastern Thailand. These yeasts were found to represent two new species of *Bullera*. In phylogenetic trees based on the SSUrDNA and D1/D2 domain of large subunit (LSU) rDNA sequences, these yeasts are located in the cluster where *B. variabilis* and *B. pseudovariabilis* are located. They are described in the present paper as *Bullera panici* sp. nov. and *Bullera siamensis* sp. nov.

MATERIALS AND METHODS

Isolation of yeast strains. Plant samples for yeast isolation were collected in a protected rain forest in Sakaerat, Nakhon Ratchasima Province, Thailand, in November 1996. Yeasts were isolated by the ballistoconidia-fall method as previously reported (11) with YM agar (Difco Lab., Detroit). The isolation was carried out at 25 ℃. Immediaterly after purification by the streaking technique, isolates were preserved at −80 ℃ suspended in YM broth supplemented with 10 % (w/v) glycerol. Four strains of yeasts employed in the present study were obtained from the following plants : TY−258 and TY−259 from leaves of *Panicum multinodes* Stapf., TY−227 from *Urena lobata* Linn. var. *sinuata* King., and TY−240 from *Ageratum conyzoides* Linn., respectively.

Examination of morphological, physiological and biochemical characteristics, ubiquinone systems, xylose in the cells, isolation and purification of nuclear DNA, and DNA base composition. All of these were carried out according to the procedures described in the previous paper (6).

Sequencing and phylogenetic analysis. The nucleotide sequences of SSUrDNA were directly determined using PCR products accrding to Sugita and Nakase (13). Reference sequences used for the phylogenetic study, shown in Fig. 1, were obtained from the DNA data bank. The nucleotide sequences of the D1/D2 domain at the 5′ end of LSU rDNA were directly determined using PCR products according to Kurztman and Robnett (8), Fell et al., (4) and Fungsin et al. (6). The sequences of SSU rDNA and the D1/D2 domain of LSU rDNA determined in this study are deposited in the GenBank databases under the following accession numbers : TY−227 (TISTR 5800 = JCM 11820) SSU rDNA (AY 188389), D1/D2 domain of LSU rDNA (AY 188388); TY−240 (TISTR 5801 = JCM 11821) D1/D2 domain of LSU rDNA (AY 188390); TY−258 (TISTR 5799 = JCM 11819) SSU rDNA (AY188386), D1/D2 domain of LSU rDNA (AY188387) ; TY−259 (TISTR 5802 = JCM 11822) D1/D2 domain of LSU rDNA (AY 188391). Generated sequences were aligned with related species in Hymenomycetous yeasts using the CLUSTAL W ver. 1.74 computer program (15). The phylogenetic tree was constructed from the evolutionary distancedata according to Kimura (7) using the neighbor−joining method (12) in the PHYLIP computer program. Sites where any gaps existed in any sequences were excluded. Bootstrap analyses (5) were performed from 1,000 random resamplings.

RESULTS AND DISCUSSION

Strains of yeasts employed in the present study produced rotationally symmetrical ballistoconidia (Fig. 4B, D), lacked non ballistoconidiogenous stalked conidia, had Q−10 as the major ubiquinone, and contained xylose in the whole cell hydrolysate. These characteristics coincided well with those of the genus *Bullera* (3). In the phylogenetic tree constructed by the neighbor−joining method based on the SSU rDNA sequences, TY−258 and TY−227 (selected as representative strains of TY−227, TY−240 and TY−259) are located at the same position that is closely related with *Bullera variabilis* and *Bullera pseudovariabilis*, and the phylogenetic distance between these isolates and the latter two known species is not short (Fig. 1). the phylogenetic tree based on the D1/D2 domain of LSU rDNA also indicated the close relationships of the four strains employed with *Bullera variabilis* and *Bullera*

