**Hannaella siamensis** sp. nov. and **Hannaella phetchabunensis** sp. nov., two new anamorphic basidiomycetous yeast species isolated from plants

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Eight strains, representing two novel anamorphic yeast species, consisted of five strains isolated from the external surfaces of rice leaves (DMKU-RP72T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156) and one from a corn leaf (DMKU-CP430T) collected in Thailand, and one strain isolated from each of a composite flower (11-1114) and a fallen dead leaf (12-301); the latter two were collected in Belize. On the basis of sequence analysis of the D1/D2 region of the large subunit rRNA gene and the internal transcribed spacer (ITS) region, they were suggested to be two novel species of the genus **Hannaella**. Seven strains (DMKU-RP72T, DMKU-RP109, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) differed from each other by 0–3 nt substitutions in the D1/D2 region and by 0–1 nt substitutions in the ITS region. In terms of pairwise sequence similarities of the D1/D2 region these seven strains were closest to **Hannaella zeae**, but with 1.2–1.7 % (7–9) nucleotide substitutions. The sequences of the ITS region of these seven strains differed from **H. zeae** by 3.7–3.9 % (16–17) nucleotide substitutions. Therefore, they were assigned to a single novel species and the name **Hannaella siamensis** sp. nov. has been proposed. The type strain is DMKU-RP72T (=BCC 69493T =NBRC 110425T =CBS 13533T).

Strain DMKU-CP430T represents the second novel species and was also most closely related to **H. zeae**, but with 1.0 % (6) nucleotide substitutions in the D1/D2 region and 3.2 % (14) nucleotide substitutions in the ITS region. It was assigned to the proposed novel species, **Hannaella phetchabunensis** sp. nov. (type strain DMKU-CP430T =BCC 69492T =NBRC 110424T =CBS 13386T).

The genus **Hannaella** is an anamorphic genus of basidiomycetous yeast in the Tremellales clade, Subphylum Agaricomycotina, Phylum Basidiomycota; it is closely related to the genera **Dioszegia** and **Derxomyces**. It was proposed on the basis of analysis of multigenes, including the small subunit (SSU) rRNA gene, the D1/D2 region of the large subunit (LSU) rRNA gene, the ITS region including the 5.8S rRNA gene and the mitochondrial cytochrome b gene to accommodate the
species in the *Bullera sinensis* clade including species of *Bullera* and *Cryptococcus* (Wang & Bai, 2008). At present, the genus *Hannaella* is composed of eight described species, namely *Hannaella coprosmensis*, *Hannaella kunmingensis*, *Hannaella luteola*, *Hannaella oryzae*, *Hannaella pagnoccae*, *Hannaella sinensis*, *Hannaella surugaensis* and *Hannaella zaee* (Wang & Bai, 2008; Boekhout et al., 2011a; Landell et al., 2014). Strains of all species of the genus *Hannaella*, except for those of *H. surugaensis*, have been reported to have been isolated from phylloplanes (Wang & Bai, 2008; Boekhout et al., 2011b; Landell et al., 2014), which refer to the external surface of plant leaves (Phaff & Starmer, 1987; Fonseca & Inacio, 2006).

Phylloplanes from various regions of the world have been found to be colonized by members of both the basidioomycteous and the ascomycetous yeasts (Nakase et al., 2001; Fonseca & Inacio, 2006; Glushakova et al., 2007; Slavikova et al., 2009; Landell et al., 2010). The most common phylloplane yeasts are members of the basidioomycteous genera, such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (Slavikova et al., 2009; de Azeredo et al., 1998; Glushakova & Chernov, 2010).

During an investigation of yeasts on the external surfaces of rice and corn leaves in Thailand, six strains, including five strains from the phylloplane of rice and one strain from the phylloplane of corn, were found, which represent two novel species of the genus *Hannaella*. In this paper, the five strains (DMKU-RP72T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156) together with two strains (11-1114 and 12-301) isolated from plants in Belize are described and proposed to represent *Hannaella siamensis* sp. nov. Strain DMKU-CP430T isolated from corn leaves is described and proposed as *Hannaella phetchabunensis* sp. nov.

