**Papiliotrema siamense f.a., sp. nov., a yeast species isolated from plant leaves**

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Two strains representing a novel species were isolated from the external surface of a sugar cane leaf (DMKU-SP85ᵀ) and tissue of a rice leaf (DMKU-RE97) collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, the two strains were determined to represent a novel species of the genus *Papiliotrema* although sexual reproduction was not observed. The sequences of the D1/D2 region of the LSU rRNA gene and ITS region of the two strains were identical, but differed from those of the type strain of *Cryptococcus nemorosus* by 0.6% nucleotide substitutions (four nucleotide substitutions out of 597 nucleotides) in the D1/D2 region of the LSU rRNA gene and 1.8% nucleotide substitutions (nine nucleotide substitutions out of 499 nucleotides) in the ITS region. The name *Papiliotrema siamense* f.a., sp. nov. is proposed. The type strain is DMKU-SP85ᵀ (=BCC 69499ᵀ = CBS 13330ᵀ).

The genus *Papiliotrema* was 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. In the same article, *Papiliotrema bandonii* was proposed as a novel species (Sampaio *et al.*, 2002) and was the only species of this genus at the time of writing (Sampaio, 2011). Phylogenetic relationships among the order Tremellales show that the genus *Papiliotrema* is located in the clade of *Bulleromyces/Papiliotrema/Auriculibuller* which is referred to as the *Bulleromyces* clade. This clade consists not only of species of the teleomorphic genera, *Bulleromyces*, *Papiliotrema* and *Auriculibuller* but also of species of the two polyphyletic anamorphic genera *Bullera* and *Cryptococcus* (Boekhout, 2011). Sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene placed *Papiliotrema bandonii* in a subclade with *Cryptococcus nemorosus* and *Cryptococcus pinnicosus* (Fonseca *et al.*, 2011; Sampaio, 2011).

While epiphytic micro-organisms inhabit the surface of various parts of plants (Andrews & Harris, 2000; Lindow & Brandl, 2003), endophytes colonize intercellular and/or intracellular healthy plant tissue without causing disease symptoms or abnormal changes in the plant (Petri, 1991; Isaeva *et al.*, 2010). Both ascomycete and basidiomycete yeasts have been found to be epiphytes and endophytes, and one yeast species can be both an epiphyte and an endophyte (Isaeva *et al.*, 2010). Some examples of epiphytic yeast species isolated from plant leaves and belong to the Ascomycota are *Candida tropicalis*, *Hanseniaspora opuntiae*, *Kodamaea olmeri* and *Meyerozyma caribbica* and *Cryptococcus laurentii*, *Rhodosporidium fluviale*, *Rhodotorula taiwanensis* and *Sporidiobolus ruineniae* belong to the Basidiomycota (de Azeredo *et al.*, 1998; Fonseca & Inacio, 2006; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010; Limtong *et al.*, 2014). Though there are reports of endophytic yeasts, they are fewer compared with those of epiphytic yeasts. Examples of endophytic yeast species which have been found in plant leaves are *Debaromyces hansenii* and *Pichia guilliermondii*, belonging to the Ascomycota and *Cryptococcus flavescens*, *Cryptococcus laurentii*, *Rhodotorula mucilaginosa*, *Sporidiobolus pararoseus* and *Sporobolomyces roseus*, belonging to the Basidiomycota (Camatti-Sartori *et al.*, 2005; Gai *et al.*, 2009).

During the investigations of epiphytic yeasts on the external leaf surfaces of sugar cane and endophytic yeasts in leaf tissue of rice in Thailand, two strains (DMKU-SP85ᵀ and DMKU-RE97) representing a novel species of the genus *Papiliotrema* were obtained.

**Abbreviations**: LSU, large subunit; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region of strain DMKU-SP85ᵀ are AB909023 and AB9115387, respectively; those for the corresponding sequences of strain DMKU-RE97 are AB863558 and AB921216, respectively. The MycoBank number for *Papiliotrema siamense* is MB808372.
Yeast isolation and characterization

A total of 267 yeast strains were isolated from the external surface of 102 samples of sugar cane (*Saccharum officinarum* L.) leaves, and 125 yeast strains were obtained from the tissue of 100 samples of surface-sterilized rice (*Oryza sativa* L.) leaves. Strain DMKU-SP85\(^T\) was isolated from the external surface of a sugar cane leaf collected from Sankhaburi district, Chai Nat province (15° 11' 14" N 100° 07' 42" E), on 2 March 2012 and strain DMKU-RE97 was obtained from tissue of a rice leaf collected from Bang Pla Ma district, Suphanburi province (14° 28' 03" N 100° 07' 01" E), on 2 March 2012.

