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ORIGINAL PAPER

Papiliotrema phichitensis f.a., sp. nov., a novel yeast species isolated from sugarcane leaf in Thailand

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Abstract Strain DMKU-SP105^T representing a novel yeast species was isolated from the external surface of a sugarcane leaf (*Saccharum officinarum* L.) collected from a sugarcane plantation field in Phichit province, Thailand. On the basis of sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, the strain DMKU-SP105^T differed by 7–16 substitutions in the D1/D2 region of LSU rRNA gene and 6–22 substitutions in the ITS region from a group of related species, *Papiliotrema aspenensis*, *Papiliotrema odontotermitis*, *Papiliotrema rajasthanensis* and *Papiliotrema laurentii*. A phylogenetic analysis based on the concatenated sequences of ITS region and the D1/D2 region of the LSU rRNA gene indicated that strain DMKU-SP105^T belongs to the *laurentii* clade of *Papiliotrema* in the *Tremellales* and is distinct from other related species in the clade. It therefore represents a novel species of the genus *Papiliotrema* although the formation of basidiospores was not observed. The name *Papiliotrema phichitensis* f.a., sp. nov. is proposed. The type is DMKU-SP105^T (= CBS 13390^T = BCC 61187^T = NBRC 109699^T).

Keywords External surface · 1 new taxon · *Papiliotrema phichitensis* sp. nov. · Sugarcane leaf · Thailand

Introduction

In 2002, the genus *Papiliotrema* was first proposed by Sampaio et al. (2002) based on an integrated analysis of morphological, ultrastuctural, physiological, and molecular data as a dimorphic and teleomorphic yeast genus in the order *Tremellales*, class *Tremellomycetes*, subphylum Agaricomycotina and phylum Basidiomycota (Sampaio et al. 2002). *Papiliotrema bandonii*, the type species, was the only species of the genus until a second species, *Papiliotrema siamensis*, was described in 2014 based on two anamorphic strains (Sampaio 2011; Surussawadee et al. 2014). Liu et al. (2015a) revised the classification of the Tremellomycetous yeasts and related dimorphic and filamentous basidiomycetes by phylogenetic analyses of seven genes consisting of the internal transcribed

The GenBank/EMBL/DDBJ accession numbers for the combined sequences of the ITS region and the D1/D2 region of the LSU rRNA gene of strain DMKU-SP105 are AB915388 and AB826437, respectively. The MycoBank number for *Papiliotrema phichitensis* f.a., sp. nov. is MB 825565.

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spacer (ITS) region rRNA gene, the D1/D2 region of the large subunit (LSU) rRNA gene, the small subunit (SSU) rRNA gene, the two subunits of RNA polymerase II (RPB1 and RPB2), the translation elongation factor 1-α (TEF1) and cytochrome b (CYTB), and Tremellomycetes have been reclassified on this basis. In consequence, the family *Rhynchogastremataceae* was proposed in the order *Tremellales* to accommodate the species in *Cryptococcus aureus*, *Auriculibuller*, *Bandoniozyma*, *Papiliotrema*, *Bullera pseudoalba* and *Cryptococcus laurentii* clades (Liu et al. 2015b). The family *Rhynchogastremataceae* consists of two emended genera namely *Rhynchogastrema*, the type genus, and *Papiliotrema*, which was emended to accommodate a monophyletic clade containing two teleomorphic species, *P. bandoni* and *Auriculibuller fuscus*, and 20 anamorphic species viz. 17 *Cryptococcus* species and three *Bullera* species (*B. hoabinhensis*, *B. japonica* and *B. pseudoalba*). In consequence, in 2015, the genus *Papiliotrema* comprised 22 accepted species including the type species (*P. bandonii*), one newly proposed species (*P. siamensis*) and 20 new combinations (Liu et al. 2015a, b). Later, the clade was enlarged with the description of *P. odontotermitis* (Handel et al. 2016), *P. leoncinii* (Pagani et al. 2016), *P. miconiae* (Pagani et al. 2016) and *P. plantarum* (Into et al. 2018). At the time of writing, the genus *Papiliotrema* consists of 26 species, of which only three species (*P. bandonii*, *P. fuscus* and *P. plantarum*) are teleomorphic species.

