Trichosporon heliocopridis sp. nov., a urease-negative basidiomycetous yeast associated with dung beetles (Heliocopris bucephalus Fabricius)

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Ninety-six yeast isolates associated with dung beetles (Heliocopris bucephalus Fabricius) were examined based on a culture-dependent method. A comparison of the colony morphology and PCR-fingerprints obtained by (GTG)5 microsatellite-primed PCR indicated that 84 of these isolates belonged to one group. Five strains (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) were selected as the representatives of this main group, where each of the five selected strains had been derived from a different dung beetle collected in northern Thailand. A comparison of the D1/D2 domain sequence of the large subunit rRNA gene (LSU D1/D2) and the internal transcribed spacer (ITS) sequences revealed that these five strains were the same and were related to the genus Trichosporon. Phylogenetic analysis based on the LSU D1/D2 plus ITS sequences placed this group within the Trichosporon brassicae clade, but it was clearly separated from any known species. In addition, physiological tests showed that this group had the unusual property of the inability to hydrolyse urea, which was distinctly different from the related taxon. Therefore a novel yeast species named Trichosporon heliocopridis sp. nov. (ex-type strain DD1-1T=TISTR 5946T=JCM 30786T=CBS 14168T) is proposed. The MycoBank number is MB812098.

The anamorphic yeasts belonging to the genus Trichosporon are commonly recognized as arthroconidia-producing yeasts with true hyphae. So far, a yeast-like phase has not been reported in some species of this genus (Fell et al., 2000). Yeast species belonging to the genus Trichosporon have been isolated from various sources, including soil, water, plants and clinical specimens (Sugita, 2011). Also, several species, such as Trichosporon mycotoxinivorans, Trichosporon veenhuysii and Trichosporon scarabaeorum, have been isolated from insects (Molnar et al., 2004; Middelhoven et al., 2000, 2004).

Insects are considered as one of the most diverse eukaryotic groups of living organisms with more than one million species currently recognized (Footit & Adler, 2009). The gut of some insects within the orders Coleoptera (beetles), Homoptera (aphids) and Hymenoptera (ants) have been reported to be a rich source of yeasts (Suh & Blackwell, 2005; Starmer & Lachance, 2011), and various novel yeast species have been recently discovered from insect guts, such as Wickerhamomyces mori, Scheffersomyces parashehatae and Wickerhamiella allomyrinae (Hui et al., 2013; Suh et al., 2013; Ren et al., 2014). However, although several studies have been reported on the yeast communities in insect guts, such as Lachance et al. (2003), Abe et al. (2010), Endoh et al. (2011) and Urbina et al. (2013), the vast...
majority of insects, including dung beetles, are still left unexplored in the context of yeast biodiversity. The dung beetle *Heliocopris bucephalus* Fabricius (Coleoptera, Scarabaeidae) plays an important role in nutrient cycling by decomposing large herbivore dung (Nichols et al., 2008). They also act as secondary seed dispersers by burying them in the brood ball, which also protects the seeds from rodent predators (Hanski & Cambefort, 1991). Due to the unique behavioural ecology of making a brood ball from dung and an interest in terms of the nutritional symbiosis, the bacterial microbiome in dung has recently been reported (Estes et al., 2013). However, no report has been made on the yeasts associated with dung beetles.

During the course of exploring the yeasts associated with *Heliocopris bucephalus*, a novel urease-negative basidioymycetous yeast belonging to the genus *Trichosporon* was found, which we propose as a new species, *Trichosporon heliocopridis* sp. nov.

