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FULL PAPER

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An ustilaginomycetous yeast, *Pseudozyma graminicola* sp. nov., isolated from the leaves of pasture plants

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Abstract Two strains belonging to a novel anamorphic species, *Pseudozyma graminicola*, were isolated from the leaves of herbaceous plants in the Moscow region (Russia). This species was genetically distinct from all known *Pseudozyma* species, based on sequence divergence in the D1/D2 domains of the large subunit rDNA and the ITS region. It is related phylogenetically to species of the genus *Sporisorium* (Ustilaginaceae, Ustilaginales). Physiological characteristics distinguishing this novel species from the other species of the genus *Pseudozyma* are presented.

Key words 26S rDNA gene sequence · Internal transcribed spacer · *Pseudozyma* · *Sporisorium*

Introduction

The anamorphic genus *Pseudozyma* Bandoni emend. Boekhout at present contains 11 described species (Wang et al. 2006). Phylogenetically, these species belong to the Ustilaginaceae (Ustilaginales, Ustilaginomycetidae, Ustilaginomycetes), and for some of them an anamorph–teleomorph connection with *Ustilago* (Pers.) Roussel or *Sporisorium* Ehremb. ex Link seems to be present (Sampaio 2004). *Pseudozyma* species are worldwide in distribution and mainly associated with plants (Boekhout and Fell 1998; Golubev and Golubeva 2004), although some species were isolated from clinical specimens (Sugita et al. 2003).

During the survey of yeast populations on the leaves of herbaceous plants in the Moscow region (Russia), we isolated two strains of *Pseudozyma* that could not be identified with any hitherto-described species (Boekhout and Fell 1998; Barnett et al. 2000; Wang et al. 2006). Sequencing of the D1/D2 region of the large subunit of rDNA (26SrDNA) and internal transcribed spacer (ITS) showed that these strains represent a novel species.

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Materials and methods

Strains

Strains VKM Y-2938^T (isolate LI-20) and LI-46 were isolated by plating on glucose-yeast extract-peptone agar with penicillin and streptomycin from the leaves of timothy grass (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.) collected in the northern part of the Moscow region (Russia) in June 2002.

Morphological, physiological, and biochemical characterization

For morphological and physiological characterization, standard methods currently employed in yeast taxonomy were used (Yarrow 1998). Identification of ubiquinone was carried out according to Nakase and Suzuki (1986).

rDNA sequence analysis

Nuclear DNA from the isolates was extracted using the method of Makimura et al. (1994). The D1/D2 region of the 26S rDNA (645 bp) and internal transcribed spacer (ITS) region of the rDNA unit were sequenced directly from polymerase chain reaction (PCR) products using the primer pairs NL-1 (forward: GCATATCAATAAGCGGAG GAAAAG) and NL-4 (reverse: GGTCCGTGTTTCA AGACGG) (Kurtzman and Robnett 1997), and pITS-F (forward: GTCGTAACAAGGTTAACCTGCGG) and pITS-R (reverse: TCCTCCGCTTATTGATATGC) (Sugita et al. 1999). The PCR products were sequenced

Table 1. Physiological characteristics that differentiate *Pseudozyma* species

Species	Growth with following conditions													
	Mel	Gal	Lac	Mgl	Rha	Eth	Ery	Rib	Man	Ino	Glu	Vfm	37°C	Eth
Pseudozyma sp. 1[a]	+	+	+	+	–	+	+	+	+	+	w	–	+	+
P. antarctica	s	s	+	+	–	+	+	–	+	+	–	–	–	w
P. aphidis	+	+	+	+	w	+	+	–	+	+	+	–	+	+
Pseudozyma sp. 2[a]	s	+	–	s	–	s	+	s	+	+	–	+	+	+
P. flocculosa	s	s	–	+	–	–	+	+	+	+	–	s	–	–
P. fusiformata	–	–	–	s	–	+	s	w	s	+	–	+	–	–
P. graminicola	+	s	s	–	–	+	w	s	s	s	–	+	–	–
P. hubeiensis	+	+	+	+	–	+	+	+	+	–	?	+	w	+
P. parantarctica	+	+	+	+	+	+	s	+	+	+	+	–	+	–
P. prolifica	–	s	s	+	w	+	s	s	s	s	–	+	–	+
P. rugulosa	+	+	–	+	+	+	+	+	+	+	+	–	+	+
P. shanxiensis	+	+	+	+	–	+	–	s	w	w	?	+	+	+
Pseudozyma sp. 3[a]	+	w	+	+	–	+	+	s	+	+	+	+	+	+
P. thailandica	+	+	–	+	–	+	+	+	+	+	–	–	+	–
P. tsukubaensis	–	w	s	+	–	+	+	–	–	+	–	s	–	+