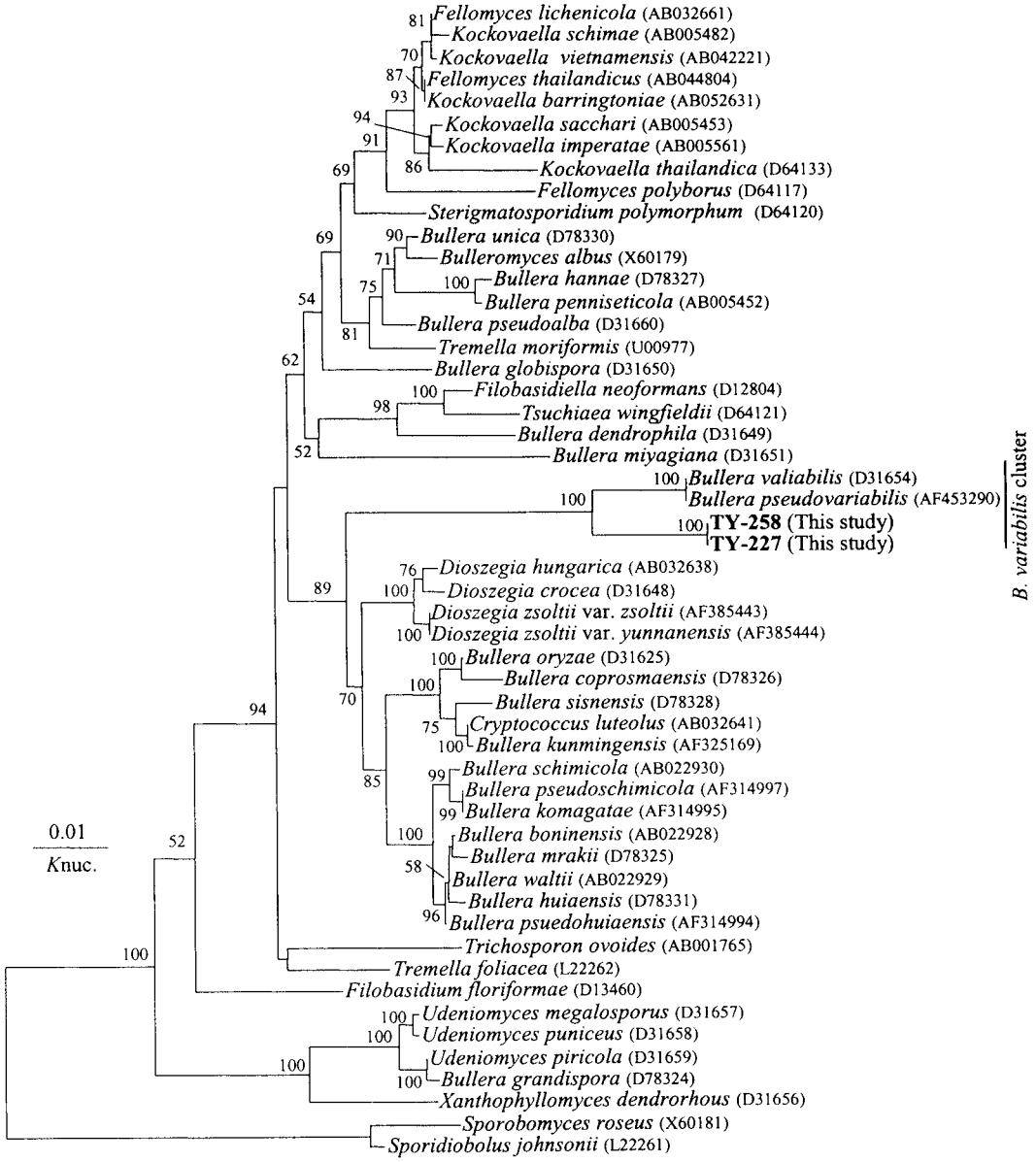

Fig. 1. Phylogenetic tree of new *Bullera* species from Thailand and related taxa on the SSU rDNA sequences.

The tree was constructed from the evolutionary distance data according to Kimura (7), using the neighbor-joining method (12) with bootstrapping (5). The numerals represent results from 1,000 replicated bootstrap samplings (a frequency of less than 50 % is not indicated).

pseudovariabilis (Fig. 2). Strains TY-227, TY-240 and TY-259 have the identical nucleotide sequence in this domain and are regarded to represent a single species. TY-258 is located at a position closely related to the former three strains but differs from the former strains by 15 nucleotides (ca. 2.8 %). This strain is considered to represent a different species from the former three strains. *Bullera variabilis*, the first described species in this cluster, differs from TY-227, TY-240 and TY-259 by 50 nucleotides (ca. 9.4 %), and from TY-258 by 59 nucleotides (ca. 10.1 %). *Bullera pseudovariabilis* differs from *B. variabilis* by one nucleotide in the D1/D2 domain. *B. arundinariae* (6) had been compared previously