Dung beetles (*Heliocopris bucephalus*) were collected from cattle pens at various locations in the north of Thailand in 2013 (Table 1). Surface sterilization was performed twice by immersing the samples in 95 % (w/v) ethanol for 5 min, 70 % (w/v) ethanol for 10 min, and then rinsing with 0.85 % (w/v) NaCl solution. To confirm the validity of the surface sterilization, the rinsed liquid was streaked on YPD agar [3 % (w/v) glucose, 0.3 % (w/v) yeast extract, 0.3 % (w/v) bacto peptone, 2 % (w/v) agar, adjusted to pH 5.0 with HCl] supplemented with 100 p.p.m. chloramphenicol and cultured for 48 h at 30 °C. Growth of yeasts was examined on YPD agar supplemented with 100 p.p.m. chloramphenicol and then incubating at 30 °C on an orbital shaker at 200 r.p.m. for 48 h. Subsequently, pure cultures were grouped on YPD agar supplemented with 100 p.p.m. chloramphenicol and then incubating at 30 °C for 48 h. Subsequently, pure cultures were grouped by observation of their colony morphology. Representative colonies were kept on a YPD agar slant for up to 6 weeks.

Yeast genomic DNA was extracted by the glass bead method as described previously (Endoh et al., 2011). Extracted DNA was used for (GTG)₅ PCR-fingerprinting and sequencing of the D1/D2 domain of the large-subunit rRNA gene (LSU D1/D2) and small-subunit rRNA gene (SSU). PCR-fingerprinting using the microsatellite primer (GTG)₅ was performed as reported by Endoh et al. (2011), and the banding patterns were visually inspected for grouping of yeast isolates. The LSU D1/D2, ITS and SSU were PCR-amplified with the primers NL1, NL4, ITS1, ITS4, NS1 and NS8 (Kurtzman & Robnett, 2003; White et al., 1990) as previously described (Endoh et al., 2008) for LSU D1/D2 and SSU. The ITS region was amplified using the primers ITS1 and ITS4 with the same PCR program as that for SSU, and was sequenced using primers ITS1 and ITS4. PCR products were directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analysed with an auto-sequencer ABI Prism 3100 following the manufacturer’s instructions. Data were edited manually using BioEdit v7.2.5 (Hall, 1999). The complete LSU D1/D2, SSU and ITS sequences were compared to those available in the DDBJ/EMBL/GenBank database using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). LSU D1/D2 plus

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**Table 1.** Isolation source locality, sampling date and GenBank accession numbers for sequences of strains of *Trichosporon heliocopridis* sp. nov. and *Trichosporon* sp. used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>GenBank accession numbers for ITS, LSU D1/D2, SSU sequences</th>
<th>Locality of dung beetle sampled</th>
<th>Date of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichosporon heliocopridis</em> sp. nov. DDI-1^T (＝TISTR 5946^T＝JCM 30786^T＝CBS 14168^T)</td>
<td>LC071456, LC034290, LC034294</td>
<td>Chiang rai, Thailand</td>
<td>August 2013</td>
</tr>
<tr>
<td>DD2-33 (＝TISTR 5947＝JCM 30787)</td>
<td>LC071457, LC034291, LC034296</td>
<td>Chiang rai, Thailand</td>
<td>August 2013</td>
</tr>
<tr>
<td>DD4-11 (＝TISTR 5948＝JCM 30788)</td>
<td>LC071458, LC034288, LC034297</td>
<td>Phaya, Thailand</td>
<td>July 2013</td>
</tr>
<tr>
<td>DD5-15 (＝TISTR 5941＝JCM 30790)</td>
<td>LC071459, LC034285, LC034299</td>
<td>Chiang mai, Thailand</td>
<td>July 2013</td>
</tr>
<tr>
<td>DD6-1 (＝TISTR 5949＝JCM 30792)</td>
<td>LC071460, LC034286, LC034301</td>
<td>Chiang mai, Thailand</td>
<td>July 2013</td>
</tr>
<tr>
<td><em>Trichosporon</em> sp. DD2-28 (＝TISTR 5943＝JCM 30793＝CBS 14169)</td>
<td>LC071461, LC034289, LC034295</td>
<td>Chiang rai, Thailand</td>
<td>August 2013</td>
</tr>
</tbody>
</table>

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http://ij.s.microbiologyresearch.org

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ITS sequences were aligned with the MUSCLE program (Edgar, 2004). Neighbour-joining (NJ) and maximum-parsimony (MP) analysis was performed using MEGA 6 software (Tamura et al., 2013). Kimura’s two-parameter model was used to calculate the nucleotide-sequence divergence for NJ tree reconstruction, where gaps were completely deleted and bootstrap values were obtained from 1000 replications (Felsenstein, 1985). The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar, 2000).