During our investigations of epiphytic yeasts on the external leaf surfaces of sugarcane in Thailand, strain DMKU-SP105^{T} representing a novel species of the genus *Papiliotrema* was obtained. In this paper, this strain is described as *Papiliotrema phichitensis* f.a., sp. nov.

Materials and methods

Yeast isolation

Strain DMKU-SP105^{T} belonged to a group of 267 strains obtained from the external surfaces of 102 samples of sugarcane (*Saccharum officinarum* L.) leaf by plating of leaf washings (Surussawadee et al. 2014). Three grams of leaf were aseptically suspended in 50 ml of 0.85% saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 25°C for 1 h to detach yeast cells from the surface. An aliquot of 0.1 ml of the washing solution was then spread on yeast extract–malt extract (YM) agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose and 2.0% agar) supplemented with 0.025% sodium propionate and 0.02% chloramphenicol and incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10% glycerol and maintained at − 80 °C.

DNA isolation, sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA extracted from yeast cells. Methods of DNA extraction and amplification were as described by Limtong et al. (2007). Amplification of the D1/D2 of the LSU rRNA gene was carried out by PCR with the forward primer NL1 (5′-GCATATCAATAAGCGG GGAAAAG-3′) and the reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1998). The ITS region was amplified with forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′) (White et al. 1990). The PCR products were checked by agarose gel electrophoresis and purified by using the HiYield^{TM} Gel/PCR Fragments Extraction Kit (RBC Bioscience, Taiwan). The purified products were sequenced commercially by Macrogen Inc. (Seoul, Korea) using primers, NL1 and NL4 for the D1/D2 and primers, ITS1 and ITS4 for the ITS region. The sequences were compared with the BLASTN search program (Altschul et al. 1997), assembled and aligned with the program MEGA version 7 (Kumar et al. 2016). Phylogenetic trees based on the combined sequences of the ITS and D1/D2 was constructed from the evolutionary distance data with Kimura's two-parameter correction, using the neighbor-joining method provided within the MEGA7 software package. Statistical significance of the clade was estimated from bootstrap analysis with 1000 replicates (Felsenstein 1985). *Kwoniella mangrovensis* CBS 8507^{T} (AF444646/AF444742) was used as outgroup in this analysis.

Phenotypic characterization

The strain DMKU-SP105 was characterised morphologically, biochemically and physiologically according to the standard methods described by Kurtzman et al. (2011). Formation of pseudohyphae and true hyphae was investigated by cultivation on potato dextrose agar (PDA; 20% potato infusion, 2% glucose and 1.5% agar) and corn meal agar (2% corn meal infusion and 1.5% agar) in slide culture at 25 °C for up to 4 weeks. Ballistospore formation was investigated on inverted PDA and corn meal agar plates and incubated at 15 and 25 °C for up to 4 weeks. Basidiospore formation was investigated by growing the strain on PDA, corn meal agar, 5% malt extract agar (5% malt extract and 1.5% agar), yeast extract-peptone glucose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) and YM agar at 15 and 25 °C for up to 6 weeks. Growth at various temperatures was determined by cultivation in YM broth. Ubiquinone was extracted from cells cultivated in 500 ml Erlenmeyer flasks containing 250 ml of yeast extract-peptone glucose (YPD) broth (1% yeast extract, 2% peptone and 2% glucose) on a rotary shaker at 28 °C for 48 h and purified according to the methods described by Yamada and Kondo (1973). Ubiquinone isoprennologues were identified by HPLC as described previously (Kuraishi et al. 1985).