Strain DMKU-SP85\(^T\) was isolated by plating of leaf washings as described by Inácio et al. (2002). A 3 g sample of leaf was 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 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% v/v glycerol and maintained at −80°C. Strain DMKU-RE97 was isolated from a surface-sterilized leaf using the method of Abdel-Motaal et al. (2009) with slight modifications. The leaf was surface sterilized by immersion in 70% ethanol for 3 min and rinsed five times for 5 min with sterilized deionized water. The effectiveness of the surface sterilization procedure was verified by spreading the final rinse water onto YM agar and direct placing of sterilized leaf pieces onto YM agar; no growth of micro-organisms indicated effective sterilization. After surface sterilization, the leaf was cut into small pieces (50 × 50 mm), slightly ground in a sterile mortar to expose the inner tissue and a few pieces of the tissue were placed directly onto YM agar supplemented with 0.02% chloramphenicol in a sterilized Petri dish. The Petri dish was incubated at 25°C until yeast colonies appeared. Yeast colonies of different morphologies were picked up, purified and maintained by the same methods as for strain DMKU-SP85.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the 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'-GCATATCAATAACCGGAGGAAG-3') and the reverse primer NL4 (5'-GTTCCGTGTTITACAGCGG-3') (Kurtzman & Robnett, 1998). The ITS region was amplified with forward primer ITS1 (5'-TCCGTAAGTGAACTCGCG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR products were checked by agarose gel electrophoresis and purified by using the HiYield Gel/PCR Fragments Extraction kit (RBC Bioscience). The purified products were sequence commercially by Macrogen (Seoul, Korea) using primers, NL1 and NL4 for the D1/D2 and primers, ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul et al., 1997) and were aligned with the sequences of related species retrieved from the GenBank database using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura’s two-parameter correction (Kimura, 1980), by the neighbour joining method (Saitou & Nei, 1987) using MEGA software version 5.0 (Tamura et al., 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). *Cryptococcus bромeliarum* Bi20\(^T\) was used as the outgroup species in the analysis.

Phenotypic characterization

The strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Kurtzman et al. (2011b). Hyphal formation was investigated on potato dextrose agar (PDA) and corn meal agar in slide cultures at 25°C for up to 14 days. Formation of ballistoconidia was determined by the method described by Kurtzman et al. (2011b) on inverted PDA plates incubated at 15°C for up to 4 weeks. Sexual processes were investigated for individual strains or strain pairs on 5% malt extract agar, corn meal agar, PDA agar and YM agar at 15°C and 25°C for up to 6 weeks. Carbon and nitrogen assimilation tests were conducted in liquid medium, and for the nitrogen assimilation test a starved inoculum was used (Kurtzman et al., 2011b). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were 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 25°C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isopenolones were identified by HPLC as described previously (Limtong et al., 2007).

Species delineation and classification

The two strains, DMKU-SP85\(^T\) and DMKU-RE97, showed identical sequences of the D1/D2 region of the LSU rRNA gene. In terms of pairwise sequence similarity, the two strains were closest to *Cryptococcus nemorosus* with 0.6% nucleotide substitutions (four nucleotide substitutions out of 597 nucleotides). The sequences of the ITS region of strains DMKU-SP85\(^T\) and DMKU-RE97 were also identical and differed by 1.8% nucleotide substitutions (nine nucleotide substitutions out of 499 nucleotides) from the corresponding sequence of *Cryptococcus nemorosus*. 
The phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene and ITS region demonstrate that the two strains of the novel species clustered with Cryptococcus nemorosus and Cryptococcus perniciosus and formed a subclade with Cryptococcus sp. CBS 8363 and Papiliotrema bandonii (Fig. 1).

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and the sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region, we concluded that the two strains represent a novel species. Due to the position of the novel species in the phylogenetic tree based on the D1/D2 region of the LSU rRNA gene, which is closer to Papiliotrema bandonii, the only species of the genus Papiliotrema, than to the other species of the two teleomorphic genera of the Bulleromyces/Papiliotrema/Auriculibullera clade, Bulleromyces and Auriculibullera, the novel species was assigned to the same genus as Papiliotrema bandonii. According to the International Code of Nomenclature for algae, fungi and plants concerning the nomenclatural rules for fungi and the adoption of ‘one fungus, one name’ (Miller et al., 2011), the novel species is assigned to the genus Papiliotrema, and the designation forma asexualis (f.a.) is included following the recommendation of Lachance (2012). The name Papiliotrema siamense f.a., sp. nov. is proposed. The other species of the genus Cryptococcus in the clade can be assigned regarding the ‘one fungus, one name’ adoption to the genera Bulleromyces, Papiliotrema or Auriculibullera depending on their phylogenetic positions.

Description of Papiliotrema siamense
Surussawadee, Khunnamwong & Limtong sp. nov.

Papiliotrema siamense (si.am.en’se. N.L. fem. adj. siamense referring to Siam, the old name of Thailand, where the two strains were isolated).