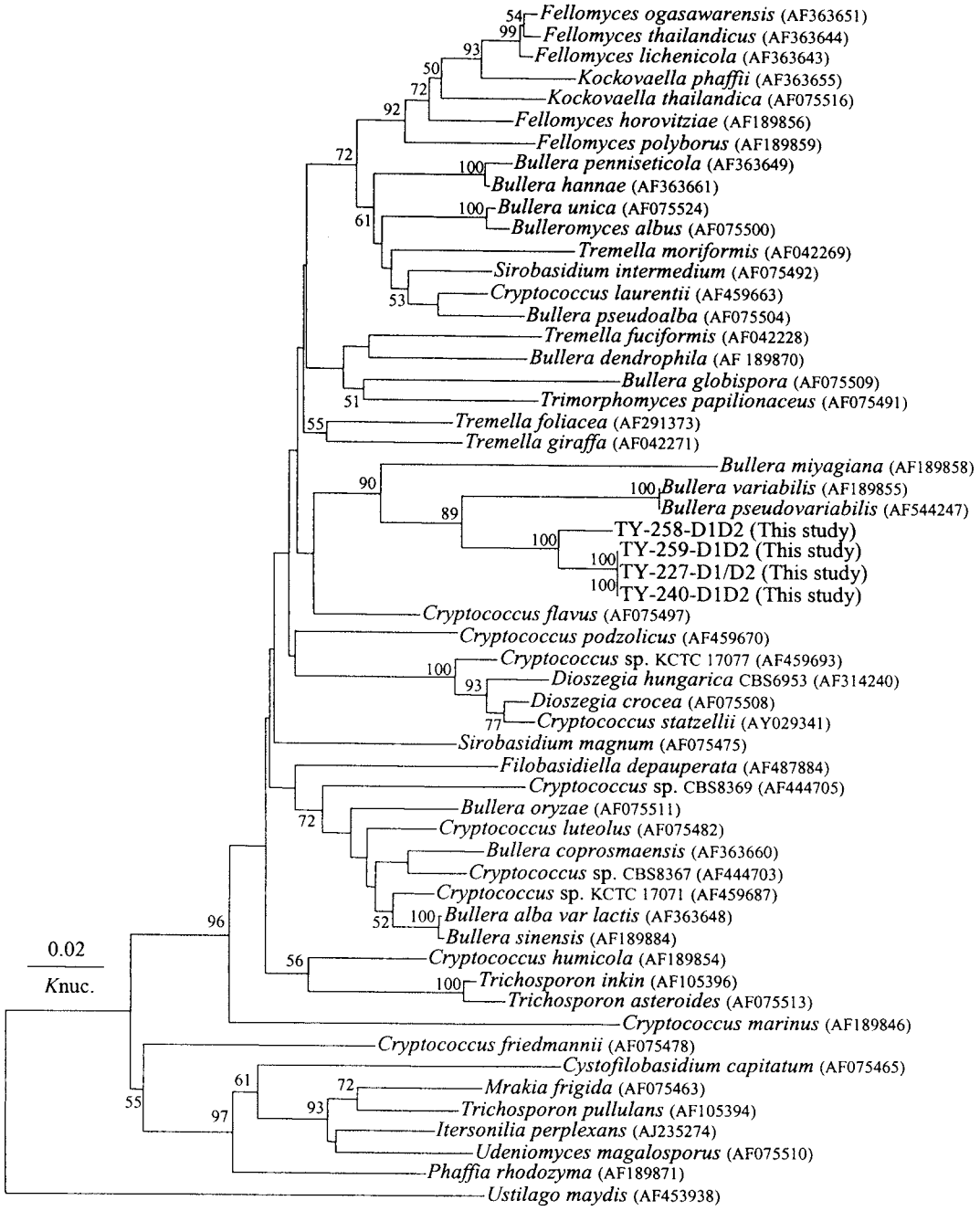

Fig. 2. Phylogenetic tree of new *Bullera* species from Thailand and related taxa on the D1/D2 domain of LSU rDNA sequences.