Results and discussion

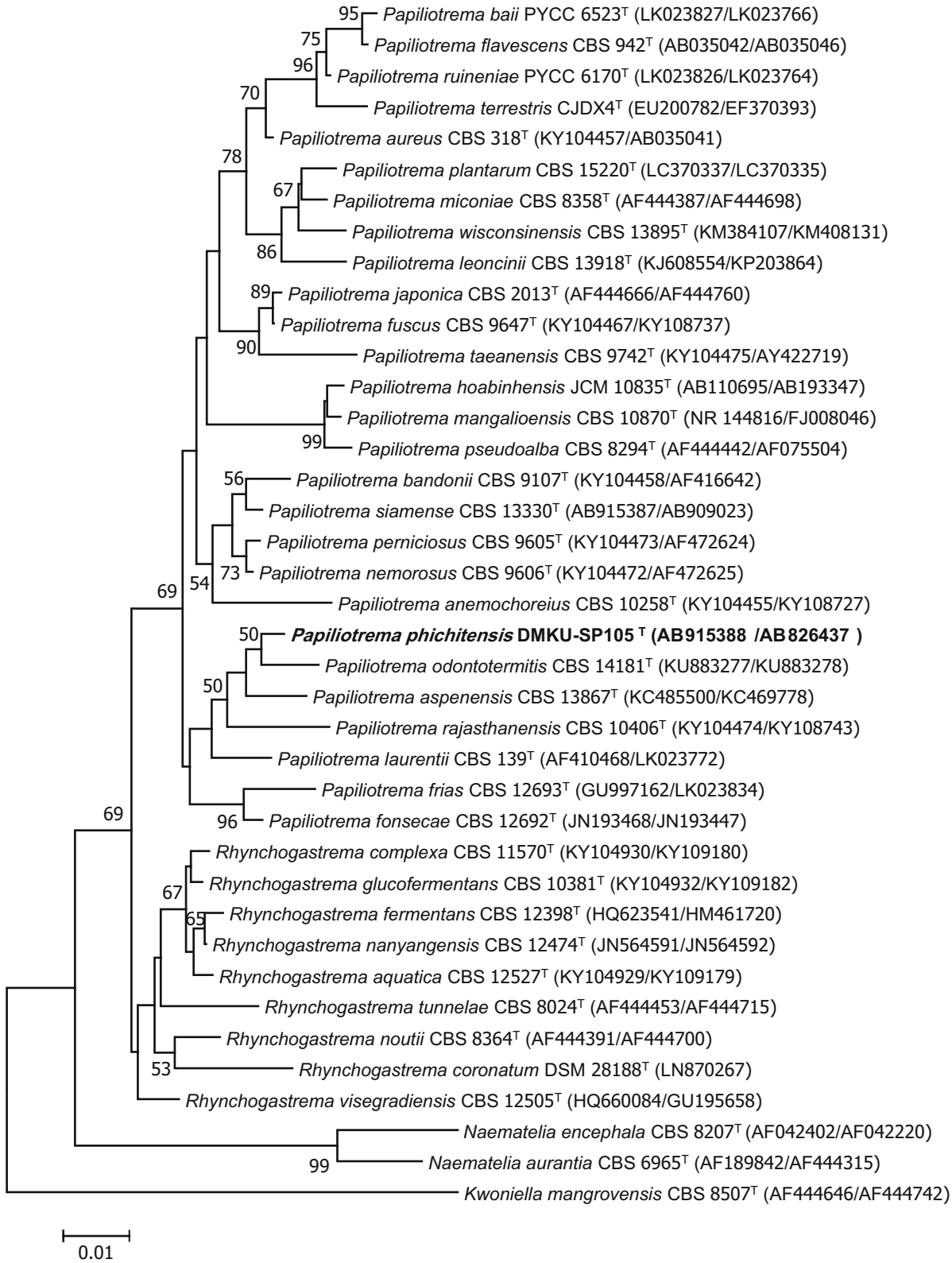
Species delineation and phylogenetic placement

Analysis of the sequence of the ITS region and the D1/D2 region of the LSU rRNA gene indicated that strain DMKU-SP105^T represents a novel *Papiliotrema* species. BLAST searches on the GenBank database revealed that strain DMKU-SP105^T is closely related to the type strains of *P. aspenensis*, *P. laurentii*, *P. odontotermitis* and *P. rajasthanensis*. In terms of pairwise sequence similarity, the strain DMKU-SP105^T differed by 7, 8, 12 and 16 nucleotide substitutions in the D1/D2 region from the known species *P. aspenensis* CBS 13867^T (KC469778), *P. laurentii* CBS 139^T (LK023772), *P. odontotermitis* CBS 14181^T (KU883278) and *P. rajasthanensis* CBS 10406^T (KY108743), respectively, and by 6, 16, 22 and 22 in ITS region from *P. odontotermitis* CBS 14181^T (KU883277), *P. aspenensis* CBS 13867^T (KC485500), *P. rajasthanensis* CBS 10406^T (KY104474) and *P. laurentii* CBS 139^T (AF410468), respectively.

