**Introduction**

In the course of a study of yeasts in the Prioksko-terrasny biosphere reserve (Moscow region, Russia) (Golubev and Golubeva, 2004) some Cryptococcus isolates could not be identified from their key characters used for species differentiation (Barnett et al., 2000; Fell and Statzell-Tallman, 1998). Mycocinotyping showed that such isolates also differed from known Cryptococcus spp. in sensitivity patterns to the mycocins (killer toxins) secreted by basidiomycetous yeasts of the orders Cystofilobasidiales, Filobasidiales and Tremellales. Sequencing of the D1/D2 domains of the 26S rDNA confirmed that these isolates were not conspecific with any other Cryptococcus species. In this paper we propose the new species Cryptococcus paraflavus to accommodate three strains isolated from steppe plants.

**Materials and Methods**

**Strains.** The strains PTZ 16A\(^{T}\) (=VKM Y-2923\(^{T}\)), 442 and 444 were isolated, by plating on inositol or glucuronate agar, from herbaceous plants collected from a steppe plot with fescue-herbaceous vegetation in the Prioksko-terrasny biosphere reserve in September 1997 and 2000. Other strains used in this study were from the Russia Collection of Microorganisms (VKM; http://www.vkm.ru/).

**Morphological and physiological characteristics.** Standard methods currently employed in yeast taxonomy were used (Yarrow, 1998). The ability to produce the brown and green color effect was examined on Guizotia abyssinica creatine agar (Staib, 1999).

**Monosaccharide analysis.** To prepare extracellular polysaccharides cultures were grown for 6 days at 20°C on a shaker (150 rpm) in a medium containing glucose, 3%; peptone, 0.2%; yeast extract, 0.1%; KH\(_{2}\)PO\(_{4}\), 0.2%. Cells were separated by centrifugation (5,000 \(\times\) g, 10 min) and extracellular polysaccharides were precipitated from the supernatant fluid and washed by ethanol. They were then dried in a stream of air. After hydrolysis of extracellular polysaccharides...
with 3 mM trifluoracetic acid (100°C, 6 h), neutral sugar composition was detected with an LG-2000 carbohydrate analyzer (Biotronic).

**DNA sequence analysis.** For sequence analysis, total DNA was extracted according to the procedures described by Sampaio et al. (2001) and amplified using primers ITS5 and LR6. Cycle sequencing of the 600–650 bp region at the 5’-end of the 26S rDNA D1/D2 domain employed forward primer NL1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and reverse primer NL4 (5’-GGTCCGTGTTTCAAGACGG-3’). The internal transcribed spacer region was sequenced using the forward primer ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and the reverse primer ITS4 (5’-TCCTCCGCTTATTGATATGC-3’). Sequences were obtained with an Amersham Pharmacia ALF express II automated sequencer using standard protocols. Alignments were made with MegAlign (DNAStar) and corrected visually. PAUP* 4.0, version b10 (Swofford, 2000), was employed to perform phylogenetic analyses using the maximum-parsimony method. Bootstrap analyses were based on 1,000 random resamplings (Felsenstein, 1985).

**Assay for sensitivity to mycocins.** Sensitivity was assayed by the previously described culture-to-culture method (Golubev et al., 2003). Mycocinogenic strains of *Bullera alba* VKM Y-2829, *B. hannae* VKM Y-2832T, *B. sinensis* var. *lactis* VKM Y-2826T, *B. unica* VKM Y-2830T, *Cryptococcus laurentii* VKM Y-1627, 1628, 1665T, *Cr. nemorosus* VKM Y-2906T, *Cr. perniciosus* VKM Y-2905T, 2907, *Cr. podzolicus* VKM Y-2247, 2249, *Cystofilobasidium bisporidii* VKM Y-2700T, and *Filobasidium capsuligenum* VKM Y-1439 were used in these tests.

### Results and Discussion

**Phenotypic affiliation**

Based on the following traits: absence of sexual state, ballisto- and arthroconidia, incapacity to ferment, positive urease reaction and glucuronate assimilation, presence of xylose-containing extracellular polysaccharides, the strains PTZ 16AT (=VKM Y-2923T), 442 and 444 were assigned to the genus *Cryptococcus* Vuillemin. They were almost identical in cultural, morphological and physiological properties. These strains vary in their ability to assimilate some polyols only. Their physiological profiles are most similar to *Cr. flavus* (Saito) Phaff et Fell but differ from the type strain of this species in the color of the cultures, the assimilation of salicin, erythritol, citrate and the absence of growth at 30°C (Table 1). In addition, *Cr. flavus* is negative in the “starch” test whereas these strains give a greenish color.

No mating reactions were observed between the type strain of *Cr. flavus* VKM Y-2232T and the novel isolates or among VKM Y-2923T (=PTZ 16A T), PTZ 442 and 444.