The tree was constructed from the evolutionary distance data according to Kimura (7), using the neighbor-joining method (12) with bootstrapping (5). The numerals represent results from 1,000 replicated bootstrap samplings (a frequency of less than 50 % is not indicated).

with the tested four strains, and it was not included in the variabilis cluster of a phylogenetic tree based on SSU rDNA. Based on these facts, we concluded that the four Thai strains represent two different new species phylogenetically related to *B. variabilis* and *B. pseudovariabilis*. These yeasts are described as

Bullera panici sp. nov. and *Bullera siamensis* sp. nov., respectively.

Phenotypically, the two new species *B. panici* and *B. siamensis* are distinguishable from *B. variabilis* and *B. pseudovariabilis* in the assimilation of citric acid, sodium nitrite, ethylamine hydrochloride and cadaverine dihydrochloride. However, they are indistinguishable from each other by the morphological, physiological and biochemical characters examined in this study. They are distinguished by D1/D2 domain sequences of 26S rDNA at positions 460-465 and 501-504 (in *Saccharomyces cerevisiae*) as shown in Fig. 3. Practically, the two new species can be distinguished from others of *Bullera* by the characteristics of carbon assimilation of L-sorbose and glycerol nitrogen assimilation of cadaverine dihydrochloride. These two species are located at a cluster that is distant from the cluster where *Bulleromyces albus*, a teleomorph of the type species of *Bullera, B. alba*, is located (Figs. 1 and 2).

Bullera panici Fungsin, Takashima et Nakase sp.nov.

In liquido "YM", post dies 3 ad 25℃, cellulae subglobosae, ovoideae aut ellipoideae, 3.8-11.2×6.2-12.5 μm, singulae aut binae. Insulae, annulus et sedimentum formantur. In agaro "YM", post unum mensem ad 17℃, cultura cremea, opaca, glabra aut crispulata, et margine undulata. Mycelium et pseudomycelium non formantur. Ballistosporae globosae or subglobosae, 6.8-10.0×6.8-10.0 μm. Fermentatio nulla. Glucosum, galactosum, L-sorbosum, sucrosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinosum, melezitosum, amylum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, erythritolum (lente et exiguum, vel nullum), ribitolum (lente et exiguum, vel nullum), ribitolum (lente et exiguum, vel unllum), galactitolum, D-mannitolum, D-glucitolum (lente et esiguum, vel nullum), α-methyl-D-glucosidum, salicinum, glucono-ä-lactonum (exiguum), acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (vel nullum), acidum D-glucuronicum, acidum D-galacturonicum, acidum succinicum et inositolum assimilantur at non lactosum, inulinum, ethanolum, glycerolum nec acidum citricum. Kalium nitricum non assimilatur. Maxima temperatura crescentiae : 27-28℃. Ad crescentiam thiaminae necessarium est. Diazonium caeruleum B positivum. Proportio molaris guanini + cytosine in acido deoxyribonucleico : 46.4 mol% (per HPLC). Ubiquinonum majus : Q-10. Teleomorphosis ignota.

Holotypus : Stirps TY-258, isoratus ex folio *Panico*

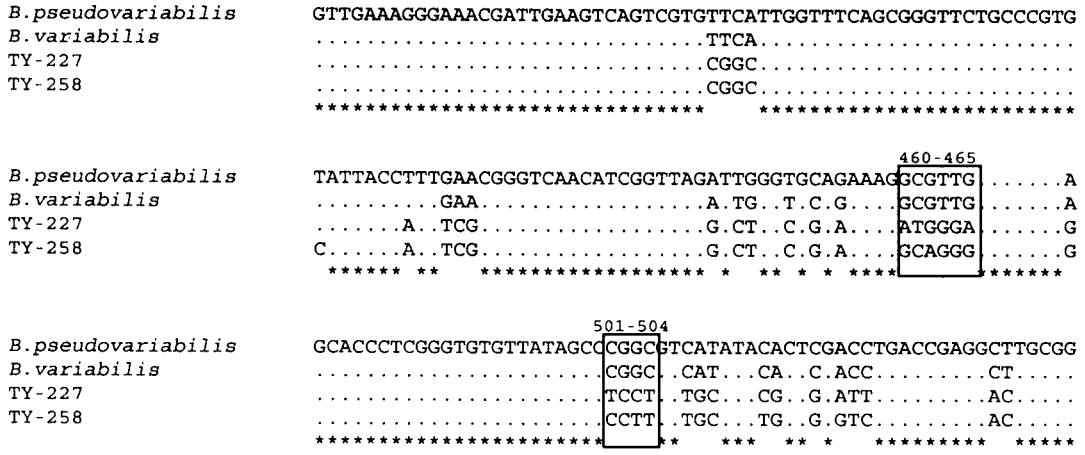

Fig. 3. Comparison in sequences of D1/D2 domain of LSU rDNA among four related species, *Bullera variabilis*, *B. pseudobariabilis*, *B. panici* (TY-258) and *B. siamensis* (TY-227). Positions are shown according to the D1/D2 domain of LSU rDNA of *Saccharomyces cerevisiae*.