Ninety-six yeast strains were isolated from the dung beetles collected from the north of Thailand. Sequencing of the LSU D1/D2 region was performed for representative strains that produced similar (GTG)3 PCR-fingerprints. Based on the comparison of the LSU D1/D2 sequences, nine different yeast species were isolated from dung beetles (Table S1, available in the online Supplementary Material). The major group (84 isolates) was assumed to be related to the genus Trichosporon with the closest sequence match (5.9 % divergence) to T. veenhuisii (Table S1). In addition, a single strain (DD2-28) also closely related to the genus Trichosporon, with 1 % sequence divergence to T. siamense (Table S1), but different from the major group was also detected. The major group was isolated from five out of the six dung beetles used for isolation. The representative strains (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) of the major group from each sample, together with strain DD2-28 were selected for phylogenetic tree reconstruction. In a preliminary study, we reconstructed an NJ phylogenetic tree using the LSU D1/D2 sequences of the strains obtained in this study together with 35 species of the genus Trichosporon. The six strains obtained in this study (five from the major group plus DD2-28) clustered with Trichosporon siamense, and the nearest branch comprised Trichosporon brassicace, Trichosporon domesticum and Trichosporon montevideense (data not shown). However, the bootstrap supports at the nodes were not very well supported (<50 %), which might be due to the significant difference in the LSU D1/D2 sequences between the strains obtained in this study and the described species of the genus Trichosporon, as stated below. Even in the more conserved SSU sequences, we found 33 substitutions from a total of 1735 nt between DD1-1T (DDBJ/GenBank accession no. LC034294) and T. domesticum (AB001754), which was inferred to be related from the BLASTN similarity search.

Therefore, we added the ITS region sequences and reconstructed a phylogenetic tree based on MP analysis of the combined LSU D1/D2 plus ITS sequences using the species of the genus Trichosporon shown in Fig. 1. The MP tree showed that the five strains from the major group (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) were distinctly separate from all known species, but located within the T. brassicace clade within the Trichosphorinales (Fig. 1). Furthermore, the five strains were placed at the neighbouring branch of DD2-28 with 59 % bootstrap support. In terms of the BLASTN similarity search of the LSU D1/D2 sequence, strain DD1-1T displayed 2.9 % divergence (17 substitutions and 18 gaps from 594 nt) from T. veenhuisii CBS 7136, and 3.5 % divergence (21 substitutions and 20 gaps from 596 nt) from the strain DD2-28. In addition, strain DD2-28 also displayed 1.0 % divergence (six substitutions from 596 nt) from T. siamense TISTR 5823T (=JCM 12478T). As a broad rule of thumb, different yeast species typically have more than six nucleotide substitutions in the LSU D1/D2 region (Fell et al., 2000). Accordingly, the five strains from the main group reported here (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) are assumed to be a novel species of the genus Trichosporon, while the DD2-28 strain is also likely to be a second novel species of the genus. Strains DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1 were different from all the related species of the genus Trichosporon in their physiological characteristics (Table 2), and especially in all five strains being urease-negative. The urease test was first performed using the standard Christensen’s urea agar (Christensen, 1946), where all five strains (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) grew well on the agar medium but did not change the medium colour at all. The urease test was also performed in liquid medium using Stuart’s urea broth (Stuart et al., 1945). As shown in Fig. S1, the broth inoculated with these five strains and Saccharomyces cerevisiae JCM 1818 (as a negative control) did not change in colour, while the broth inoculated with Trichosporon asahii JCM 2466 (as a positive control) and strain DD2-28 changed to bright pink within 6 days. Therefore, we concluded that whilst strain DD2-28 is urease-positive, the five strains from the main group (DD1-1T, DD2-33, DD4-11, DD5-15 and DD6-1) are urease-negative. This is unusual since urease activity is a universal physiological characteristic for basidiomycetous yeasts, with most basidiomycetous yeasts able to hydrolyse urea (Kurtzman et al., 2011). In contrast, urease-negative basidiomycetous yeasts, such as Filobasidium elegans and Sporobolomyces clavatus, are exceptional (Bandoni et al., 1991; Wang & Bai, 2004). No member of the genus Trichosporon has been reported previously to be urease-negative (Sugita, 2011). We could not find any commonality among Filobasidium elegans, Sporobolomyces clavatus and the five strains obtained in this study with respect to the isolation source, locality and phylogeny. Thus, it is unknown how or whether their urease-negative status could be related to their ecological niche or not. It is important to note that no yeasts were isolated from the beetle washings after sterilization and prior to homogenization, supporting that these are gut-associated yeasts.