Six yeasts were isolated from the surfaces of rice and corn leaves collected in Thailand by the plating of leaf washes. Leaves (3 g) were aseptically suspended in 50 ml of 0.85 % (w/v) saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 150 rpm and room temperature (27 ± 3 °C) for 1 h to detach yeast cells from surfaces. An aliquot of 0.1 ml of the washing solution was then spread on yeast extract malt extract (YM) agar (0.3 % (w/v) yeast extract, 0.3 % (w/v) malt extract, 0.5 % (w/v) peptone, and 2.0 % (w/v) agar) supplemented with 0.025 % (w/v) sodium propionate and 0.02 % (w/v) chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by streaking on YM agar plates. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C. Five strains (DMKU-RP72T, DMKU-RP109, DMKU-RP119, YE-124 and YE-156) were obtained from five samples of rice leaves and strain DMKU-CP430T from a corn leaf sample (Table 1). Strain 11-1114 was isolated from a composite flower and strain 12-301 from fallen dead leaves, both collected in Belize. The sample was macerated in sterile water and the macerated plant material was placed on yeast extract peptone glucose (YPG) agar (1.0 % yeast extract, 1.0 % peptone, 2.0 % glucose and 2.0 % agar). After incubation at 25 °C for 7 days, the yeast colonies were isolated and purified by streaking on YPG agar plates. The purified clones were maintained on YPG agar.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) (ITS1-5.8S-ITSS2) region were determined from PCR products, which had been amplified from genomic DNA. Genomic DNA was isolated from cultures grown overnight on YM agar or in YPG broth. The isolated DNA was used for the amplification of the D1/D2 region of the LSU rRNA gene and the ITS region. The primers used were NL-1 and NL-4 for the D1/D2 region (O’Donnell, 1993), and ITS1 and ITS4 for the ITS region (White et al., 1990). The PCR products were checked by agarose gel electrophoresis, purified and sequenced with NL1 and NL4 primers for the D1/D2 region, and with the ITS1 and ITS4 primers 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 GenBank using the multiple alignment program CLUSTAL X version 2.0 (Larkin et al., 2007). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura’s two-parameter correction (Kimura, 1980), with 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).

The strains from Thailand were characterized morphologically, biochemically, and physiologically according to the standard methods described by Kurtzman et al. (2011). Formation of hyphae was investigated on potato dextrose agar (PDA) and YPG agar in slide cultures at 25 °C for up to 28 days with cultures examined microscopically at intervals of a few days. Basidiospore formation was investigated individually or in pairs on YM agar, YPG agar, 5 % malt extract agar, acetate agar, corn meal agar and Gorodkowa agar at 25 °C for up to 4 weeks. Formation of ballistoconidia was investigated by the ballistoconidia-fall method using corn meal agar. Fermentation was tested with Durham tubes in 5 ml of medium at 25 °C. Carbon and nitrogen assimilation tests were conducted in liquid medium or solid medium. Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of YPG broth on a rotary shaker at room temperature for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC, as described previously (Limtong et al., 2007).

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the eight strains (DMKU-RP72T, DMKU-RP109, DMKU-RP119, DMKU-CP430T, YE-124, YE-156, 11-1114 and 12-301) represented two novel yeast species. The first species consisted of seven strains, of which four strains (DMKU-RP72T, DMKU-RP119, YE-124 and...
Table 1. Strains of *Hannaella siamensis* sp. nov. and *Hannaella phetchabunensis* sp. nov.

