RESEARCH ARTICLE

Torulaspora maleeae sp. nov., a novel ascomycetous yeast species from Japan and Thailand

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Received 28 June 2007; revised 14 August 2007; accepted 15 September 2007.
First published online 6 November 2007.

DOI:10.1111/j.1567-1364.2007.00324.x

Editor: Cletus Kurtzman

Keywords
Torulaspora maleeae sp. nov.; novel ascomycetous yeast; Japan; Thailand.

Abstract

Nine strains of a new *Torulaspora* species were isolated from natural samples collected in Japan and Thailand including one strain obtained from a leaf of *Rhizophora stylosa* (NBRC 11061^T), one strain from soil (NBRC 11062), six strains from mosses (ST-14, ST-266, ST-510, ST-511, ST-513 and ST-581) and one strain from sediment in mangrove forest (RV-51). On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and the sequence analyses of the D1/D2 domain of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) (ITS1–5.8S rRNA gene–ITS2) region, the nine strains were found to represent a single novel species of the genus *Torulaspora*, which were named *Torulaspora maleeae* sp. nov. The type strain is NBRC 11061^T (BCC 25515^T = CBS 10694^T). In the phylogenetic trees based on the sequences of the D1/D2 domain of the LSU rRNA gene, *T. maleeae* showed a close relationship with the five recognized species of the genus *Torulaspora, Torulaspora delbrueckii*, *Torulaspora franciscae*, *Torulaspora globosa*, *Torulaspora microellipsoides* and *Torulaspora pretoriensis*. *Torulaspora maleeae* differed from the five recognized species of the genus *Torulaspora* by six to 12 nucleotide substitutions (1.1–2.1%) in the D1/D2 domain of the LSU rRNA gene and by 6.4–11.7% nucleotide substitutions in the ITS (ITS1–5.8S rRNA gene–ITS2) region.

Introduction

The ascomycetous yeast genus *Torulaspora* is closely related to the genera *Saccharomyces* and *Zygosaccharomyces*. The type strain of the genus was first described as *Saccharomyces delbrueckii* by Lindner in 1895. In 1904, Lindner established the new genus *Torulaspora* and transferred *S. delbrueckii* to this genus as *Torulaspora delbrueckii* (van der Walt, 1970). The genus *Torulaspora* was merged into the genus *Saccharomyces* together with the genus *Zygosaccharomyces* by Lodder & Kreger van Rij (1952) and subsequently redefined by van der Walt & Johanssen (1975). In the third and fourth editions of '*The Yeasts, A Taxonomic Study*,' *Torulaspora* accommodated three species: *T. delbrueckii, Torulaspora globosa* and *Torulaspora pretoriensis* (Yarrow, 1984; Kurtzman, 1998). Then, Kurtzman & Robnett (2003) resolved the family *Saccharomycetaceae* into 11 well-supported clades by multigene sequence analysis consisting of an rRNA gene repeat [small subunit (SSU), large subunit (LSU), internal transcribed spacer (ITS) between the SSU and the LSU], single-copy nuclear genes (translation elongation factor 1α, actin-1, RNA polymerase II) and mitochondrially encoded genes (SSU rRNA gene, cytochrome oxidase II). From the multigene phylogenetic analysis five species, *T. delbrueckii*, *T. globosa*, *Torulaspora franciscae*, *Torulaspora microellipsoides* and *T. pretoriensis*, were assigned to the genus *Torulaspora* (Kurtzman, 2003).

Strains of *T. delbrueckii* could be isolated from both natural and manmade habitats such as insect frass, slime flux, rotten tree stump, soil invertebrates, above-ground plant parts, ragi, sorghum brandy, wine and grape musts (Byzov *et al*., 1993; Kurtzman, 1998; Vaughan-Martini *et al*., 1999; Barnett *et al*., 2000; Yurkov & Chernov, 2005). The species *T. globosa* was reported to isolate from soil,

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loog-pang, feces of humans and spoiled meat-based entomophaga diet (Kurtzman, 1998; Vaughan-Martini *et al.*, 1999; Barnett *et al.*, 2000; Limtong *et al.*, 2002; Inglis & Cohen, 2004). Strains of *T. pretoriensis* were found in soil (Kurtzman, 1998; Vaughan-Martini *et al.*, 1999; Barnett *et al.*, 2000), and slime flux of orange tree (Kodama *et al.*, 1964). *Torulaspora microellipsoides* were found in apple juice, berries of black currants, exudates of sandalwood and tea-beer (Kurtzman, 1998).

