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.01848^T (=CBS 10922^T =NRRL Y-48475^T) and NCAIM Y.01849^{IT} (=CBS 10923^{IT} =NRRL Y-48476^{IT}), 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 endomycete-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 *Trichomonascus* 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

The GenBank/EMBL/DBJ accession number for the D1/D2 domain of the 26S rRNA gene sequence of strain NCAIM Y.01848^T (=CBS 10922^T =NRRL Y-48475^T) is EU790643.

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

CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

Strain	Source of isolation	Mating type
NCAIM Y.01848 ^T (=CBS 10922 ^T =NRRL Y-48475 ^T)	Mouldy honeycomb, Hungary	h ^T
NCAIM Y.01849 ^{IT} (=CBS 10923 ^{IT} =NRRL Y-48476 ^{IT})	Mouldy honeycomb, Hungary	h ^I
NCAIM Y.01850	Mouldy honeycomb, Hungary	h ^T
NCAIM Y.01851	Mouldy honeycomb, Hungary	h ^I

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.01848^T is indicated on the phylogenetic tree depicted in Fig. 1) and differ significantly from the genetically most closely related species, *B. (Sympodiomyces) 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.01848^T) was designated h^T and the

isotype strain (Y.01849^{IT}) as h^I, whilst the mating types of the other two investigated strains were defined in reference to them (Table 1). Sexual reproduction of this species resembles that of the former *Stephanoascus* species, rather than the peculiar manner of ascospore formation of *T. mycophagus*, the type species of the genus, or the recently described *T. petasosporus*. The two mating types of the novel species show some morphological dimorphism: mating type h^T produces white/cream colonies on 5 % malt extract agar after 3 days at 25 °C. Its filamentous character is more pronounced and it produces fewer blastoconidia. Mating type h^I produces cream-coloured colonies under the same conditions, its filamentous character is less pronounced and it produces more blastoconidia.

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, 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. (Sympodiomyces) 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, *Blastobotrys (Sympodiomyces) 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 standard 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, Tornai-Lehoczki & Dlačny sp. nov.

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

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.

Hyphae septatae, ramosae (1.5–3.5 µm) *et blastoconidia ellipsoideae, ovoideae vel elongatae* (2.5–5.0 × 5–10 µm) *formantur*. In agaro malti post dies tres in 25 °C cultura est offirmata, membranacea, crenea vel alba, pilosa, et opaca. Centrum coloniae sublatum est. In agaro Zea maydis confecto post dies 7 in 25 °C mycelium verum formantur. Species heterothallica. Asci subglobosae, ellipsoideae, obpyriformae aut obclavatae, cellula apicali coronata, 1–4 ascoparas ellipsoideas, non liberas habent. D-Glucosum (lente) et D-galactosum (lente) fermentantur. Maltosum, sucrosus, lactosum, raffinsum et trehalosum non fermentantur. D-Glucosum, D-galactosum, L-sorbosum, D-glucosaminum, N-acetyl-D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum (lente), L-rhamnosum, sucrosus, maltosum, trehalosum, α-methyl-D-glucosidum, cellobiosum, salicinum (exiguum, lente), arbutinum (lente, variabile), melibiosum (lente), lactosum (lente, variabile), raffinsum (lente, variabile), melezitsum (valde exiguum, variabile), amyllum solubile, glycerolum, meso-erythritolum, ribitolum, xylitolum, L-arabinitolum, D-glucitolum, D-mannitolum, galactitolum, myo-inositolum, glucono-δ-lactonum (lente, variabile), 2-ketogluconicum (variabile), D-gluconicum, D-glucuronicum, D-galacturonicum (lente), DL-lactatum (lente), succinatum, citratum (lente), ethanolum, propane-

1,2-diolum (lente), butane-2,3-diolum (exiguum, lente, variabile) et hexadecanum (lente, variabile) assimilantur, at non inulinum, methanolum et saccharatum. Ethylaminum hydrochloricum, lysinum, cadaverinum dihydrochloricum et glucosaminum assimilantur, at non kalium nitricum, natrium nitrosus, creatinum, creatininum, imidazolium. Materia amyloidea iodophila non formantur. Vitamina externa crescentiae sunt necessaria. Crescere potest (variabile) in 30 °C, at non in 35 °C. In agaro extracto fermenti confecto 50 partes glucosi per centum crescit, at non in agaro extracto fermenti confecto 60 partes glucosi per centum. Parte una cycloheximidi per mille crescit. Ureum non finditur. Diazonium caeruleum B est negativum. Typus stirps NCAIM Y.01848^T (=CBS 10922^T =NRRL Y-48475^T). Isolata ex favo apis, Hungaria. Isotypus stirps NCAIM Y.01849^{IT} (=CBS 10923^{IT} =NRRL Y-48476^{IT}). Isolata ex favo apis, Hungaria.

Description of *Trichomonascus apis* Péter, Tornai-Lehoczki & Dlačhy 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).

Fig. 2. Septate hyphae, blastoconidia and sympodia of *Trichomonascus apis* NCAIM Y.01848^T (malt extract, 3 days, 25 °C). Bar, 10 µm.

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 ascospore formation 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. Asci 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

Fig. 3. Septate hyphae and blastoconidia of *Trichomonascus apis* NCAIM Y.01848^T (CM agar, 7 days, 25 °C). Bar, 10 µm.

Fig. 4. Ascospore-forming culture of *Trichomonascus apis* NCAIM Y.01848^T × NCAIM Y.01849^{IT} (CM agar, 14–16 days, 15 °C). Bar, 10 µm.

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-arabinitol, D-glucitol, D-mannitol, galactitol, myo-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.01848^T (=CBS 10922^T =NRRL Y-48475^T) and NCAIM Y.01849^{IT} (=CBS 10923^{IT} =NRRL Y-48476^{IT}) in the National Collection of Agricultural and Industrial Microorganisms in Budapest (Hungary). NCAIM Y.01848^T and NCAIM Y.01849^{IT} represent opposite mating types.

Acknowledgements

This research was partly supported by the Bolyai János Research Scholarship of the Hungarian Academy of Sciences.

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