Trichomonascus apis sp. nov., a heterothallic yeast species from honeycomb

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Four strains of a novel heterothallic yeast species were isolated from pollen-storing cells of a honeycomb of honeybee (Apis mellifera) in Hungary. Analysis of the D1/D2 domain of the large-subunit (26S) rRNA gene sequences placed the strains in the Trichomonascus clade. The four strains share identical D1/D2 sequences and differ by 24 substitutions and nine indels from the genetically most closely related species, Blastobotrys attinorum. The name Trichomonascus apis sp. nov. is proposed for the novel species. The carbon-source assimilation spectrum of T. apis sp. nov. is rather broad. Unlike B. attinorum, it assimilates sucrose, trehalose, D-glucuronate and succinate and does not grow at 37 °C, thus enabling the two taxa to be distinguished. The type and isotype strains of Trichomonascus apis are NCAIM Y.01848T (=CBS 10922T =NRRL Y-48475T) and NCAIM Y.01849IT (=CBS 10923T =NRRL Y-48476T), respectively.

Trichomonascus mycophagus was described by Jackson (1947). The species appeared to be mycoparasitic on Corticium confluens growing on the bark of Abies balsamea. Jackson was unable to cultivate T. mycophagus on laboratory media; however, the attempt was made several months after the collection of the fungal material. T. mycophagus is only known from dried herbarium collections (Malloch & de Hoog, 1998). Jackson (1947) placed the genus in the family Aspergillaceae. Benny & Kimbrough (1980) were of the opinion that the taxonomic relationship of this fungus is uncertain and fresh material would be needed to determine whether Trichomonascus is related to any other known taxon. Malloch & de Hoog (1998) listed Trichomonascus among endomycte-like genera. The second Trichomonascus species, Trichomonascus rutilus, was described from two herbarium specimens collected from wood in 1961 and 1967 in Denmark (Hauerslev, 1987). The type material of T. rutilus is not axenic. Besides T. rutilus, which exhibits clear ascomycetous characters, spores and basidia similar to those of Radulomyces confluens were found in the type material. Hauerslev (1987) concluded that T. rutilus may be a parasite, like T. mycophagus. Kurtzman (2004) described Trichomonascus petasosporus, the third species of the genus. T. petasosporus is a culturable, heterothallic yeast species of the order Saccharomycetales and the three strains mentioned in the description were isolated from insect frass, collected from different trees in the USA. Kurtzman’s efforts to isolate DNA from the herbarium specimen of T. mycophagus, the type species of the genus, were unsuccessful, preventing him from performing a genetic comparison of the two taxa. However, he argued that the nearly identical unique morphology of the sexual states of T. mycophagus and T. petasosporus leaves little doubt that they are closely related. A multigene phylogenetic analysis revealed that T. petasosporus is related closely to Stephanoascus ciferrii, the type species of the genus Stephanoascus (Kurtzman & Robnett, 2007). As Stephanoascus H. S. Jackson (1947) has taxonomic priority over Stephanoascus M. Th. Smith, van der Walt & E. Johannsen (1976), S. ciferrii and the related species Stephanoascus farinosus were transferred to the genus Trichomonascus (Kurtzman & Robnett, 2007). Similarly, the same authors transferred most of the anamorphic species of the Trichomonascus clade to the genus Blastobotrys von Klopotek (1967), which has taxonomic priority over Sympodiomyces Fell & Statzel (1971) and Arxula van der Walt, M. Th. Smith & Y. Yamada (1990). Since then, the Trichomonascus clade has been expanded considerably by the description of seven novel Blastobotrys species (Middelhoven & Kurtzman, 2007; Kurtzman, 2007). In this paper, we report the isolation of four yeast strains representing haploid mating types of a novel Trichomonascus species, and propose a novel species with the name Trichomonascus apis sp. nov. to accommodate these strains.

The four strains used in this study (Table 1) were isolated on rose–bengal chloramphenicol (RBC) agar from pollen-storing cells of the same mouldy honeycomb of honeybee (Apis mellifera) in Hungary in 1994. Strains representing different colony morphologies were picked, purified and characterized by using standard methods described by Yarrow (1998). Sexual reactivity was studied by mixing...
actively growing cultures of the four investigated strains on acetate (1.4% sodium acetate, 0.04% glucose), cornmeal (CM), potato–glucose (PDA), 2% malt extract, glucose–peptone–yeast extract (GPY), yeast extract–malt extract (YM) and V8 agars. The mixture was incubated at 15 and 25 °C and examined regularly by microscopy up to 21 days. Mating types were determined by pairwise mixing of actively growing cultures of the investigated strains in all possible combinations on CM agar at 15 °C. The D1/D2 domain of the large-subunit (26S) rRNA gene from selected strains was sequenced as described by Kurtzman & Robnett (1998). A sequence-similarity search was performed against GenBank by using the BLAST 2.2.18 database-search program (Altschul et al., 1997). The sequences generated during this study, along with sequences of related species retrieved from GenBank, were aligned and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL_X 1.81 program (Thompson et al., 1997). Bootstrap support for the tree was determined from 1000 replications.