During the investigations of yeasts in various natural habitats in Thailand seven strains, six strains from mosses and one from sediment in mangrove forest, were found to belong to the same species as two strains isolated in Japan, the one from a leaf and the other from soil, and maintained in the NITE Biological Resource Center (NBRC) as *Torulaspora* sp. NBRC 11061 and 11062. In this study, the new species *Torulaspora maleeae* sp. nov. is described.

Materials and methods

Yeast strains

Selected properties of the strains described in this study are summarized in Table 1. Two strains have been maintained at NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan. Six strains (ST-14, ST-266, ST-510, ST-511, ST-513 and ST-581) were isolated from mosses by means of an enrichment technique. A small amount of each moss sample was added to yeast extract malt extract (YM) broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) supplemented with 0.2% sodium propionate and $100\,\mu g\,mL^{-1}$ chloramphenicol in a test tube and incubated at 25 °C for 3–4 days. An aliquot of the enrichment culture was then streaked on YM agar. When necessary, colonies were restreaked onto YM agar for purification. A strain RV-51 was isolated using the dilution spread plate technique on the same medium as used for enrichment isolation.

Examination of taxonomic characteristics

The 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).

Ubiquinone system

Ubiquinones were extracted from intact cells cultivated in YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). The isoprenologues

Table 1. Strains of *Torulaspora maleeae* used in this study

	Accession number			GenBank accession number		Sample for isolation		
T. maleeae	BCC	NBRC	CBS	D1/D2	ITS	Sources	Locality	Date
Strains isolated from Japan								
NBRC 11061^{T}	25515^{T}	11061^{T}	10694	AB087395	AB304152	A leaf of *Rhizophora stylosa*	Iriomote Island, Okinawa Prefecture	Unknown
NBRC 11062	25516	11062	10695	AB303866	AB304153	Soil, rhizosphere of *Bruguiera gymnorrhiza*	Iriomote Island, Okinawa Prefecture	Unknown
Strains isolated from Thailand								
RV-51	17685	103199	–	AB303868	AB304155	Sediment	Magrove forest, Lame Son National Park, Ranong Province	13 December 1998
ST-14	7714	103204	–	AB303873	AB304160	Moss	Locality Khao-Yai National Park, Nakhon-Ratchasima Province	3 November 2000
ST-266	15018	103198	–	AB303867	AB304154	Moss	Hala-Bala Wild Life Sanctuary, Narathiwat Province	10 March 2001
ST-510	15195	103200	–	AB303869	AB304156	Moss	Tong Pha Phum, Kanchanaburi Province	19 February 2003
ST-511	15196	103201	–	AB303870	AB304157	Moss	Tong Pha Phum, Kanchanaburi Province	19 February 2003
ST-513	15198	103202	–	AB303871	AB304158	Moss	Tong Pha Phum, Kanchanaburi Province	19 February 2003
ST-581	15265	103203	–	AB303872	AB304159	Moss	Tong Pha Phum, Kanchanaburi Province	20 February 2003

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were identified by HPLC using a Cosmosil (Waters C18) 4.6 mm × 50 mm column and methanol : isopropanol (2 : 1) at 1 mL min^{-1} as the elution system with spectrophotometric detection (wavelength 275 nm).

DNA sequencing and phylogenetic analysis

Genomic DNA was prepared using Dr GenTLE for Yeast (Takara) according to the manufacturer's protocol. PCR was performed according to 'Materials and methods' described previously for the amplification of the D1/D2 domain of the LSU rRNA gene (Kurtzman & Robnett, 1997) or the ITS region (White *et al*., 1990). PCR products were purified with AMpure (Beckman Coulter) following the manufacturer's protocol and directly used for sequence analysis. The sequences were determined using the BigDye Terminator version 3 (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol.

The sequences were compared pairwise by BLAST search (Altschul *et al*., 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment-program CLUSTAL_X version 1.81 (Thompson *et al*., 1997). A phylogenetic tree was constructed from the evolutionary distance data corrected by two-parameter transformation of Kimura (1980), using the neighbor-joining method (Saitou & Nei, 1987). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Determination of DNA base composition and DNA–DNA hybridization

Cells for genomic DNA preparation were prepared as described previously by Imanishi *et al*. (2007). The genomic DNA was prepared according to the protocol of Holm *et al*. (1986), as modified by Kaneko & Banno (1991), and the base composition was determined by HPLC as described previously by Tamaoka & Komagata (1984). DNA–DNA hybridization was performed by the photobiotin microplate-hybridization method of Kaneko & Banno (1991).