Mel, melibiose; Gal, D-galactose; Lac, lactose; Mgl, α-methyl-D-glucoside; Rha, L-rhamnose; Eth, ethanol; Ery, erythritol; Rib, ribitol; Man, D-mannitol; Ino, *i*-inositol; Glu, glucarate; Vfm, vitamin-free medium; 37°C, temperature; Eth, ethylamine; +, positive; s, slow; w, weak; –, negative

[a]Species that have not formally described (Sugita et al., unpublished results)

using an ABI 310 DNA sequencer and an ABI PRISM BigDye Terminator Cycle Sequencing kit version 3.1 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The phylogenetic analyses included sequence data of about 100 species mainly of genera *Sporisorium*, *Pseudozyma*, and *Ustilago*. The sequences were aligned using Clustal W version 1.8 software (Thompson et al. 1994). For the neighbor-joining analysis (Saitou and Nei 1987), the distances between sequences were calculated using Kimura's two-parameter model (Kimura 1980). A bootstrap analysis was conducted with 100 replications (Felsenstein 1985).

Results and discussion

Phenotyic affiliation of isolates

The isolates Ll-20 and Ll-46 were identical in their phenotypic characteristics. They formed yeastlike colonies fringed with pseudohyphae and septate hyphae without clamp-connections at the margin and produced fusiform blastoconidia on denticles. Arthroconidia and ballistoconidia were not produced. No mating reactions or sexual structures were observed either in cultures of single strains or between the strains Ll-20 and Ll-46. Judging from the absence of alcoholic fermentation and production of starchlike compounds but positive urease and DBB tests, assimilation of D-glucuronate and/or *i*-inositol as single carbon sources, utilization of nitrate, and molecular analysis, we identified these isolates as belonging to the genus *Pseudozyma.* The presence of Q-10 as a major ubiquinone is consonant with this placement.

However, our isolates could not be identified from their physiological characteristics used for species differentiation (Barnett et al. 2000; Boekhout and Fell 1998), and they differed also in their physiological profiles from the *Pseudozyma* species described recently (Sugita et al. 2003; Wang et al. 2006). Rather numerous dissimilarities suggest that these strains might represent a novel species. Considering the most clearly distinctive characters, some physiological tests can be used for species differentiation (Table 1).

Morphologically, our isolates are similar to the yeastlike fungi classified in the Ustilaginales. From a phenotypic point of view, they, similar to other *Pseudozyma* species, cannot easily be differentiated from the recently described *Acaromyces ingoldii* Boekhout, Scorzetti, Gerson & Sztejnberg (Boekhout et al. 2003). We can only point out that D-glucosamine and L-lysine are assimilated as sole nitrogen sources by *Pseudozyma* species but not by *A. ingoldii*.