The dominant yeast strain recovered from the pollen stored in the investigated honeycomb was identified as *Candida magnoliae*. It was accompanied by two minor ones, represented by only a few colonies. One of them was identified as *Zygosaccharomyces rouxii*, whilst the other proved to be a thus-far-undescribed species. Four strains of this novel species were isolated. Analysis of the D1/D2 large-subunit rRNA gene sequences placed them in the *Trichomonascus* clade (Fig. 1). The phylogenetic tree exhibits an arrangement of the included species similar to that of the tree derived from the analysis of multigene sequences by Kurtzman & Robnett (2007). The four strains share identical D1/D2 sequences (the GenBank accession number of strain NCAIM Y.01848T is indicated on the phylogenetic tree depicted in Fig. 1) and differ significantly from the genetically most closely related species, B. (Sympodiomycetes) *attinorum*. Twenty-four substitutions and nine indels were detected in the D1/D2 region, indicating that the two taxa are phylogenetically distinct. As the novel species clusters near to the known *Trichomonascus* species and sexual reproduction was observed, we assign it to the genus *Trichomonascus*.

The isolates of *T. apis* represented opposite mating types. The type strain (Y.01848T) was designated hT and the isotype strain (Y.01849T) as hI, whilst the mating types of the other two investigated strains were already weak and uncertain. The mating types of the two investigated strains were determined from 1000 replications.

### Table 1. *Trichomonascus apis* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source of isolation</th>
<th>Mating type</th>
</tr>
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<tbody>
<tr>
<td>NCAIM Y.01848T (=CBS 10922T = NRRL Y-48475T)</td>
<td>Mouldy honeycomb, Hungary</td>
<td>hT</td>
</tr>
<tr>
<td>NCAIM Y.01849T (=CBS 10923T = NRRL Y-48476T)</td>
<td>Mouldy honeycomb, Hungary</td>
<td>hI</td>
</tr>
<tr>
<td>NCAIM Y.01850</td>
<td>Mouldy honeycomb, Hungary</td>
<td>hT</td>
</tr>
<tr>
<td>NCAIM Y.01851</td>
<td>Mouldy honeycomb, Hungary</td>
<td>hI</td>
</tr>
</tbody>
</table>

The four strains of *T. apis* were isolated from pollen-storing cells of a mouldy honeycomb. The ability of the strains to grow at reduced water activity [with 50% glucose and with 10 or 16% (w/v) NaCl] seems to be in accord with the conditions provided by the source of isolation. However, the conditions provided by the source of isolation. However, subsequent efforts to isolate additional strains of the species from the same (honeycomb with pollen) or related (honey with or without honeycomb, pollen collected by honeybees, dead honeybees) sources were unsuccessful. The genetically most closely related species, B. (Sympodiomycetes) *attinorum*, was also recovered from an insect-associated habitat: the four strains of that species were isolated from laboratory nests of the leaf-cutting ant (*Atta sexdens*) in Brazil (Carreiro et al., 2004). In contrast, *Blastobotrysts* (Sympodiomycetes) *parvus*, which is also related to *T. apis*, may be endemic to marine waters (Statzell-Tallman & Fell, 1998). The carbon-source assimilation spectrum of *T. apis* is rather broad. Except for inulin, methanol and saccharate, all investigated carbon sources were assimilated (some of them weakly and/or slowly or variably). It can be separated easily from *B. attinorum* by several general physiological characteristics, e.g. by assimilation of sucrose, trehalose, D-glucuronate and succinate. In addition, *B. attinorum* grows at 37 °C (Carreiro et al., 2004), whereas growth of the *T. apis* strains is already weak and variable at 30 °C.

**Latin diagnosis of Trichomonascus apis Péter, Tomai-Lehoczki & Dlauchy sp. nov.**

*In extracto mali post dies tres in 25 °C annulus incompletus et sedimentum floccosum formantur, pellicula non formantur.*

**Description of Trichomonascus apis Péter, Tornai-Lehoczki & Dlauchy sp. nov.**

Trichomonascus apis (‘a’pis. L. gen. fem. sing. n. apis of a bee, referring to the honeybee-associated isolation source of the investigated strains).