multinodes Stapf., in pluvial sylva tropica, Nakhon Ratchasima Province inThailandia, cultura vita ex Holotypus huius speciei conservatur in collectionibus culturarum in 'Thailand Institute of Scientific and Technological Research (TISTR)', Chatuchak, Bangkok, Thailandia ut TISTR 5799 in statu lyophilo, item in collectionibus culturarum ut JCM 11819 in statu kyophilo quas 'Japan Collection of Microorganisms (JCM) RIKEM', Wako, Saitama, Japonia sustentat.

Growth in YM broth : After 3 days at 25 ℃ cells are subglobose, ovoidal, or ellipsoidal 3.8–11.2 × 6.2–12.5 μm (Fig. 4A) After 1 month at 17 ℃, islets ring and a sediment are present.

Growth on YM agar : After 1 month at 17 ℃, the streak culture is cream to dark cream, dull, smooth and delicately wrinkled near the buttom and has an undulate margin.

Dalmau plate culture on corn meal agar : Mycelium and pseudomycelium are not formed.

Production of ballistoconidia : Ballistoconidia are rarely produced on the cornmeal agar. They are globose or subglobose, 6.8–10.0 × 6.8–10.0 μm (Fig. 4B).

Fermentation : Absent

Assimilation of carbon compounds :

Glucose	+	Ethanol	–
Galactose	+	Glycerol	–
L-Sorbose	+	Erythritol	+
		(latent & weak) or	–
Sucrose	+	Ribitol	+
		(latent & weak) or	–
Maltose	+	Galactitol	+
Cellobiose	+	D-Mannitol	+
Trehalose	+	D-Glucitol	+
		(latent & weak) or	–
Lactose	–	α-Methyl-D-glucoside	+
Melibiose	+	Salicin	+
Raffinose	+	Goucono-δ-lactone	+
			(weak)
Melezitose	+	2-Ketogluconic acid	+
Inulin	–	5-Ketogluconic acid	+
Soluble starch	+	DL-Lactic acid	+ or –
D-Xylose	+	D-Glucuronic acid	+
L-Arabinose	+	D-Galacturonic acid	+
D-Arabinose	+	Succinic acid	+
D-Ribose	+	Citric acid	–
L-Rhamnose	+	Inosital	+

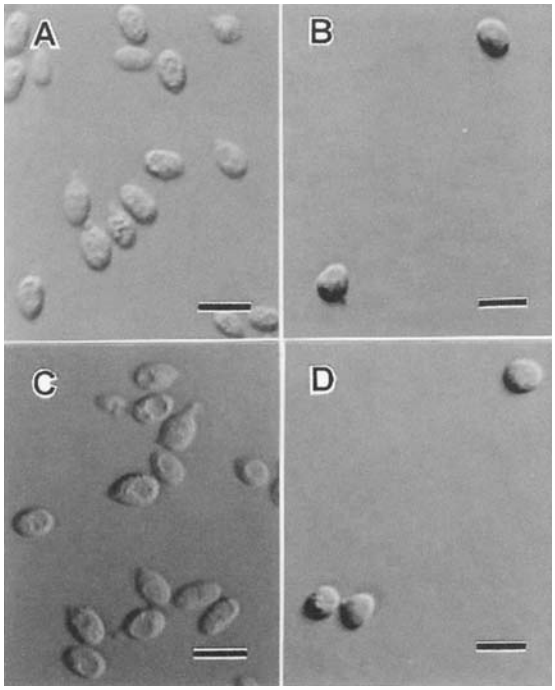

Fig. 4. Morphology of *Bullera panici* and *Bullera siamensis*.