<table>
<thead>
<tr>
<th>Strain (accession number)</th>
<th>GenBank accession number</th>
<th>Source/locality</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hannaella siamensis</em> sp. nov.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMKU-RP72T (BCC 69493T, NBRC 110425T, CBS 13533T)</td>
<td>AB922844, AB922850</td>
<td>Rice leaves (<em>Oryza sativa</em>)/Nakhon Pathom province, Thailand</td>
<td>12 January 2012</td>
</tr>
<tr>
<td>DMKU-RP119 (BCC 69494)</td>
<td>AB922845, AB922851</td>
<td>Rice leaves (<em>Oryza sativa</em>)/Suphan Buri province, Thailand</td>
<td>12 January 2012</td>
</tr>
<tr>
<td>YE124 (BCC 63420)</td>
<td>AB922847, AB922853</td>
<td>Rice leaves (<em>Oryza sativa</em>)/Nonthaburi province, Thailand</td>
<td>13 February 2012</td>
</tr>
<tr>
<td>YE156 (BCC 63452)</td>
<td>AB922848, AB922854</td>
<td>Rice leaves (<em>Oryza sativa</em>)/Suphan Buri province, Thailand</td>
<td>13 February 2012</td>
</tr>
<tr>
<td>11-1114</td>
<td>KM206722</td>
<td>Unknown composite flower/Bird Island, Belize City, Belize</td>
<td>Unknown</td>
</tr>
<tr>
<td>12-301</td>
<td>KM206725</td>
<td>Dead fallen leaf of unknown tree/Benque Viejo del Carmen, Belize City, Belize</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Hannaella phetchabunensis</em> sp. nov.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMKU-CP430T (BCC 69492T, NBRC 110424T, CBS 13386T)</td>
<td>AB922849, AB922855</td>
<td>Corn leaves (<em>Zea mays</em>)/Phetchabun Province, Thailand</td>
<td>24 May 2012</td>
</tr>
</tbody>
</table>

YE-156 had identical D1/D2 region sequences of the LSU rRNA gene, and these differed by two nucleotide substitutions from strain DMKU-RP109 and by three nucleotide substitutions from the two strains (11-1114 and 12-301) that had identical sequences. The ITS region sequences of the six strains (DMKU-RP72T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) were identical and differed from strain DMKU-RP109 by only one nucleotide substitution. In terms of pairwise sequence similarity of the D1/D2 region of the LSU rRNA gene the closest species was *H. zeae*. From the type strain of *H. zeae*, the four strains (DMKU-RP72T, DMKU-RP119, YE-124 and YE-156) differed by 1.2 % nucleotide substitutions (7 nt substitutions out of 576 nt), strain DMKU-RP109 differed by 1.6 % nucleotide substitutions (9 nt substitutions out of 576 nt), and the two strains (11-1114 and 12-301) differed by 1.7 % nucleotide substitutions (10 nt substitutions out of 576 nt). The sequences of the ITS region of the six strains (DMKU-RP72T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) were identical and differed to that of strain DMKU-RP109 by only one nucleotide substitution. The sequence difference in the ITS region between the seven strains and *H. zeae* was 3.7 % nucleotide substitutions (16 nt substitutions and three gaps out of 438 nt) for the six strains (DMKU-RP72T, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) and 3.9 % nucleotide substitutions (17 nt substitutions and two gaps out of 438 nt) for strain DMKU-RP109. The strain of the second novel species, strain DMKU-CP430T, was closest to *H. zeae*, but with 1.0 % nucleotide substitution (6 nt substitutions out of 576 nt) in the D1/D2 region of the LSU rRNA gene. The ITS region sequences of strain DMKU-CP430T and *H. zeae* differed by 3.2 % nucleotide substitutions (13 nt substitutions and four gaps out of 437 nt). The two novel species differed from each other by 1.2–1.7 % in their nucleotide substitutions (7–10 nt substitutions out of 597 nt) in the D1/D2 region of the LSU rRNA gene and by 5.0–5.3 % nucleotide substitutions (22–23 nt substitutions and three gaps out of 437 nt) in the ITS region.

