***Bullera penniseticola* sp. nov. and *Kockovaella sacchari* sp. nov., two new yeast species isolated from plants in Thailand**

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Two strains of ballistoconidium-forming yeasts, isolated from plants collected in the south-east seacoast of Bangkok, Thailand, were described. The strains (K-272^T and K-337^T) were assigned to the genera *Bullera* and *Kockovaella*, respectively, based on morphological and chemotaxonomical characteristics. Phylogenetically, strain K-272^T is close to *Bullera hanna*e, and strain K-337^T is close to *Kockovaella thailandica* and *Kockovaella imperatae*. These two strains represent new species based on DNA–DNA reassociation experiments. *Bullera penniseticola* Takashima et Nakase sp. nov. and *Kockovaella sacchari* Takashima et Nakase sp. nov. are proposed for K-272^T (= JCM 9857^T) and K-337^T (= JCM 9858^T), respectively.

Keywords: yeast, *Bullera penniseticola* sp. nov., *Kockovaella sacchari* sp. nov., Thailand

INTRODUCTION

In 1990, two ballistoconidium-forming yeasts, K-272^T and K-337^T, were isolated from plant leaves collected in Thailand. Strain K-272^T, assigned to the genus *Bullera*, was characterized by coenzyme Q-10, the presence of xylose in the cells, and proliferation by ballistoconidia and budding cells. Strain K-337^T, assigned to the genus *Kockovaella*, was characterized by Q-10 as major ubiquinone, the presence of xylose in the cells, and proliferation by stalked conidia, ballistoconidia and budding cells. *Bullera* sp. K-272^T showed physiological and biochemical characteristics similar to *Bullera hanna*e, while *Kockovaella* sp. K-337^T was found to be similar to *Kockovaella thailandica* and *Kockovaella imperatae*. DNA–DNA reassociation experiments, however, revealed that these yeasts represented new species of the genera *Bullera* and *Kockovaella*. They are described as *Bullera penniseticola* Takashima et Nakase sp. nov. and *Kockovaella sacchari* Takashima et Nakase sp. nov.

METHODS

Yeast strains. The strains K-272^T and K-337^T reported in this paper were isolated from leaves of *Pennisetum* sp. and

Saccharum officinarum, respectively, which were collected from the south-east seacoast of Bangkok, Thailand, using the ballistoconidium-fall method with YM agar (Difco) plates (Nakase & Takashima, 1993).

Examination of morphological, physiological and biochemical characteristics. Most of the morphological, physiological and biochemical characteristics were examined according to the methods of van der Walt & Yarrow (1984). Assimilation of nitrogen compounds was investigated on solid media using starved inocula according to the method of Nakase & Suzuki (1986). Vitamin requirements were investigated according to the method of Komagata & Nakase (1967). The maximum growth temperature was determined in YM broth (Difco) using metal block baths.

Ubiquinone system. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the early stationary growth phase. The cells were washed with distilled water. Extraction, purification and identification of ubiquinones were carried out according to the method of Nakase & Suzuki (1986).

Xylose in the cells. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the early stationary growth phase. The cells were washed with distilled water and dried with acetone. Xylose in the cells was analysed as described by Suzuki & Nakase (1988).

DNA base composition. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the exponential growth phase. The cells were washed with distilled water and 0.067 M phosphate buffer containing 1 mM EDTA (pH

Abbreviation: SSU, small subunit.

The GenBank/EMBL/DBJ accession number for the sequences reported in this paper are AB005452 (*Bullera penniseticola* JCM 9857^T), AB005453 (*Kockovaella sacchari* JCM 9858^T) and AB005561 (*K. imperatae* JCM 7826^T).

7-4). Isolation and purification of DNA were carried out according to the methods of Nakase & Suzuki (1986). The DNA base composition was determined by HPLC after enzymic digestion of DNA to deoxyribonucleosides. The DNA-GC Kit (Yamasa Shoyu) was used as a quantitative standard.

DNA-DNA relatedness. DNA-DNA reassociation experiments were carried out using a membrane-filter method according to the method of Hamamoto & Nakase (1995).