A. Vegetative cells of *B. panici* TY-258 grown in YM broth for 3 days at 25 ℃.

B. Ballistoconidia of *B. panici* TY-258 produced on corn meal agar after 7 days at 25 ℃.

C. Vegetative cells of *B. siamensis* TY-227 grown in YM broth for 3 days at 25 ℃.

D. Ballistoconidia of *B. siamensis* TY-227 produced on corn meal agar after 7days at 25 ℃.

Scale bars indicate 10 μm.

Assimilation of nitrogen compounds:

Ammonium sulfate	+	Ethylamine hydrochloride	+
Potassium nitrate	–	L-Lysine hydrochloride	+
			(latent & weak)
Sodium nitrite	+	Cadaverine dihydrochloride	+

Maximum growth temperature : 27–28 ℃.

Vitamin required for growth : Thiamine.

Production of starch-like substances : Positive (weak).

Growth on 50% (w/w) glucose yeast extract agar : Negative.

Urease : Positive.

Liquefaction of gelatin: Negative.

Diazonium Blue B reaction : Positive.

G + C content of nuclear DNA: 46.4 mol% (by HPLC).

Major ubiquinone : Q-10.

Xylose in the whole cell hydrolysate : Present.

Nucleotide sequences of the D1/D2 domain of 26S rDNA at positions 460-465 and 501-504 (in *Saccharomyces cerevisiae*): GCAGGG and CCTT, respectively.

Holotype : TY-258, isolated by B. Fungsin, M. Takashima and T. Nakase from a leaf of *Panicum multinodes* Stapf., which was collected at a tropical rain forest in Sakaerat Environmental Research Station, Nakhon Ratchasima Province, Thailand, in November 1996. A living culture of ex-holotype of this species is deposited at the culture collection of the Thailand Institute of Scientific and Technological Research, Bangkok as TISTR 5799, and in the culture collection of Japan Collection of Microorganisms, Wako, Saitama, as JCM 11819.

Etymology : The specific epithet of this species is derived from the generic name of a plant from which this species was isolated.

It is distinguished by D1/D2 domain sequences of 26S rDNA at positions 460-465 and 501-504 (in *Saccharomyces cerebisiae*) as shown in Fig. 3.

Bullera siamensis **Fungsin, Takashima et Nakase, sp. nov.**

In liquido "YM" post dies 3 ad 25 ℃, cellulae subglobosae, ovoideae aut ellipsoideae, 3.0-11.2 × 6.2-12.0 μm. Sedimentum formatur. In agaro "YM", cultura cremea, glabra, opaca, magine glabra. Mycelium et pseudomycelium non formantur. Ballistoconidiae globosae aut napiformes 6.3-10.0 × 6.3-10.0 μm. Fermentatio nullum. Glucosum, galactosum, L-sorbosum (latent et exiguum), sucrosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinosum, melezitosum, amylum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, erythritolum (lente et exiguum, vel lente), ribitolum (lente et exiguum, vel lente), galactiolum, D-mannitolum, D-glucitotum (lente et exiguum, vel lente), α-methyl-D-glucosidum, salicinum, glunono-δ-lactonum (fortasse exiguum), acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente et exiguum), acidum D-glucuronicum, acidum D-galacturonicum, acidum succinicum, acidum citricum (lente et exiguum, vel nullum) et inositolum assimilantur at non lactosum, inulinum, ethanolum nec glycerolum. Kalium nitricum non assimilatur. Maxima temperatura crescentiae : 28-29 ℃. Ad crescentiam thiaminae necessarium est. Diazonium caeruleum B positivum. Proportio molaris guanini + cytosine in acido deoxyribonucleico : 48.4 mol% (per HPLC). Ubiquinonum majus : Q-10. Teleomorphosis ignota.

Holotypus : Stirps TY-227 isolata ex folio *Ureana lobata* Linn. var . *sinuate* King in pluvial sylva tropica, Nakhon Ratchasima Province in Thailandia, cultura viva ex Holotypo huius speciei conservatur in collectionibus culturarum in 'Thailand Institute of Scientific and Technological Research (TISTR)', Chatuchak, Bangkok, Thailandia ut TISTR 5800 in statu lyophilo, item in collectionibus culturarum quas 'Japan Collection of Microorganisms (JCM) RIKEN', Wako, Saitama, Japonia ut JCM 11820 sustentat.