From the evidence presented here, we propose a novel species, Trichosporon heliocopridis sp. nov. At the time of writing, we have not found any other isolate related to strain DD2-28 and so retain this potential new species as undescribed.

As mentioned above, Trichosporon heliocopridis sp. nov. was frequently (five out of six beetles) isolated from surface-sterilized dung beetles collected from geographically
Trichosporon heliocopridis DD1-1T (LC071456/LC034290)
Trichosporon heliocopridis DD2-33 (LC071457/LC034291)
Trichosporon heliocopridis DD6-1 (LC071460/LC034286)
Trichosporon heliocopridis DD5-15 (LC071459/LC034285)
Trichosporon heliocopridis DD4-11 (LC071458/LC034288)
Trichosporon sp. DD2-28 (LC071461/LC034286)
Trichosporon brassicae CBS 6382T (AF444436/AF075521)
Trichosporon montevideense CBS 6721T (AF444422/AF105397)
Trichosporon domesticum CBS 8280T (AF444414/AF075512)
Trichosporon siamense ST318T (AB164370/AB164370)
Trichosporon scarabaeorum CBS 5601T (AF444446/AF444710)
Trichosporon mycotoxinivorans HB1175T (AJ601389/AJ601388)
Trichosporon loubieri CBS 7065T (AF444438/AF075522)
Trichosporon veenhouisi CBS 7136T (AF105400/AF414693)
Trichosporon vadense CBS 8901T (AY093425/AY093426)
Trichosporon multisporum CBS 2495T (AF139984/AF414695)
Trichosporon laibachii CBS 5790T (AF444421/AF075514)
Trichosporon gracile CBS 8180T (AF444440/AF105399)
Trichosporon dulcitum CBS 8257T (AF444428/AF075517)
Trichosporon wieringae CBS 8903T (AY315666/AY315667)
Trichosporon gamsii CBS 8246T (AF444424/AF444708)
Trichosporon lignicola var. undulatum CBS 222.34T (AF444482/AF363658)
Trichosporon sporotrichoides CBS 8246T (AF444470/AF189885)
Trichosporon porosum CBS 2040T (AF189833/AF414694)
Trichosporon dehoogii CBS 8686T (AF444476/AF444718)
Vanrija humicola CBS 571T (AB035572/AB189836)

Fig. 1. Phylogenetic tree generated from MP analysis of the LSU D1/D2 plus ITS sequences showing the placement of Trichosporon heliocopridis sp. nov. and the related taxa. Numbers next to branches indicate the bootstrap percentage from 1000 replications. One of the five parsimonious trees (length=364, the consistency index=0.53) is shown. GenBank accession numbers are given in parentheses as left: ITS, right: LSU D1/D2. Vanrija humicola served as the outgroup. Bar, 10 changes per nucleotide position.
Table 2. Salient physiological characteristics between *Trichosporon heliocopridis* sp. nov. and related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>L (-)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melezitose</td>
<td>-</td>
<td>-</td>
<td>V</td>
<td>V</td>
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</tr>
<tr>
<td>Ribitol</td>
<td>-</td>
<td>-</td>
<td>V</td>
<td>V</td>
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<td>L-Rhamnose</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>Methyl α-D-glucoside</td>
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<td>-</td>
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<tr>
<td>Salicin</td>
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<td>-</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Saccharic acid</td>
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<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Growth at 37 °C</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease activity</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+*, Positive; *−*, negative; *L*, latent; *V*, variable; *N*, not determined.