### Mycocinotyping

Significant differences were found between the strains under study and *Cr. flavus* in sensitivity to the mycocins produced by basidiomycetous yeasts. The type strain of the latter is sensitive to the mycocins of *Cryptococcus laurentii* VKM Y-1627, 1665T and *Cr. nemorosus* VKM Y-2906T but the novel isolates are also sensitive to the mycocins of *Bullera alba* VKM Y-2829, *B. unica* VKM Y-2830T, *Cr. laurentii* VKM Y-1628 and *Cr. podzolicus* VKM Y-2247, 2249. In contrast to *Cr. flavus* VKM Y-2232T they are insensitive to *B. hannae* VKM Y-2832T mycocin (Table 1).

### Table 1. Salient characteristics of *Cryptococcus flavus* and *Cr. paraflavus*.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>Cr. flavus</em> VKM Y-2232T</th>
<th><em>Cr. paraflavus</em> VKM Y-2923T, PTZ 442, 444</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assimilation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Erythritol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ribitol</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
<td>s</td>
</tr>
<tr>
<td>Growth at 30°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>Sensitivity to the mycocins of</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bullera alba</em> VKM Y-2829</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>B. hannae</em> VKM Y-2832T</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. unica</em> VKM Y-2830T</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td><em>Cryptococcus laurentii</em> VKM Y-1628</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Cr. podzolicus</em> VKM Y-2247, 2249</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Characteristics are scored as: +, positive; s, slow; w, weak; –, negative. *Cr. flavus* VKM Y-2232T = CBS 331T.
Fig. 1. Phylogenetic trees of Cryptococcus paraflavus and related taxa, of the Tremellomycetidae. Percentage bootstrap values of 1,000 replicates are given at each node (values under 50% are not shown). GenBank accession numbers are indicated after strain numbers. Sequences determined in this study are indicated in bold face. A. Maximum-parsimony analysis of an alignment of the D1/D2 region of the 26S rDNA. The topology was rooted with the three species of Filobasidium. B. Unrooted maximum parsimony ITS tree.
**Molecular phylogenetic analysis**

Sequencing of the D1/D2 domain of the 26S rDNA revealed that the strain VKM Y-2923<sup>T</sup> belongs to the Tremellales and the closest species was *Cr. flavus* (Fig. 1A). However, seven nucleotide differences and eleven gaps in the D1/D2 region were found between them. It should be noted from sequencing of both the 18S and 26S rDNA that *Cr. flavus* holds an isolated position in the Tremellales and it does not belong to any the well-supported clades based on topologies of phylogenetic trees and bootstrap values (Scorzetti et al., 2002; Takashima and Nakase, 1999). Moreover, its phylogenetic position was changeable depending on the number, sets of taxa studied and the methods phylogenetic analyses.

Although sequencing of the ITS region confirmed the relationship between *Cr. flavus* and the new species (Fig. 1B), there are even more differences in this region (20 nucleotide differences and eight gaps). Both analyses showed that the strain PTZ 16A<sup>T</sup> (=VKM Y-2923<sup>T</sup>) was not conspecific with the closest phylogenetic relative, *Cr. flavus*, but represents a separate species.

On the basis of these data, the mycocin-sensitivity profile and physiological characteristics (Table 1), a new species, *Cr. paraflavus*, is proposed for the isolates studied.

**Latin diagnosis and standard description of Cryptococcus paraflavus Golubev et Sampaio sp. nov.**

In aqua glucosum et extractum fermenti et peptonum continent, post dies 3 cellulae subglobosae et ovoidae (3.4–6.0×4.2–7.7 μm (mean 4.3×5.6 μm)) with small capsules, single or in pairs. After a month, a sediment and a pellicle are formed.

Growth on yeast morphology agar (Difco): After a month, the streak cultures are cream-colored, smooth, glistening and slimy; the border is entire. No ballistoconidia are observed.

Slide cultures on corn meal agar: Neither pseudomycelium nor true mycelium is produced.

Fermentation: Absent.

Assimilation of carbon compounds:
- Glucose +
- Galactose + slow
- Sorbose –
- Glucosamine + weak
- N-Acetylglucosamine +
- Ribose +
- Xylose +
- L-Arabinose +
- D-Arabinose + weak
- Rhamnose + weak
- Sucrose +
- Maltose +
- Trehalose +
- α-Methylglucoside + slow
- Cellobiose +
- Salicin –
- Inulin –
- Starch + slow
- Glycerol + weak
- Erythritol –
- Ribitol +
- Xylitol +
- Arabinitol + slow
The type strain, PTZ 16A, isolated from plants in the Prioksko-terrasny reserve (Russia) has been deposited in the Russia Collection of Microorganisms, Pushchino, Russia, as VKM Y-29293 and in the Portuguese Yeast Culture Collection, Monte de Caparica, Portugal, as PYCC 5829.  

Etymology: para near, from the Gr. pr. παρά. The name *paraflavus* is proposed because the yeast described closely resembles *Cr. flavius*.

### References