26S rDNA and internal transcribed spacer analysis

Sequencing of the D1/D2 region of the 26S rDNA showed that strains Ll-20 and Ll-46 have the identical sequences. The results of molecular study demonstrated that these two strains were not conspecific with any known *Pseudozyma* species but represent a new species, for which the name *Pseudozyma graminicola* is proposed. The phylogenetic tree also demonstrated that species of the anamorphic genus *Pseudozyma* are phylogenetically distributed among the genera *Sporisorium*, *Ustilago*, *Moesziomyces* Vánky, and *Cintractia* Cornu (Ustilaginaceae) (Sampaio 2004; Sugita, unpublished data), and they appear to be saprobic phases of these phytoparasitic species occurring on Poaceae, Cyperaceae, and Eriocaulaceae. The analyses based on the D1/D2 region of the 26S rDNA sequence showed that *P. graminicola* is located in the *Sporisorium* 1 of the *Sporisorium* clade (Stoll et al. 2005). It was most closely related to *Sporisorium holwayi* (G.P. Clinton & Zundel) Vánky (Fig. 1). Phylogenetic similarity between *P. graminicola* and

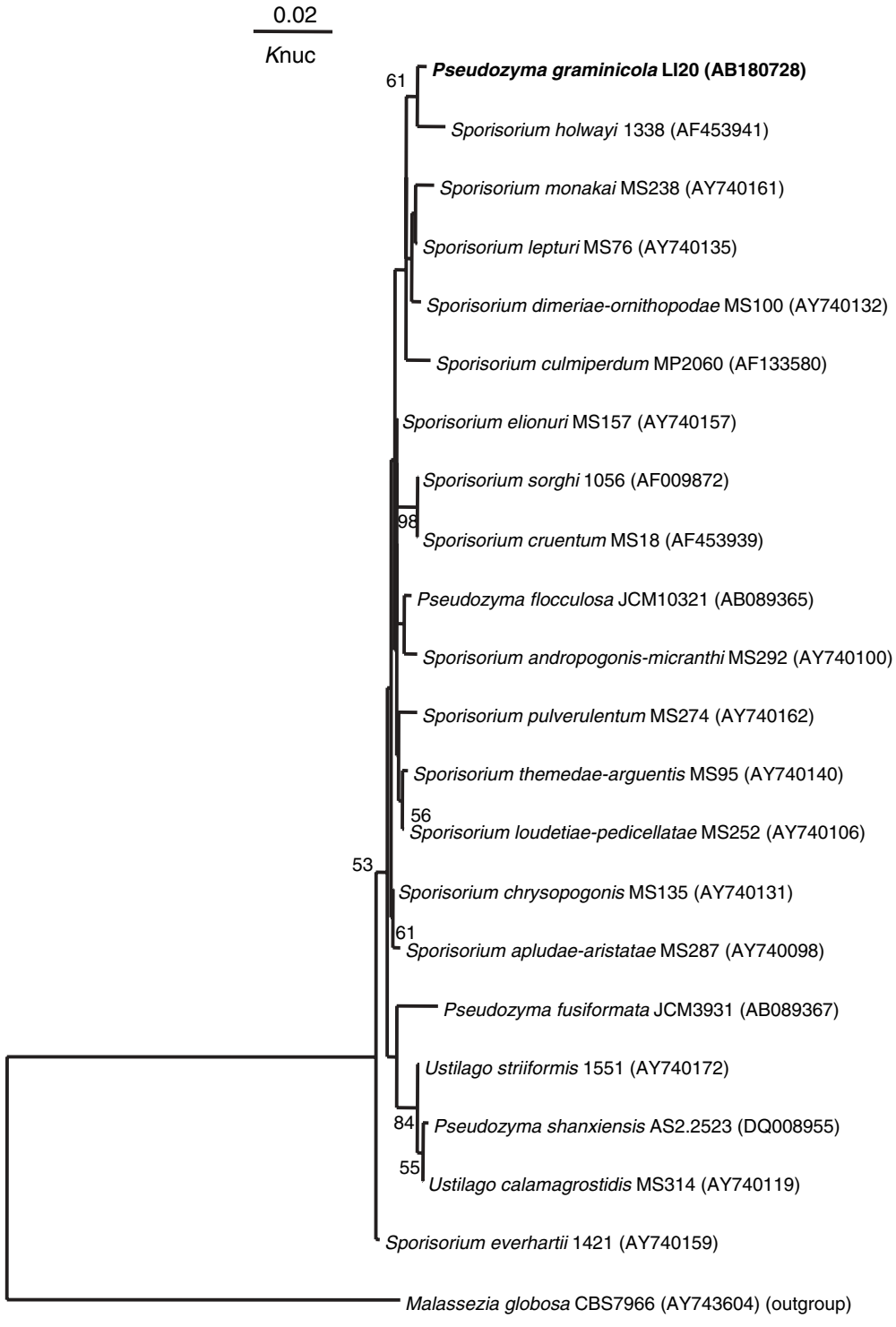

Fig. 1. Molecular phylogenetic trees constructed using the D1/D2 region of the 26S rDNA sequences of a new *Pseudozyma* species and related *Sporisorium* spp. Only closely related species are shown. The DDBJ/GenBank/EMBL accession numbers are indicated in *parentheses*. The *numerals* indicate the confidence level from 100 replicate bootstrap samplings (frequencies below 50% are not indicated). *K*nuc, Kimura's parameter (1980)

S. holwayi was also shown with neighbor-joining analysis of the combined sequences of the ITS region (including 5.8S rDNA) and the 26S rDNA D1/D2 domain (Wang et al. 2006). However, *P. graminicola* differed from its teleomorphic relative by 6 base differences in the D1/D2 domain (600 bp) and up to 28 base differences in the ITS region, which does not allow suggesting an anamorph–teleomorph connection between these two species.