Growth is observed on YM broth after 3 days at 25 °C, cells are globose to oval (2–6.5 x 2–8 μm) and occur singly or in pairs. (Fig. 2) Budding is polar. Colonies are butryous, white to cream, with a smooth surface and an entire margin. After 14 days in Dalmau plate cultures on corn meal agar and PDA agar at 25 °C, pseudohyphae and true hyphae are not formed. Ballistospores are not produced on PDA agar after culture at 15 °C for up to 4 weeks. Sexual reproduction is not observed on 5 % malt extract agar, corn meal agar, PDA agar or YM agar after incubation at 15 °C and 25 °C for up to 6 weeks. Fermentation of D-glucose is negative. D-Glucose, D-galactose, L-sorbose,
Strain DMKU-SP85T is the holotype of *Papiliotrema siamense*. Members of the *LSU rRNA gene and the ITS region* but also some species distinguished from *Cryptococcus* nemorosus and *Papiliotrema* f.a., sp. nov. from the closest species, *Cryptococcus* nemorosus

Species: 1, *Papiliotrema siamense* sp. nov.; 2, *Cryptococcus nemorosus* (data from Kurtzman et al., 2011a). +, Positive; w, weakly positive; s, slow; l, latent; −, negative.

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<tr>
<th>Characteristic</th>
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<td>Assimilation of carbon compounds</td>
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<tr>
<td>L-Sorbos</td>
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<td>w</td>
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<td>N-Acetylglucosamine</td>
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<td>D-Ribose</td>
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<td>Maltose</td>
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<td>Cellobiose</td>
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<td>Salicin</td>
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<td>Inulin</td>
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<td>Glycerol</td>
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<td>Galactitol</td>
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<td>2-Ketogluconic acid</td>
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<td>Ethanol</td>
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<td>Assimilation of nitrogen compounds</td>
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<td>Potassium nitrate</td>
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<td>Sodium nitrate</td>
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<td>Growth in/at:</td>
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<td>60 % Glucose</td>
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<td>30 °C</td>
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N-acetylglucosamine, D-ribose (weakly), D-xylene, L-arabinose, D-arabinose (slowly), L-rhamnose (latent), sucrose, trehalose, methyl α-D-glucoside (slowly), melibiose, lactose, raffinose, melezitose, soluble starch (weakly), ribitol, D-mannitol, myo-inositol, D-glucono-1,5-lactone, 5-ketogluconic acid, D-glucuronic acid, D-galacturonic acid, succinate, citrate, xylitol, ethylamine HCl, L-lysine and cadaverine are assimilated, but nitrate and nitrite are not assimilated. Growth at 25 and 30 °C is positive, but growth is negative at 35 °C. No growth is observed in vitamin-free medium. Growth on medium containing 50 % glucose, 60 % glucose (weakly) and 10 % sodium chloride/5 % glucose are assimilated, but nitrate and nitrite is negative. No growth is observed with 0.01 % cycloheximide. Starch-like compounds are produced. Diazonium blue B colour and urease reaction are positive. The major ubiquinone is Q-10.

Strain DMKU-SP85T is the holotype of *Papiliotrema siamense*. The strain was isolated from the external surface of a sugar cane (*Saccharum officinarum* L.) leaf collected from Chai Nat province, Thailand. The living culture from the type has been deposited at BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand (Pathumthani, Thailand) as BCC 69499T and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 13330T. The Mycobank registration number is MB808372.

**Concluding remarks**

In practice, *Papiliotrema siamense* sp. nov. can be distinguished from *Cryptococcus nemorosus*, its closest relative, on the basis of not only the sequences of the D1/D2 regions of the LSU rRNA gene and the ITS region but also some phenotypic characteristics, as shown in Table 1. Members of the *Bulleromyces/Papiliotrema/Auriculibuller* clade comprised ballistoconidia-producing and non-ballistoconidia-producing species. Plant leaves are the main habitat of ballistoconidia-producing species, *Auriculibuller fuscus*, *Bullera albus*, *Bullera hannae*, *Bullera hoabinhensis*, *Bullera japonica*, *Bullera pennisetocola*, *Bullera pseudoalba* and *Bullera unica* (Boekhout, 2011). On the other hand, non-ballistoconidia-producing species, *Cryptococcus anemochorus*, *Cryptococcus aureus*, *Cryptococcus flavescent*, *Cryptococcus laurentii*, *Cryptococcus nemorosus*, *Cryptococcus perniciosus*, *Cryptococcus rajarshani* and *Cryptococcus taeamensis* have been identified from various sources such as plant parts, atmosphere, rhizosphere, soil, fermenting soy beans sauce, caterpillar gut and wild rabbit faeces (Fonseca et al., 2011), and a single strain of *Papiliotrema bandonii* was obtained from a fruiting body that was found to be associated with pyrenomycetous ascomycetes at the base of the inflorescences of the pampas grass (*Sampaio, 2011*). Among non-ballistoconidia-producing species, *Cryptococcus flavescent*, *Cryptococcus laurentii* and *Cryptococcus rajarshani* have been reported from the phylloplane of sugar cane in Thailand (Limityong et al., 2014). In this study, one strain of the novel species was isolated from the phylloplane of sugar cane and the other from rice leaf tissue. Therefore, not only phylloplane but also leaf tissue is a habitat that warrants further investigation for non-ballistoconidia-producing species in this clade. It should be noted that strains of the novel species, *Cryptococcus nemorosus*, *Cryptococcus perniciosus* and *Papiliotrema bandonii*, which formed a subclade with high bootstrap value...
(94 %), were obtained from herbaceous plants (Fonseca et al., 2011; Sampaio, 2011). Therefore, herbaceous plants and yeast species in this subclade seems to have ecological association.

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References