A phylogenetic analysis based on the concatenated sequences of the ITS region and the D1/D2 region of the LSU rRNA gene demonstrated that strain DMKU-SP105 formed a subclade with the other *Papiliotrema* species in the *laurentii* clade, a group recognized by Liu et al. (2015a), including *P. aspenensis*, *P. laurentii*, *P. odontotermitis* and *P. rajasthanensis*, but the strain DMKU-SP105 was placed in a position different from the other *Papiliotrema* species (Fig. 1), which confirmed its recognition as a novel species. Delineation of the novel species was based primarily on the analysis of the concatenated sequences of the genes encoding the ITS region and the D1/D2 region of the LSU rRNA gene.

We decided to describe the novel species based on a single strain because the description of species based on a single strain will add to understanding of yeast phylogeny and species diversity (Kurtzman 2010). Although formation of basidiospores was not observed, in conformity with the International Code of Nomenclature for Algae, Fungi and Plants, which permits related anamorphic or teleomorphic species to be assigned to the same genus (McNeill et al. 2012), the species is therefore assigned to the genus *Papiliotrema*. The designation *forma asexualis* (f.a.) was added to indicate the lack of a sexual cycle in the species assigned to the genus (Lachance 2012). The name *Papiliotrema phichitensis* f.a. sp. nov. is proposed to accommodate this yeast strain. The type of the novel species was found during a comprehensive survey of epiphytic yeasts on sugarcane leaves in Thailand. Although 267 yeast strains were obtained, upon molecular identification no additional strains of this novel species were found. Strain DMKU-SP105^T was isolated from a sample collected from Bueng Na Rang district, Phichit province (16°10′18″N 100°7′36″E), on 2 March 2012. Among the 267 strains, some new yeast species such as *Papiliotrema siamense* (Surussawadee et al. 2014), *Wickerhamiella siamensis* (Khunnamwong et al. 2014), *Occultifur tropicalis* (Khunnamwong et al. 2015) and *Hannaella phyllophila* (Surussawadee et al. 2015) were described.

Papiliotrema phichitensis f.a. sp. nov. can be clearly distinguished from the closely related *Papiliotrema* species by the analysis of the sequence of the


95
Papiliotrema baii PYCC 6523T (LK023827/LK023766)
75
Papiliotrema flavescens CBS 942T (AB035042/AB035046)
96
Papiliotrema ruineniae PYCC 6170T (LK023826/LK023764)
70
Papiliotrema terrestris CJDX4T (EU200782/EF370393)
Papiliotrema aureus CBS 318T (KY104457/AB035041)
78
Papiliotrema plantarum CBS 15220T (LC370337/LC370335)
67
Papiliotrema miconiae CBS 8358T (AF444387/AF444698)
Papiliotrema wisconsinensis CBS 13895T (KM384107/KM408131)
86
Papiliotrema leoncinii CBS 13918T (KJ608554/KP203864)
89
Papiliotrema japonica CBS 2013T (AF444666/AF444760)
Papiliotrema fuscus CBS 9647T (KY104467/KY108737)
90
Papiliotrema taeanensis CBS 9742T (KY104475/AY422719)
Papiliotrema hoabinhensis JCM 10835T (AB110695/AB193347)
Papiliotrema mangalioensis CBS 10870T (NR 144816/FJ008046)
99
Papiliotrema pseudoalba CBS 8294T (AF444442/AF075504)
56
Papiliotrema bandonii CBS 9107T (KY104458/AF416642)
Papiliotrema siamense CBS 13330T (AB915387/AB909023)
Papiliotrema perniciosus CBS 9605T (KY104473/AF472624)
73
Papiliotrema nemorosus CBS 9606T (KY104472/AF472625)
69
54
Papiliotrema anemochoreius CBS 10258T (KY104455/KY108727)
50
Papiliotrema phichitensis DMKU-SP105T (AB915388 /AB826437)
Papiliotrema odontotermitis CBS 14181T (KU883277/KU883278)
50
Papiliotrema aspenensis CBS 13867T (KC485500/KC469778)
Papiliotrema rajasthanensis CBS 10406T (KY104474/KY108743)
Papiliotrema laurentii CBS 139T (AF410468/LK023772)
Papiliotrema frias CBS 12693T (GU997162/LK023834)
69
96
Papiliotrema fonsecae CBS 12692T (JN193468/JN193447)
Rhynchogastrema complexa CBS 11570T (KY104930/KY109180)
Rhynchogastrema glucofermentans CBS 10381T (KY104932/KY109182)
67
65
Rhynchogastrema fermentans CBS 12398T (HQ623541/HM461720)
Rhynchogastrema nanyangensis CBS 12474T (JN564591/JN564592)
Rhynchogastrema aquatica CBS 12527T (KY104929/KY109179)
Rhynchogastrema tunnelae CBS 8024T (AF444453/AF444715)
Rhynchogastrema noutii CBS 8364T (AF444391/AF444700)
53
Rhynchogastrema coronatum DSM 28188T (LN870267)
Rhynchogastrema visegradiensis CBS 12505T (HQ660084/GU195658)
Naematelia encephala CBS 8207T (AF042402/AF042220)
99
Naematelia aurantia CBS 6965T (AF189842/AF444315)
Kwoniella mangrovensis CBS 8507T (AF444646/AF444742)
0.01