Pseudozyma graminicola W. Golubev, Sugita, et N. Golubev, sp. nov.

In liquido cum dextroso et peptono et extracto fermenti, post 3 dies ad 20°C, cellulae elongatae, ellipsoideae vel fusiformes, guttatae, 2–3.5 × 4–13 (medietas 3–7.5) µm; post unum mensem, pellicula crassa et rugosa, et sedimentum formantur. In medio agaro morphologia fermenti, post

unum mensem ad 20°C, cultura plana, alba, hebetata, laevis vel rugosa, margine fimbriata. In corn meal agar (CMA) in lamina vitrea, pseudohyphae et hyphae septatae sine colligatione unciformi formantur. Blastoconidia stipitata, fusiformia. Non ballistosporae nec arthrosporae nec chlamydosporae formantur. Fermentatio nulla. Glucosum, saccharosum, raffinosum, melibiosum, galactosum (lente), lactosum (lente), trehalosum (lente), maltosum, melezitosum, amylum solubile (lente), cellobiosum (exiguum), salicinum (exiguum), L-sorbosum (exiguum), D-xylosum, L-arabinosum, D-ribosum (exiguum), ethanolum, glycerolum (lente), erythritolum (exiguum), ribitolum (exiguum), L-arabitolum (exiguum), D-mannitolum (lente), D-glucitolum (lente), *i*-inositolum (exiguum), D-glucuronatum, DL-lactatum (lente), succinatum (lente), citratum (lente), D-gluconatum (lente), 5-ketogluconatum (exiguum), acidum quinicum, *N*-acetyl-D-glucosaminum, et hexadecanum assimilantur, autem inulinum, α-methyl-D-glucosidum, L-rhamnosum, D-arabinosum, methanolum, xylitolum, galactitolum, saccharatum, et D-glucosaminum non assimilantur. Kalium nitrosum, kalium nitricum, L-lysinum, L-tryptophanum, D-glucosaminum, et cadaverinum assimilantur. Creatinum, creatininum, et ethylaminum non assimilantur. Maxima temperatura incrementi 35°C. Vitaminarum addendum no necessarium est. In medio cum 10% NaCl exigue crescit; non crescit in medio cum 50% glucoso aut 1% acido acetico. Materia amyloidea iodophila non formatur. Ureum hydrolysatur. Commutatio colori per diazonium caeruleum B positiva. Ubiquinonum majus: Q-10.