Sequencing and phylogenetic analysis. The small subunit (SSU) rDNA sequence was determined after amplifying the gene using PCR. Both strands were sequenced by the dideoxy method as described by Takashima & Nakase (1996). The SSU rDNA sequences of *Bullera penniseticola* JCM 9857^T (K-272^T), *Kockovaella sacchari* JCM 9858^T (K-337^T) and *K. imperatae* JCM 7826^T were determined in this study. Generated sequences were aligned with other unicellular and filamentous basidiomycetes using the CLUSTAL W computer program (Thompson *et al.*, 1994). *Sporidiobolus johnsonii* and *Sporobolomyces roseus* were used as outgroups. The fixed gap and floating gap penalty values used in CLUSTAL were 10. The alignment is available upon request. The phylogenetic tree was constructed from the evolutionary distance data according to Kimura (1980) using neighbour-joining method (Saitou & Nei, 1987) in the CLUSTAL W computer program. Sites where gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were performed from 1000 random resamplings.

RESULTS AND DISCUSSION

DNA base composition and DNA-DNA relatedness

Fifteen species are now recognized in the genus *Bullera*. Suh & Nakase (1995) and Suh *et al.* (1996) described the phylogenetic heterogeneity of the genus *Bullera* based on SSU rDNA sequences. The wide range of the DNA base composition, namely 39–62 mol% G + C, also predicts the heterogeneity of the genus.

The DNA base composition of *Bullera* sp. K-272^T was 44.2 mol% G + C, and similar to that of *B. hanna*e and *B. huiaensis* (Hamamoto & Nakase, 1996). In the neighbour-joining tree based on SSU rDNA sequences, however, *Bullera* sp. K-272^T showed close relationship (99.6% sequence similarity) to *B. hanna*e JCM 8937 and was located in a cluster different from that of *B. huiaensis* JCM 8933 (Fig. 1). DNA-DNA reassociation experiments, carried out between *Bullera* sp. K-272^T and *B. hanna*e, revealed DNA relatedness values of 23 and 26%. This suggests that *Bullera* sp. K-272^T is a species different from *B. hanna*e.

Two species, *K. thailandica* and *K. imperatae*, have been described in the genus *Kockovaella* (Nakase *et al.* 1991). The DNA base composition of *Kockovaella* sp. K-337^T was found to be 48.0 mol% G + C, and similar to those of the described species in the genus. As shown in Fig. 1, *Kockovaella* sp. K-337^T constituted a cluster with *K. thailandica* JCM 7824^T and *K. imperatae* JCM 7826^T in the neighbour-joining tree based on SSU rDNA sequences. DNA-DNA reassociation experiments carried out between *Kockovaella* sp. K-337^T, *K. thailandica* and *K. imperatae* showed low DNA relatedness values (5–15%) (Table 1).

Latin diagnosis of *Bullera penniseticola* Takashima et Nakase sp. nov.

In 'YM', post dies 5 ad 25 °C, cellulae vegetativae ovoideae, ellipsoidales aut elongatae, (2.0–4.0) × (3.3–10.1) µm, singulae aut binae. Sedimentum formatur. Post unum mensem ad 17 °C, annulus imperfectus, insulae et sedimentum formantur. In agar 'YM', post unum mensem ad 17 °C, cultura subflava, glabra, nitida, mollis et margine glabra. Mycelium et pseudomycelium non formantur. Ballistosporae globosae vel napiformes, (2.4–3.5) × (2.6–4.1) µm. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinose, melezitose, amyllum solubile, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum (lente), glycerolum (lente), ribitolum (lente et exiguum), galactitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum (lente et exiguum), glucono-δ-lactonum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum succinicum (exiguum), acidum citricum (exiguum) et inositolum assimilantur at non L-sorbosum, inulinum, ethanolum, erythritolum nec acidum DL-lacticum. Kalium nitricum, natrium nitrosum, ethylaminum, L-lysinum et cadaverinum non assimilantur. Maxima temperatura crescentiae: 30–31 °C. Ad crescentiam thiaminum necessarium est. Diazonium caeruleum B positivum. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 44.2 mol% (per HPLC). Ubiquinonum majus: Q-10. Xylosum in cellulis praesens. Holotypus: Isolatus ex folio *Pennisetum* species, JCM 9857^T (originaliter ut K-272^T) conservatur in collectionibus culturarum quas 'Japan Collection of Microorganisms', Wako, Saitama sustentat.

Description of *Bullera penniseticola* Takashima et Nakase sp. nov.