Results and discussion

Phylogenetic analysis

The sequences of the D1/D2 domain of the LSU rRNA gene of four strains (NBRC 11061, NBRC 11062, ST-266 and ST-581) were identical and differed from those of the other four strains (ST-510, ST-511, ST-513 and RV-51) by only one nucleotide substitution in 573 nucleotides (nt). Strain ST-14 differed from the first four strains by two nucleotide substitutions and one gap in 574 nt and from the second four strains by one nucleotide substitution and one gap in 574 nt. In the phylogenetic analysis based on the D1/D2 domain of the LSU rRNA, gene all nine strains clustered together in a separate branch and connected with the five known species of the genus *Torulaspora*: *T. delbrueckii*, *T. franciscae*, *T. globosa*, *T. microellipsoides* and *T. pretoriensis* (Fig. 1). The nine strains differed by six to eight nucleotide substitutions (1.1–1.4%) from *T. pretoriensis*, the closest species in terms of pairwise sequence similarity, and from the other four recognized species of the genus *Torulaspora*, *T. globosa*, *T. delbrueckii*, *T. franciscae* and *T. microellipsoides*, by 1.2–1.6%, 1.4–1.6%, 1.4–1.6% and 1.9–2.1% nucleotide substitutions, respectively. According to Kurtzman & Robnett (1998), yeast strains showing nucleotide substitutions $> 1\%$ in the D1/D2 domain of the LSU rRNA gene are usually different species. As the difference in the D1/D2 domain of the LSU rRNA gene was relatively small, therefore, the ITS regions was determined to confirm the novelty of *T. maleeae*. In this region, the nucleotide sequence of the nine strains exhibited 99–100% similarity. The closest species in terms of pairwise sequence similarity to *T. maleeae* was *T. globosa* but with 44–47 nucleotide substitutions (6.4–6.8%). *Torulaspora maleeae* is also related to the other four recognized species of the genus *Torulaspora*, but fairly huge differences were found in nucleotide substitutions: 8.5–9% from *T. pretoriensis*, 8.9–9% from *T. delbrueckii*, 10.3–10.8% from *T. franciscae* and 11.4–11.7% from *T. microellipsoides*. These results lend further support to the conclusion that the nine strains represent a single novel species of the genus *Torulaspora*.

DNA base composition (mol% G+C) and DNA–DNA reassociation

Torulaspora maleeae has a G+C content of 43–45 mol%, which is lower than for *T. pretoriensis* and *T. globosa*, similar to *T. delbrueckii* and higher than *T. microellipsoides* (Table 2).

The DNA relatedness among some strains of *T. maleeae* and *T. globosa* is summarized in Table 2. The strains of *T. maleeae* showed DNA relatedness of 80–123% among them, indicating that they are conspecific. On the other hand, they gave values of 11–37% with *T. globosa*, the most closely related known species according to the D1/D2 and ITS sequences. These results clearly indicated that *T. maleeae* examined in the present study represents a single novel species.

Phenotypic characteristics

Torulaspora maleeae proliferated by multilateral budding (Fig. 2), and formed one to four spheroidal ascospores in a persistent ascus that may be produced parthenogenetically or by conjugation between a cell and its bud or between independent cells (Fig. 2). *Torulaspora maleeae* differed from the other species of *Torulaspora* only in a few phenotypic