Holotypus: VKM Y-2938 in statu lyophilo ex stripe Ll-20, cultura ex foliis Phlei pratensis, Junius 2002, Moscow, Russia in collectione culturae Russia Collection of Microorganisms (VKM, Pushchino, Russia) conservatus. Isotypus: JCM 12496 in Japan Collection of Microorganisms (Saitama, Japan) et CBS 10092 in Centraalbureau voor Schimmelcultures (Utrecht, Hollandia).

Etymology: *Pseudozyma graminicola* (L. gramen, a grass; L. suffix -cola, a dweller; graminicola, a dweller of a grass plant).

After 3 days at 20°C in glucose-yeast extract-peptone broth, the vegetative cells are elongate, ellipsoidal, or fusiform [width/length ratio 1.2–7.5 (mean, 2.7)], 2–3.5 × 4–13 (mean, 3 × 7.5) μm, and contain oil droplets (Fig. 2). After 1 month, a thick wrinkled pellicle and sediment are formed. On yeast morphology agar (Difco), after 1 month at 20°C, the streak culture is flat, white, dull, smooth to wrinkled, with a fringed margin. On slide culture on corn meal agar, pseudomycelia and septate mycelia without clamp-connections are produced. The hyphae have denticles on which fusiform blastoconidia are formed. No ballistoconidia, arthrospores, or chlamydospores were observed. Fermentation is absent. Glucose, sucrose, raffinose, melibiose, galactose (slow), lactose (slow), trehalose (slow), maltose, melezitose, soluble starch (slow), cellobiose (weak), salicin (weak), L-sorbose (weak), D-xylose, L-arabinose, D-ribose (weak), ethanol, glycerol (slow), erythritol (weak), ribitol (weak), L-arabitol (weak), D-mannitol (slow), D-glucitol (slow), inositol (weak), D-glucuronate, DL-lactate (slow), succinate (slow), citrate (slow), D-gluconate (slow), 5-ketogluconate (weak), quinic acid, *N*-acetyl-D-glucosamine (slow), and hexadecane are assimilated, but inulin, α-methyl-D-glucoside, L-rhamnose, D-arabinose, methanol, xylitol, galactitol, saccharate, and D-glucosamine are not assimilated as sole carbon sources. Nitrate, nitrite, L-lysine, L-tryptophan, D-glucosamine, and cadaverine are utilized as sole nitrogen sources, but creatine, creatinine, and ethylamine are not utilized. Maximum temperature of growth (on malt agar) is 35°C. Growth in vitamin-free medium in the presence of 10% NaCl weak. Growth on 50% (w/w) glucose-yeast extract agar in the presence of 1% acetic acid or 10% ethanol negative. Starchlike compounds are not produced. Hydrolysis of urea and DBB reaction are positive. The major ubiquinine is Q-10. The type strain, VKM Y-2938^T (= CBS 10092^T = JCM 12496^T) is isolated from the leaves of *Phleum pratense* collected in the Moscow region (Russia) in June 2002.

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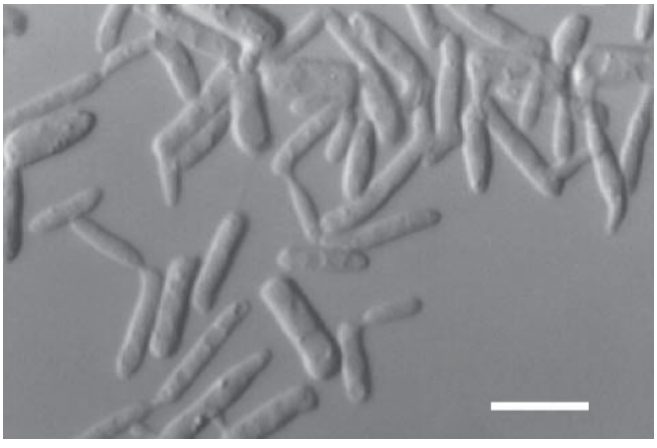

Fig. 2. Vegetative cells of *P. graminicola* VKM Y-2938^T grown in Yeast extract-malt extract broth (YM) broth at 25°C for 3 days. *Bar* 10 μm

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