Culminating, the related species *T. siamense* was isolated from insect frass in Thailand (Nakase *et al.*, 2006), and *T. scarabaeorum* was isolated from scarabaeid beetle (Middelhoven *et al.*, 2004). It is possible that there is some relationship between these yeast species and coleopteran beetles, but further investigation is required to reveal any actual function of *Trichosporon heliocopridis* sp. nov., especially in the context of symbiosis, with the dung beetle.

**Description of *Trichosporon heliocopridis* sp. nov. Kunthiphun, Endoh, Takashima, Ohkuma, Tanasupawat & Akaracharanya**

*Trichosporon heliocopridis* (he.li.o.co’pri.dis. N.L. gen. n. *heliocopridis* named after the genus of the isolation source *Heliocopris bucephalus*).

After 3 days of growth in YM broth at 25 °C, cells are subglobose to narrowly ellipsoidal and filiform (1.0–4.0 × 2.0–22.0 μm). Cells occur singly or in chains with sediment formed; budding is multilateral at narrow base (Fig. 2a). After growth for 3 days on YM agar at 25 °C, colonies are cream, butyrous and wrinkled on the top with a filamentous edge. After growth for 3 days in Dalmau plate culture on corn meal agar at 20 °C, pseudohyphae and arthroconidia are observed under a coverslip (Fig. 2b). Aerobic growth is white with a filamentous margin. Basidiospores are not detected from both individual and mixed cultures on YM agar and corn meal agar at 25 °C after 6 weeks. Glucose is not fermented. Glucose, galactose (variable, may be weak or slow), sucrose, maltose (weak), trehalose, lactose (latent, seldom negative), raffinose (weak), D-arabinose (seldom negative), D-ribose, ethanol, glycerol, D-mannitol (may be weak or slow) D-glucitol (weak), salicin, 2-keto-D-gluconic acid, succinic acid, citric acid, *myo*-inositol (may be slow), D-glucuronic acid, D-galacturonic acid, propylenglycol (seldom weak), N-acetyl-D-glucosamine and butane-2,3-diol are assimilated as a sole carbon source, but L-sorbitose, cellobiose, melibiose, melezitose, inulin, soluble starch, D-xylene, L-arabinose, L-rhamnose, meso-erythritol, ribitol, galactitol, mehanol, methyl α-D-glucoside, glucono-δ-lactone, DL-lactic acid (seldom weakly positive), D-glucuronic acid, D-galacturonic acid,
D-glucosamine (seldom weakly positive), D-glucuronolactone, L-arabinitol, quinic acid, saccharic acid, xyitol, xylo-oligosaccharide, n-decane and n-hexadecane are not assimilated as sole carbon sources. Ammonium, ethylamine, L-lysine, cadaverine and D-tryptophan are assimilated as a sole nitrogen source, while nitrate, nitrite, creatine, creatinine, D-glucosamine and imidazole are not. Growth at 37 °C in YM broth is positive, but negative at 40 °C. Arbutin is not split. Growth in 0.01 % (w/v) cycloheximide is positive, but negative or weak in 0.1 % (w/v) cycloheximide. Growth in vitamin-free medium is weak. Growth in 10 % (w/v) NaCl/0.5 % (w/v) glucose medium is negative. Growth on 50 % (w/v) glucose and 1 % (v/v) acetic acid are negative. Acid production is negative. The diazonium blue B colour reaction is positive. Extracellular amyloid compound is not produced. Gelatin is not liquefied. Urease activity is negative.

The ex-type strain is DD1-1 T (=JISTR5946T=JCM 30786 T=CBS 14168 T) isolated from Helicopris bucephalus, a dung beetle, in a cattle pen, Chiang rai, Thailand. The type is preserved by lyophilization under the number JCM 30786 T (=DD1-1 T=JISTR5946T=CBS 14168 T) in the Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan. The Myco-Bank number is MB812098.

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References