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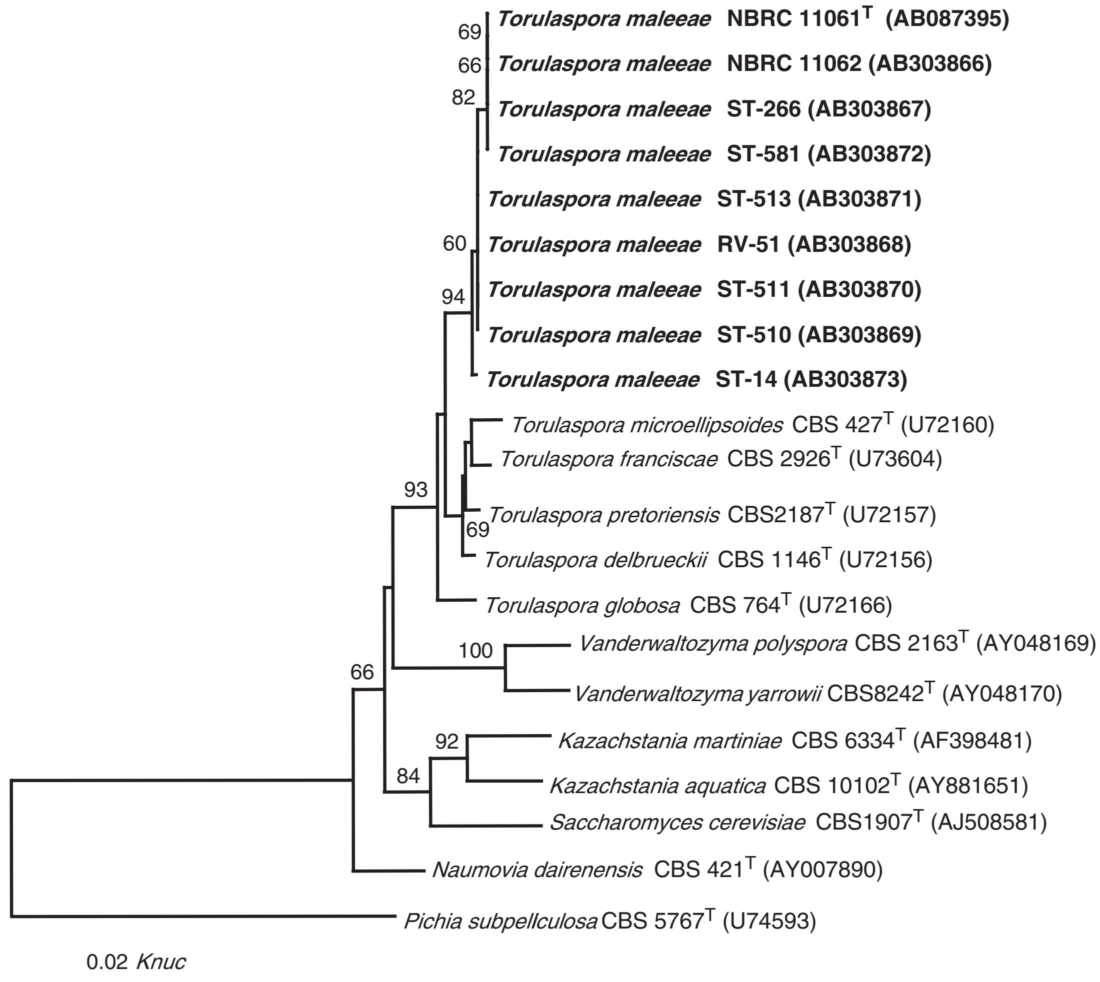

Fig. 1. Phylogenetic tree based on the sequences of the D1/D2 domain of the LSU rRNA gene, showing positions of the nine strains of the novel species, *Torulaspora maleeae* sp. nov., with respect to closely related species. The phylogenetic tree was constructed from the evolutionary distance data corrected by two-parameter transformation of Kimura (1980), using the neighbor-joining method. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples.

characteristics. The nine strains were negative for diazonium blue B and urease reactions and had Q-6 as the major ubiquinone as do other members of the genus *Torulaspora*.

On the basis of the data reported above, it is therefore concluded that the nine strains represent a single novel species of *Torulaspora*. The name *Torulaspora maleeae* sp. nov. is proposed for these strains.

Latin diagnosis of *Torulaspora maleeae* Limtong, Imanishi, Jindamorakot, Ninomiya, Yongmanitchai et Nakase sp. nov.

In medio liquido: 'YM', post dies 3 ad 25 °C cellulae globosae, singulae, binae vel cum gemma, 2.5–8.5 μm in diam. Post unum mensem ad 15 °C, annulus et Sedimetun formantur. In agaro 'YM', post unum mensem ad 25 °C, cultura griseoalbida, glabra, nitida, butyrosa et margine glabra. Hyphae et pseudohyphae non formantur. Ascus formatur per parthenogenesis vel conjunctio. Ascosporae globosae, 1–4 in asco, 1.9–4.3 μm in diam.