◀**Fig. 1** Phylogenetic tree based on the concatenated sequence of the ITS region and the D1/D2 region of the LSU rRNA gene, showing positions of a novel species with respect to closely related species. The phylogenetic tree was constructed using the neighbor-joining method provided within the MEGA7 software package. Numbers at the node indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers in the order of ITS/D1/D2. *Kwoniella mangrovensis* CBS 8507^T (AF444646/AF444742) was used as outgroup in these analyses. *Bar*, patristic distance of 0.01

D1/D2 region of the LSU rRNA gene and the ITS region. Only a few phenotypic characteristics of the novel species are different from each of these closely related species (Table 1). *Papiliotrema phichitensis* f.a. sp. nov. differ from *P. laurentii* by only a result of starch formation, which is negative for the novel species but positive for *P. laurentii*. The novel species is different form *P. odontotermitis* by its assimilation of L-sorbose and D-arabinose, and grows at 37 °C, but it does not grow on 10% (w/v) sodium chloride 5% (w/v) glucose. When comparing with *P. rajasthanensis*, the novel species assimilates soluble starch, glycerol (slow) and DL-lactate, and grows on 50% glucose medium, while *P. rajasthanensis* does not. The novel species has similar phenotypic characteristics to *P. aspenensis*, however, only a few phenotypic characteristics of *P. aspenensis* are available for comparison. Therefore, clear separation of these species by only phenotypic criteria might not be reliable.

Description of *Papiliotrema phichitensis* P. Khunnamwong, J. Surussawadee, N. Srisuk, C. Boonmak and S. Limtong f.a. sp. nov.

Cell morphology and sexual state

After 3 days at 25 °C on YM agar, cells are subglobose to ovoid (2–7 × 3–8 μm) and occur singly or in pairs (Fig. 2). Budding is polar. The colony is cream, smooth and has an entire margin. Pseudohyphae or true hyphae are not formed in slide culture on PDA within 4 weeks at 25 °C. Ballistoconidia are not produced on PDA or corn meal agar for 4 weeks. Sexual reproduction is not observed on PDA, corn meal agar, 5% malt extract agar, YPD agar and YM agar at 15 and 25 °C for 6 weeks.

Phenotypic and growth characteristics

Fermentation of glucose is absent. Glucose, galactose, sorbose, *N*-acetyl glucosamine, D-ribose, xylose, L-

Table 1 Phenotypic characteristics that differentiate *Papiliotrema phichitensis* sp. nov. from the other *Papiliotrema* species in the *laurentii* clade

Characteristics	1	2	3	4	5
Assimilation of carbon compounds					
L-Sorbose	+	–	+	w	–/s
D-Arabinose	+	–	+	+	+
Soluble starch	+	+	nd	–	+
Glycerol	s	w	nd	–	–/s
DL-Lactate	+	w	+	–	s/+
Growth with					
Growth 35 °C	+	w	nd	–	nd
Growth 37 °C	+	–	nd	nd	v
50% Glucose	+	–	nd	–	v
10% NaCl/5% glucose	–	+	nd	nd	–
Starch formation	–	–	nd	w	+
Pseudohypha formation	Absent	Present	Absent	Absent	Absent

Species: 1 *P. phichitensis* sp. nov., 2 *P. odontotermitis*, 3 *P. aspenensis*, 4 *P. rajasthanensis*, 5 *P. laurentii*. Data for species 1 is from the present study, for species 2 from Handel et al. (2016), for species 3 from Ferreira-Paim et al. (2014), for species 4 from Saluja and Prasad (2007) and for species 5 from Fonseca et al. (2011)