In YM broth, after 5 d at 25 °C, the vegetative cells are ovoidal, ellipsoidal or elongate, (2.0–4.0) × (3.3–10.1) µm, single or in pairs. A sediment is formed. After 1 month at 17 °C, an incomplete ring, islets and a sediment are present. On YM agar, after 1 month at 17 °C, the streak culture is pale yellow, shining, smooth and delicately wrinkled near the bottom, and has an entire margin. On Dalmau plate culture on corn meal agar, mycelia and pseudomycelia are not observed. Ballistoconidia are abundantly produced on corn meal agar. They are globose to napiform, (2.4–3.5) × (2.6–4.1) µm (Fig. 2). Does not ferment glucose. Assimilates glucose, galactose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose (slow), glycerol (latent), ribitol (latent and weak), galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin (latent and weak), glucono-δ-lactone, 2-ketogluconic acid, 5-ketogluconic acid, succinic acid (weak), citric acid (weak) and inositol. Does not assimilate L-sorbose, inulin, ethanol, erythritol or DL-lactic acid. Does not assimilate potassium nitrate, sodium nitrite, ethylamine hydrochloride, L-lysine hydrochloride, or cadaverine

Fig. 1. Phylogenetic tree of *Bullera*, *Kockovaella*, *Udeniomyces* and related taxa based on SSU rDNA sequences. The tree was constructed from the evolutionary distance data according to Kimura (1980) using the neighbour-joining method (Saitou & Nei, 1987) with bootstrapping (Felsenstein, 1985). The numerals represent the percentages from 1000 replicate bootstrap samplings. Sequences were retrieved from the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers indicated.

Table 1. DNA–DNA relatedness of *Kockovaella* sp. K-337^T and other *Kockovaella* species

Species	Strain	G + C mol%	Percentage relative binding of DNA from:		
			K-337 ^T	JCM 7824 ^T	JCM 7826 ^T
<i>Kockovaella</i> sp.	K-337 ^T	48.0	100	5	12
<i>K. thailandica</i>	JCM 7824 ^T	47.5	15	100	13
<i>K. imperatae</i>	JCM 7826 ^T	47.7	12	8	100

Fig. 2. *Bullera penniseticola*. (a) Vegetative cells grown in YM broth for 5 d at 25 °C. (b) Ballistospores produced on corn meal agar after 5 d at 25 °C. Bars, 10 µm.

dihydrochloride. Maximum growth temperature is 30–31 °C. Thiamine is required for growth. No starch-like substances are produced. Growth does not occur on 50% (w/w) glucose-yeast extract agar. Urease-positive. Does not liquefy gelatin. Does not hydrolyse fat. The diazonium blue B reaction is positive. The G+C content of nuclear DNA is 44.2 mol% as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the cells. The type strain of *B. penniseticola*, K-272^T, was isolated by T. Nakase and B. Fungsin in December 1990 from a leaf of *Pennisetum* sp. collected along the south-east seacoast of Bangkok, Thailand. This strain has been deposited in Japan Collection of Microorganisms (JCM), Wako, Saitama, as JCM 9857^T. The physiological and biochemical characteristics of *B. penniseticola* are similar to those of *B. hanna*, but the maximum growth temperature, 30–31 °C for *B. penniseticola* and 22–23 °C for *B. hanna*, can clearly distinguish between these species.

Latin diagnosis of *Kockovaella sacchari* Takashima et Nakase sp. nov.

In liquido 'YM', post dies 5 ad 25 °C, cellulae vegetativae sphaerical vel ovoideae, (2.0–6.3) × (3.3–7.3) µm, singulae, binae, in catenis aut in fasciculis, propagantes formatione conidiorum stipitatorum et gemmarum blasticarum. Conidia stipitata ex cellula conidiogena multilateraliter. Sedimentum formatur. Post unum mensem ad 17 °C, anulus, insulae et sedimentum formantur. In agar 'YM', post unum mensem ad 17 °C, cultura griseo-flava, glabra, nitida, butyracea et margine glabra. Mycelium et pseudomycelium non formantur. Ballistospores globosae vel napiformes, (2.2–4.0) × (3.1–4.6) µm. Fermentatio nulla. Glucosum, galactosum, saccharosum, maltosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinsum, meleztosum, amyllum solubile, D-xylosum, L-arabiosum, D-arabiosum (lente), D-ribo-

Fig. 3. *Kockovaella sacchari*. (a) Vegetative cells grown in YM broth for 5 d at 25 °C. (b) Ballistospores produced on corn meal agar after 5 d at 25 °C. Bars, 10 µm.