D-Glucosum, D-galactosum (variabile), sucrosum, maltosum, lactosum (variabile) et raffinosum fermentatur at non trehalosum, D-xylosum nec cellobiosum. D-glucosum, galactosum (variabile), sucrosum, maltosum, trehalosum (variabile) melibiosum (variabile), raffinosum, melezitosum, inulinum (variabile), amylum solubile (variabile), ethanolum (exigue et lente, variabile), glycerolum (exigue et lente, variable), D-mannitolum (lente, variabile), glucitolum (lente, variable), α-methyl-D-glucosidum, acidum gluconicum (variabile), acidum 2-ketogluconicum (exigue

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Table 2. DNA base composition (mol% G+C) and DNA–DNA reassociation of some strains of *Torulaspora maleeae* and *Torulaspora globosa*

		% Relatedness binding with labelled DNA from						
Strains	G+C content (mol% G+C)	NBRC 11061	NBRC 11062	ST-14	ST-511	ST-513	ST-581	*T. globosa* NBRC 1160
NBRC 11061	45	100	103	81	84	115	84	36
NBRC 11062	45	99	100	ND	ND	97	ND	21
ST-14	43	90	90	ND	84	96	ND	32
ST-266	45	97	ND	85	ND	ND	100	18
ST-510	ND	97	84	ND	100	108	ND	32
ST-511	ND	96	ND	ND	100	120	ND	37
ST-513	ND	ND	97	ND	ND	100	ND	15
ST-581	ND	84	123	80	ND	ND	100	16
RV-51	44	96	97	96	94	100	ND	11
T. globosa NBRC 1160	48	30	35	37	38	47	ND	100

ND, not determined.

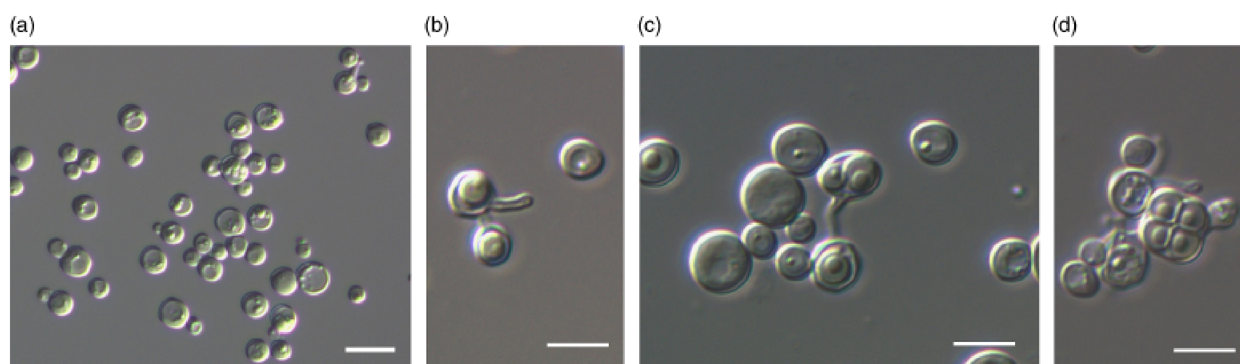

Fig. 2. *Torulaspora maleeae* sp. nov. NBRC 11061^T. (a) Vegetative cells grown in YM broth for 3 days at 25 °C. Scale bar = 10 μm. (b–d) Asci and ascospores formed on corn meal agar after 11 days at 25 °C. Scale bar = 5 μm. (b) Conjugation tube on an ascus is formed but no conjugation using it; (c) Ascus formed by conjugation with the conjugation tube; (d) Ascus with four ascospores.

et lente), acidum DL-lacticum (exigue et lente, variabile), et xylitolum (exigue et lente, variabile) assimilatur at non L-sorbosum, cellulobiosum, lactosum, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, methanolum, erythritolum, ribitolum, galactitolum, salicinum, acidum succinicum, acidum citricum, inositolum, D-glucosaminum, *N*-acetyl-D-glucosaminum, acidum 5-Keto-D-gluconicum, hexadecanum, propanum-1,2-diolum, butanum-2,3-diolum nec D-glucono-1,5-lactonum. L-Lysinum (variabile) assimilatur at non nitricum, nitrosum, ethylaminum, cadaverinum nec D-glucosaminum. Crescit in 10% NaCl/5% glucosum. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Ubiquinonum majus: Q-6. Proporti molaris guanine+cytosini in acido deoxyribonucleico 43–45 mol% (per HPLC).