The phylogenetic tree, based on the combined sequences of the ITS and D1/D2 regions of the LSU rRNA gene, demonstrated further that all eight strains were in the *Hannaella* clade. The four strains (DMKU-RP72T, DMKU-RP119, YE-124 and YE-156) were located in the same position and connected to strain DMKU-RP109 and strain 11-1114 with high bootstrap support. They clustered with the type strain of *H. zeae*, their most closely related species, with high bootstrap values (Fig. 1). Strain DMKU-CP430T was also connected to *H. zeae* (Fig. 1).

The novel species delineation was based primarily on the analysis of the combined sequences of the ITS region and D1/D2 region of the LSU rRNA gene, which indicated the reciprocal monophyletic nature of the species in this clade; therefore, a phylogenetic species concept applies. Consequently, on this basis, we concluded that the eight strains represent two novel species of the genus *Hannaella*. The name *Hannaella siamensis* sp. nov. (MB809601) is proposed for seven of the strains (DMKU-RP72T, DMKU-RP109, DMKU-RP119, YE-124, YE-156, 11-1114 and 12-301) and the name *Hannaella phetchabunensis* sp. nov. (MB809602) is assigned to the remaining single strain DMKU-CP430T.

In practice, *H. siamensis* sp. nov. and *H. phetchabunensis* sp. nov. can be distinguished from their closest species, *H. zeae*, on the basis not only of the sequences of the D1/D2
region of the LSU rRNA gene and the ITS region, but also from some phenotypic characteristics, as shown in Table 2. Members of the genus *Hannaella* are found in diverse habitats. *H. coprosmaensis*, for example, was isolated from a dead leaf and the fruit of *Coprosma tenuifolia* and *H. kunmingensis* was isolated from a dead leaf of a climbing plant, but some strains were isolated from a snail and a bee. *H. oryzae* was isolated from the surfaces of grasses, such as rice, bamboo and Chinese silver grass, *Hannaella sinensis* var. *sinensis* was isolated from the living and dead leaves of the grasses: rice, Chinese silver grass and wheat. *Hannaella sinensis* var. *lactis* was isolated from a ladybird beetle (Boekhout et al., 2011b), while *H. luteola* was isolated from various habitats, such as air, plant leaves, seawater and acidic sludge; *H. surugaensis* was isolated from deep-sea sediment (Fonseca et al., 2011) and *H. zeae* was isolated from corn and corn pests (Molnár & Prillinger, 2006). Strains of the latest species to be proposed, *H. pagnoccae*, were obtained from plants and soil (Landell et al., 2014). In this study, the six strains were isolated in Thailand from rice and corn leaf surfaces, while two strains were isolated in Belize from a composite flower and a fallen dead leaf. Therefore, plant materials, especially leaf surfaces, appear to be the natural habitats of yeasts in this genus.

**Description of *Hannaella siamensis***

*Kaewwichian, Jindamorakot, Am-in, Sipiczki & Limtong sp. nov.*

*Hannaella siamensis* (si'am.en'sis. N.L. fem. adj. *siamensis* referring to Siam, the old name of Thailand, where the type strain was isolated.)