sum, L-rhamnosum (lente), ribitolum (lente), galactitolum (lente), D-mannitolum (lente), D-glucitolum (lente), methyl α-D-glucosidum, salicinum, glucono-δ-lactonum (exiguum), acidum succinicum (lente), acidum citricum (exiguum) et inositolum (lente et exiguum) assimilantur at non L-sorbosum, inulinum, ethanolum, glycerolum, erythritolum, acidum 2-ketogluconicum, acidum 5-ketogluconicum nec acidum DL-lacticum. L-Lysinum assimilatur at non kalium nitricum, natrium nitrosum, ethylaminum nec cadaverinum. Maxima temperatura crescentiae: 31–32 °C. Ad crescentiam thiaminum necessarium est. Diazonium caeruleum B positivum. Proportio molaris guanini+cytosini in acido deoxyribonucleico: 48.0 mol% (per HPLC). Ubiquinonum majus: Q-10. Xylosum in cellulis praesens. Holotypus: Isolatus ex folio Saccharum officinarum, JCM 9858^T (originaliter ut K-337^T) conservatur in collectionibus culturarum quas 'Japan Collection of Microorganisms', Wako, Saitama sustentat.

Description of *Kockovaella sacchari* Takashima et Nakase sp. nov.

In YM broth, after 5 d at 25 °C, the vegetative cells are spherical to ovoidal (2.0–6.3) × (3.3–7.3) µm, single, in pairs or in clusters, reproduced by budding and stalked conidia. Stalks develop multilaterally from a conidiogenous cell. A sediment is formed. After 1 month at 17 °C, a complete ring and islets, and a sediment are present. On YM agar, after 1 month at 17 °C, the streak culture is greyish-yellow, shining, smooth, butyrous and has an entire margin. On Dalmau plate culture on corn meal agar, mycelia and pseudomycelia are not formed. Ballistoconidia are abundantly produced on corn meal agar. They are globose to napiform (2.2–4.0) × (3.1–4.6) µm (Fig. 3). Does not ferment glucose. Assimilates glucose, galactose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, meleztose, soluble starch, D-xylose, L-ar-

Table 2. Salient characteristics of *Kockovaella sacchari* and other *Kockovaella* species

+, Positive; L, latent; W, weak; LW, latent and weak; –, negative.

Species	Strain	Assimilation of:				
		Galactitol	Methyl α -D-glucoside	Glucono- δ -lactone	2-Ketogluconic acid	5-Ketogluconic acid
<i>K. sacchari</i>	JCM 9858 ^T	L	+	W	–	–
<i>K. thailandica</i>	JCM 7824 ^T , JCM 7825	–	L/–	–	+	+
<i>K. imperatae</i>	JCM 7826 ^T	L	–	L	LW	LW

abinose, D-arabinose (latent), D-ribose, L-rhamnose (latent), ribitol (latent), galactitol (latent), D-mannitol (latent), D-glucitol (latent), methyl α -D-glucoside, salicin, glucono- δ -lactone (weak), succinic acid (latent), citric acid (weak), and inositol (latent and weak). Does not assimilate L-sorbose, inulin, ethanol, glycerol, erythritol, 2-ketogluconic acid, 5-ketogluconic acid, or DL-lactic acid. Assimilates L-lysine hydrochloride. Does not assimilate potassium nitrate, sodium nitrite, ethylamine hydrochloride, or cadaverine dihydrochloride. Maximum growth temperature is 31–32 °C. Thiamine is required for growth. No starch-like substances are produced. Growth does not occur on 50% (w/w) glucose-yeast extract agar. Urease positive. Does not liquefy gelatin. Does not hydrolyse fat. The diazonium blue B reaction is positive. The G+C content of nuclear DNA is 48.0 mol% as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the cells. The type strain of *K. sacchari*, K-337^T, was isolated by T. Nakase and B. Fungsin in December 1990 from a leaf of *Saccharum officinarum* collected along the south-east seacoast of Bangkok, Thailand. This strain has been deposited in Japan Collection of Microorganisms, Wako, Saitama, as JCM 9858^T. *Kockovaella sacchari* is distinguished from *K. thailandica* by the assimilation of galactitol, 2-ketogluconic acid, 5-ketogluconic acid, and from *K. imperatae* by the assimilation of methyl α -D-glucoside (Table 2). This is the third species of the genus *Kockovaella*.

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