Growth reactions: –, no growth; w, weak growth; d, delayed growth; s, slow growth; +, strong growth; nd, not determined; v, variable

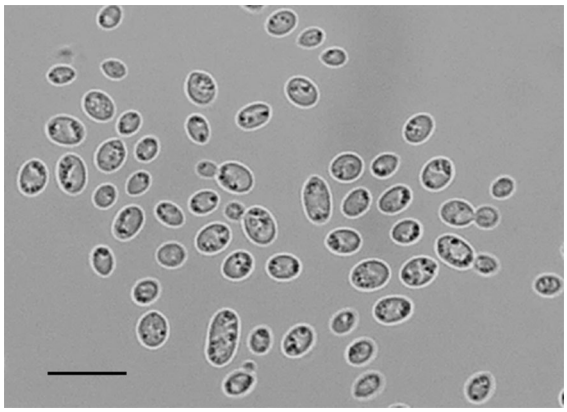


Fig. 2 Budding cells of *Papiliotrema phichitensis* sp. nov. DMKU-SP105^T on YM agar after 3 days at 25 °C (bar, 10 μm)

arabinose, D-arabinose, L-rhamnose, sucrose, maltose, trehalose, cellobiose, salicin, methyl-α-D-glucoside, melibiose, lactose, raffinose, melezitose, soluble starch, glycerol (slow), erythritol, ribitol, D-glucitol, D-mannitol, galactitol (weak), myo-inositol, gluconoδ-latone, 2-ketogluconate (weak), 5-ketogluconate (weak), glucuronate (weak), galacturonic acid, DL-lactate, succinate, citrate, xylitol and ethanol are assimilated, but inulin, gluconate and methanol are not assimilated. Ethylamine and L-lysine are assimilated, but nitrate, nitrite and cadaverine are not assimilated.

Growth at 15 and 37 °C is positive, but negative at 40 °C. No growth occurs on media containing 10% (w/v) sodium chloride 5% (w/v) glucose, 16% (w/v) sodium chloride/5% (w/v) glucose and 60% (w/v) glucose, and in the presence of 0.01% cycloheximide and 0.1% cycloheximide. Growth occurs on medium containing 50% (w/v) glucose and in vitamin free medium. Acid formation is absent. Starch-like compounds are not produced. Diazomium blue B color and urease reaction are positive. The major ubiquinone is Q-7.

Type

DMKU-SP105^T is designated as the type of *Papiliotrema phichitensis*. The strain was isolated from the external surface of sugarcane (*Saccharum officinarum* L.) leaf collected from Phichit province. It has been preserved as a metabolically inactive state in the culture collection of the Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand (DMKU).

The ex-type has been deposited in the Thailand Bioresource Research Center (TBRC), National Center for Genetic Engineering and Biotechnology, Thailand as BCC 61187^T, the CBS collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (CBS) as CBS 13390^T, and the NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan (NBRC) as NBRC 109699^T. The MycoBank number is MB 825565.

Etymology

The species epithet *phi.chit.en'sis* N.L. fem. adj., *phichitensis*, refers to Phichit province, Thailand, where this yeast was found.

Ecology

The members of the genus *Papiliotrema* have been isolated from diverse habitats. Although the ecology of the novel species is unknown because only a single strain has been isolated, the species related to *P. phichitensis* have been obtained from plants. *P. aspenensis* was proposed as novel species based on strains isolated from the bark of a trembling aspen (*Populus tremuloides*) in the USA (Ferreira-Paim et al. 2014). *P. rajasthanensis* was isolated from inflorescences of false amaranth (*Digera* sp.) and false water willow (*Andrographis echioides*) in India (Saluja and Prasad 2007). *P. laurentii* was first isolated from palm wine in Congo and the additional strains in this species were found from various sources including plants (Fonseca et al. 2011). However, another species closely related to *P. phichitensis*, *P. odontotermitis*, was isolated from the gut of the higher termite *Odontotermes obesus* in India (Handel et al. 2016). In this study, the novel species, *P. phichitensis*, was isolated from the external surface of sugarcane leaf. Therefore, the yeasts belonging to this *Papiliotrema* subclade seem to be associated with plants.

Author contributions PK: performed research and wrote the paper; JS: performed research; NS: analyzed data; CB: analyzed data; SL: designed study and wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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