Growth in YM broth: after 3 days at 25 °C, cells are oval, subglobose, ellipsoidal (3–5 μm × 5–7 μm) and occur singly, in pairs or in groups (Fig. 2). Budding is polar budding. Growth on YM agar: after 3 days at 25 °C, the streak culture is yellowish-cream, semi-glistening, soft, low convex and has an entire margin. Pseudohyphae and true hyphae are not formed in slide cultures on PDA. Basidiospores are not produced by any of the strains either individually or when paired on YM agar, 5% malt extract agar, corn meal agar, acetate agar and Gorodkowa agar after 4 weeks at 25 °C. Ballistoconidia are absent. Fermentation of D-glucose,
D-galactose, maltose, sucrose, trehalose, lactose, melezitose, raffinose and xylene are absent. D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose (slow), melibiose, raffinose, melezitose, soluble starch, D-xylene, L-arabinose, D-arabinose, L-rhamnose, D-ribose, ethanol (weak), glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, DL-lactic acid (weak), succinic acid, citric acid, inositol, D-glucuronic acid, D-galacturonic acid, xylitol, L-arabinitol, D-gluconic acid, D-glucosamine, N-acetyl-D-glucosamine (delay) and propane-1, 2-diol (weak) are assimilated, but methanol, butane-2,3-diol and hexadecane are not assimilated. Ethylamine, L-lysine and cadaverine are assimilated, but nitrate and nitrite are not assimilated. Growth in vitamin-free medium is positive. Growth on medium containing 50% (w/v) glucose, 60% (w/v) glucose, 10% (w/v) sodium chloride/5% (w/v) glucose, 15% (w/v) sodium chloride/5% (w/v) glucose is positive. Growth with 0.01% cycloheximide is weakly positive, but with 0.1% cycloheximide is negative. Growth at 25 and 30°C is positive, but at 35, 37, 40 and 42°C is negative. Acid formation is absent. Starch-like compounds are not produced. The diazonium blue B colour and urease reactions are positive. The major ubiquinone is Q-10.

Strain DMKU-RP72T is the holotype of Hannaella siamensis (MB809601). The strain was isolated from the phylloplane of rice (Oryza sativa) collected from Nakhon Pathom Province, Thailand.

**Description of Hannaella phetchabunensis**

Kaewwichian, Jindamorakot, Am-In & Limtong sp. nov.

Hannaella phetchabunensis (phet.cha.bun.en’sis. N.L. fem. adj. phetchabunensis referring to Phetchabun Province, where the strain was isolated.

Growth in YM broth: after 3 days at 25°C, cells are ovoid to ellipsoid (2–3 × 3–6 μm) and occur singly, in pairs or in groups (Fig. 3). Budding is polar. Growth on YM agar after 3 days at 25°C: the streak culture is yellowish-cream, semi-glistening, soft, low convex and has an entire margin. Pseudohyphae and true hyphae are not formed in slide cultures on PDA. Basidia and spores are not produced on YM
aggar, 5% malt extract agar, aceate agar, corn meal agar or Gorodkowa agar after 4 weeks at 25°C. Ballistoconidia are absent. The fermentation of D-glucose, D-galactose, sucrose, maltose, lactose, melezitose and raffinose is absent. D-Glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, D-ribose, ethanol (slow), glycerol, erythritol, ribitol (slow), galactitol, D-mannitol, D-glucitol, α-D-methyl-D-glucoside (slow), salicin (slow), 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, DL-lactic acid (slow), succinic acid, citric acid, inositol (slow), D-glucurononic acid, D-galacturonic acid, xylitol (slow), L-arabinitol, D-glucuronic acid, D-galactosamine (weak), and N-acetyl-D-glucosamine are all assimilated, but lactose, inulin, methanol, propane-1,2-diol, butane-2,3-diol and hexadecane are not assimilated. Nitrate is not assimilated. Growth in vitamin-free medium is positive. Growth on medium containing 50% (w/v) glucose, 10% (w/v) sodium chloride/5% (w/v) glucose and 15% (w/v) sodium chloride/5% (w/v) glucose (weakly) is positive. Growth with 0.01% cycloheximide is slow, but positive; with 0.1% cycloheximide it is negative. Growth at 25 and 30°C is positive, but at 35, 37, 40 and 42°C is negative. Acid formation is absent. Starch-like compounds are not produced. The diazonium blue B colour and urease reactions are positive. The major ubiquinone is Q-10.

Strain DMKU-CP430T is the holotype of Hannaella phetchabunensis (MB809602). The strain was isolated from the phylloplane of corn (Zea mays) collected from Phetchabun Province, Thailand.

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References