Holotypus: Stirps NBRC 11061^T isolatus folio *Rhizophora stylosae* in insula Iriomote, Okinawa, Japonia *et conservatus* in Collectionie Culturarum in NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japonia; BIOTEC Culture Collection (BBC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand ut BCC 25515^T et Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands ut CBS 10694^T.

Description of *Torulaspora maleeae* Limtong, Imanishi, Jindamorakot, Ninomiya, Yongmanitchai & Nakase sp. nov.

Growth in YM broth: After 3 days at 25 °C, cells are spherical, occur singly or in pairs, often have several buds, 2.5–8.5 μm in diameter. After 1 month at 15 °C, traces of a ring and sediment are present. Growth on YM agar: After 1 month at 25 °C, the streak culture is grayish white, smooth, shining, and butyrous with an entire margin.

Formation of ascospores: Ascospores are produced on corn meal agar and YM agar after 11 days at 25 °C. Usually, the ascus is formed by heterogamic conjugation between a cell and its bud, and sometimes between independent cells. Conjugation tubes are often seen on the cells, but they rarely seem to be involved in the conjugation process.

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Ascospores are spherical with a smooth wall, one to four (some appear slightly warty), usually two per ascus, 1.9–4.3 μm in diameter.

Slide culture on corn meal agar: Mycelium and pseudomycelium are not produced.

Fermentation

D-Glucose	+
D-Galactose	+, −
Sucrose	+
Maltose	+
Lactose	Weak, −
Trehalose	−
Raffinose	+, Weak
D-Xylose	−
Cellobiose	−

Assimilation of carbon compounds

D-Glucose	+
D-Galactose	+, −
L-Sorbose	−
Sucrose	+
Maltose	+
Cellobiose	−
Trehalose	+, Slow, −
Lactose	−
Melibiose	+, −
Raffinose	+
Melezitose	+, Slow
Inulin	Delayed, −
Soluble Starch	Weak, −
D-Xylose	−
L-Arabinose	−
D-Arabinose	−
D-Ribose	−
L-Rhamnose	−
D-Glucosamine	−
N-Acetyl-D-glucosamine	−
Methanol	−
Ethanol	+, Weak and slow, −
Glycerol	+, Weak and slow, delayed, −
Erythritol	−
Adonitol (Ribitol)	−
Galactitol	−
D-Mannitol	+, Weak, −
D-Glucitol	+, Weak, −
α-Methyl-D-glucoside	+
Salicin	−
D-Gluconic acid	Weak, −
2-Keto-D-gluconate	Weak and slow
5-Keto-D-gluconate	−
DL-Lactic acid	+, Weak and slow, −
Succinic acid	−
Citric acid	−
Inositol	−
Hexadecane	−
Propane-1,2-diol	−
Bitane-2,3-diol	−
D-Glucono-δ-lactone	−
Xylitol	Weak and slow, slow, −

Assimilation of nitrogen compounds

Nitrate	−
Ethylamine	−
L-lysine	+, −
Cadaverine	−
D-glucosamine	−
Growth in vitamin-free medium:	+
Growth on NaCl 10% and Glucose 5%	+
Growth at 34 °C	+, −
Growth at 37 °C	+, −
Diazonium blue B color reaction	−
Urease	−
Major ubiquinone	Q6
G+C content of nuclear DNA:	43–45 mol % (by HPLC)

Holotype: NBRC 11061 is the holotype of *Torulaspora maleeae*. The strain was isolated from a leaf of *Rhizophora stylosa* in Iriomote Island, Okinawa Prefecture, Japan. The living culture from type was deposited at the NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 11061^T; BIOTEC Culture Collection (BBC), National Center for Genetic Engineering and Biotechnology (BIOTE), Pathumthani, Thailand, as BCC 25515^T; and Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands as CBS 10694^T.

Etymology: The specific epithet *maleeae* (ma.lee.ae. N.L. gen.n) was chosen for this novel yeast in honor of Dr Malee Suwana-adth for her early contribution to yeast sciences in Thailand including taxonomy and biodiversity.

It should be noted that before this study, none of the strains in the genus *Torulaspora* have been reported from moss or sediment in mangrove forest. Therefore, this is the first report on the discovery of the strains in this genus from these habitats.

Acknowledgements

The authors thank Mr Toshikazo Sakamoto, the NITE Biological Resource Center and the students in the laboratory of Associate Prof. Dr Savitree Limtong for their help with several experiments.

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