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**Fig. 1.** Phylogenetic tree showing the placement of *Trichomonascus apis* and some related species based on analysis of the D1/D2 domain of the large-subunit (26S) rRNA gene. Sequences not generated during this study were obtained from GenBank; accession numbers are shown in parentheses. The tree was constructed by neighbour-joining analysis of aligned sequences. *Schizosaccharomyces pombe* was used as an outgroup. Numbers at nodes indicate percentages of bootstrap sampling from 1000 replications. Bar, 0.02 sequence divergence.
In 5 % malt extract after 3 days at 25 °C, growth mainly consists of clusters of true, branching hyphae (1.5–3.5 μm in diameter). The hyphae give rise to ellipsoid, ovoid or elongated blastoconidia (2.5–5.0 × 5–10 μm). Conidia are produced singly or in small clusters, mostly on denticles, pedicels, protuberances or sympodia (Fig. 2). Incomplete ring and loose flocculent sediment are present. On 5 % malt extract agar after 3 days at 25 °C, the streak culture is tough, membranous, moderately raised, cream-coloured or white, fine-hairy and dull. On slide culture with CM agar after 7 days at 25 °C, the growth is snow-white and filamentous; septate hyphae and blastoconidia are formed (Fig. 3). The species is heterothallic. Individual strains at 15 or 25 °C or a mixture of strains at 25 °C formed no ascospores after 3 weeks incubation on acetate, CM, PDA, 2 % malt extract, GPY, YM or V8 agars. However, when compatible mating types were mixed, good ascoporumlation was observed on CM agar at 15 °C within 2 weeks. Conjugation of hyphal cells preceding ascus formation was observed in the cases of some asci. Ascii are persistent and variable in shape even in the same cross (Fig. 4). They are subglobose, ellipsoid, obpyriform and obclavate, measure 4–8 × 6–13 μm and bear a cylindrical or conical apical cell, from which buds sometimes arise. Rarely, the apical cell is divided by a transversal septum. One to four ellipsoid ascospores are formed per ascus. One side of the ascospores is often depressed; their lower surface is sometimes even concave and may contain a refractive droplet. D-Glucose (slow) and D-galactose (weak and slow) are fermented; maltose, sucrose, lactose, raffinose and α, α-trehalose are not fermented. Carbon compounds D-glucose, D-galactose, L-sorbose, D-glucosamine, N-acetyl-D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose (slow or weak slow), L-rhamnose, sucrose, maltose, α, α-trehalose, methyl α-D-glucoside, cellobiose, salicin (weak, slow or very weak, slow), arbutin (slow and variable), melibiose (slow or weak, slow), lactose (slow and variable), raffinose (slow and variable), melezitose (very weak and variable), starch, glycerol, meso-erythritol, ribitol, xylitol, L-arabininitol, d-glucitol, d-mannitol, galactitol, inositol, glucono-δ-lactone (slow and variable), 2-keto-D-gluconate (variable), D-gluconate, D-glucuronate, D-galacturonate (slow or weak, slow), DL-lactate (slow or very weak, slow), succinate, citrate (slow), ethanol, propane 1,2 diol (slow or weak, slow), butane 2,3 diol (weak, slow and variable) and hexadecane (slow and variable) are assimilated; no growth occurs on inulin, methanol or saccharate. Ethylamine hydrochloride, L-lysine, cadaverine dihydrochloride and glucosamine (as nitrogen source) are assimilated; potassium nitrate, sodium nitrite, creatine, creatinine and imidazole are not assimilated. The formation of amyloid material is negative. Growth in vitamin-free medium is negative. Growth occurs at 25 °C; it is weak and variable at 30 °C and negative at 35 °C. Growth is present on 50 % (w/w) glucose–yeast extract agar, with 10 % NaCl and with 16 % NaCl (weak). No growth occurs on 60 % (w/w) glucose–yeast extract agar or with 1 % acetic acid. Growth with 0.1 % cycloheximide is positive. Urea hydrolysis and colour reaction with diazonium blue B are negative.

The type and isotype strains were recovered from mouldy honeycomb in Hungary, and are maintained respectively as NCAIM Y.01848T (=CBS 10922T =NRRL Y-48475T) and NCAIM Y.01849IT (=CBS 10923IT =NRRL Y-48476IT) in the National Collection of Agricultural and Industrial Microorganisms in Budapest (Hungary). NCAIM Y.01848T and NCAIM Y.01849IT represent opposite mating types.
Acknowledgements

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