Growth in YM broth : After 3 days at 25 ℃ cells are subglobose, ovoidal, or ellipsoidal 3.0-11.2 × 6.2-12.0 μm (Fig. 4C). After 1 month at 17 ℃, ring and a sediment are present.

Growth on YM agar : After 1 month at 17 ℃, the streak culture is cream to browny cream, smooth, dull and has an entire margin.

Dalmau plate culture on corn meal agar : Mycelium and pseudomycelium are not formed.

Production of ballistoconidia : Ballistoconidia are rarely produced on the corn meal agar. They are globose, or napiform 6.3-10.0 × 6.3-10.0 μm (Fig. 4D).

Fermentation : Absent

Assimilation of carbon compounds :

Glucose	+	Ethanol	−
Galactose	+	Glycerol	−
L-Sorbose	+ (latent & weak)	Erythritol	+ (latent & weak) or −
Sucrose	+	Ribitol	+ (latent & weak) or −

Maltose	+	Galactitol	+
Cellobiose	+	D-Mannitol	+
Trehalose	+	D-Glucitol	+
		(latent & weak) or	−
Lactose	−	α-Methyl-D-glucoside	+
Melibiose	+	Salicin	+
Raffinose	+	Glucono-δ-lactone	+
		(may be weak)	
Melezitose	+	2-Ketogluconic acid	+
Inulin	−	5-Ketogluconic acid	+
Soluble starch	+	DL-Lactic acid	+
		(latent & weak)	
D-Xylose	+	D-Glucuronic acid	+
L-Arabinose	+	D-Galacturonic acid	+
D-Arabinose	+	Succinic acid	+
D-Ribose	+	Citric acid	+
		(latent & weak) or	−
L-Rhamnose	+	Citric acid	+
		(latent & weak) or	−
		Inositol	+

Assimilation of nitrogen compounds :

Ammonium sulfate	+	Ethalamine hydrochloride	+
Potassium nitrate	−	L-Lysine hydrochloride	+
Sodium nitrite	+	Cadaverine dihydrocholoride	+

Maximum growth temperature : 28-29 ℃

Vitamin required for growth : Thiamine.

Production of starch-like substances : Positive (weak).

Growth on 50% (w/w) glucose yeast extract agar : Negative.

Urease : Positive.

Liquefaction of gelatin : Negative.

Diazonium Blue B reaction : Positive.

G + C content of nuclear DNA : 48.4 mol% (by HPLC).

Major ubiquinone : Q-10.

Xylose in the whole cell hydrolysate : Present .

Nucleotide sequences of the D1/D2 domain of LSU rDNA at positions 460-465 and 501-504 (in *Saccharomyces cerevisiae*): ATGGGA and TCCT, respectively.

Holotype : Strain TY-227, isolated by B. Fungsin, M. Takashima and T . Nakase, from a leaf of *Urena lobata* Linn. var. *sinuata* King. which was collected at a tropical rain forest in Sakaerat Environmental Research Station, Nakhon Ratchasima Province, Thailand, in November 1996, was chosen as the holotype of this species. A living culture of ex-holotype of this species was deposited in the culture collection of the Thailand Institute of Scientific and Technological Research, Bangkok as TISTR 5800, and Japan Collection of Microorganisms, Wako, Saitama, as JCM 11820. Strain TY-240 and TY-259 were also deposited in the culture collection mentioned above as TISTR 5801 = JCM 11821 and TISTR 5802 = JCM 11822, respectively.

Etymology : The specific epithet "*siamensis*" is derived from the old name of Thailand where this species was isolated.

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Bullera variabilis cluster に位置するタイ国産射出胞子形成酵母の2新種 *Bullera panici* sp. nov. および *Bullera siamensis* sp. nov.

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タイの保護熱帯雨林で採集した植物から分離した，キシロースを含有し，主要ユビキノンがQ−10である射出胞

子形成酵母4株は2新種に区別された．分類学的性状は*Bullera*属に一致したので*Bullera panici* sp. nov．および*Bullera siamensis* sp.nov.と命名した．この2種はSSU rDNAおよびLSU rDNAのD1/D2領域の配列に基づき作成した分子系統樹では*Bullera variabilis*および*Bullera pseudovariabilis*を含む系統枝に位置しており，菌蕈綱に属する他の射出胞子形成酵母および関連菌類から離れた位置にあった．