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EXPERIMENTAL ARTICLES

Mrakia curviuscula sp. nov.: A New Psychrophilic Yeast from Forest Substrates

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Abstract—Seven strains with similar characteristics from the laboratory collection of yeasts isolated from forest substrates collected in the central part of European Russia corresponded to none of the known yeast species. Based on the study of their life cycle, physiological characteristics, and the nucleotide composition of their DNA and taking into account the data of PCR analysis with universal primers, the strains were ascribed to a new psychrophilic yeast species, *Mrakia curviuscula* sp. nov.

Key words: psychrophilic yeasts, UP-PCR, new yeast species, *Mrakia.*

Our multiple-year investigations of yeasts inhabiting soil and plant substrates in various ecosystems, from subtropics to polar deserts, showed that the yeasts are most diverse in forest ecosystems [1–3]. Recently, we have described two new yeast species isolated from the fruiting bodies of forest macromycetes [4, 5]. Plant substrates, particularly mosses, lichens, and berries, are more appropriate for the isolation of diverse yeasts than forest soils and the forest floor [6].

Seven yeast strains isolated from mosses and the surface of bilberries were found to be close to the species *Cryptococcus huempii* (Ramirez and Gonzalez) Roejmans et al. in morphology and the range of utilizable substrates and to homothallic yeasts of the genus *Mrakia* Yamada and Komagata in the life cycle characterized by the formation of teliospores.

A comparison of these strains with the type strains of related psychrophilic yeast species *Mrakia frigida* and *C. huempii* by means of PCR analysis with universal primers (UP-PCR) showed that their genomes have a similar organization (this indicates that the seven strains under study belong to one species) and differ from the genomes of the reference species.

These data, together with the results of the morphological, physiological, and biochemical studies of the seven strains, allowed us to classify them into a new species, *Mrakia curviuscula* sp. nov.

MATERIALS AND METHODS

Samples of plant substrates were collected in forests of the central part of European Russia, namely, on the territories of the Central State Forest Biosphere Reserve (CSFBR) in the Tver region and the Oka Reserve in the Ryazan region. Yeasts were isolated by routine procedures using slightly acidic malt agar plates, which were incubated after inoculation at 20°C.

The type strains of *Mrakia frigida* CBS 5270 (VKM Y-1455) and *Cryptococcus huempii* CBS 8186 (VKM Y-2637) were used as reference species.

Pure cultures were identified according to the modern identification schemes [7]. The formation and germination of teliospores were investigated as follows. Potato–glucose agar plates inoculated with strain Oz-358 were incubated at 20°C for 1.5 month and then cut into small agar blocks with teliospore-containing mycelium inside. The blocks were placed in sterile distilled water and kept at 11°C for 3 months to check the germination of teliospores.

DNA was isolated by the Marmur procedure [8]. The G+C content of DNA was determined from the thermal denaturation curves recorded using a Pye-Unicam SP 1800 spectrophotometer. The G+C content was calculated by the formula: G+C (mol %) = 2.08*T* – 106.4 [9].

Samples of yeast DNA for PCR analysis were prepared by a new rapid procedure [10], which allows tens of DNA samples for PCR analysis to be prepared as soon as within 25–30 min. UP-PCR was carried out using three universal primers: (5'–3')GGATCCGAGGGTGGCGGTTCT (no. 21); (5'–3')GTAAAACGACGGCCAGT (no. 45); and (5'–3')GAGGGTGGCGGCTAG (no. 15/19) [11]. PCR amplifications (30–35 cycles) were run in a Perkin-Elmer–Cetus model 480 thermocycler (United States) with DNA denaturation at 93°C for 30 s, primer annealing at 50°C (or 55°C in the case of the primer no. 21) for 30 s, and DNA synthesis at 72°C for 60 s.

The products of PCR amplification were separated in 1% agarose gel with the standard Tris–acetate buffer containing ethidium bromide [12].

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Table 1. Sources from which the seven strains under study have been isolated

Original designation	Designation in KBP	Substrate, ecosystem, location, and sampling data
Oz-130a	KBP Y-3614	*Vaccinium myrtillus* bilberries, bilberry–spruce forest, Oka Reserve, June 1993
Oz-132	KBP Y-3615	The same
Oz-233	KBP Y-3616	*Polytrichum commune* moss, crab apple–spruce forest, Oka Reserve, August 1993
Oz-240	KBP Y-3617	The same
Oz-358	KBP Y-3618(t)	*Bryum* sp. moss, white moss–pine forest, Oka Reserve, August 1993
Lz-207p	KBP Y-3717	*Sphagnum* sp. moss, bilberry–sphagnum–spruce forest, CSFBR, October 1996
Lz-230	KBP Y-3718	The same

RESULTS

Of the seven yeast strains under study, five were isolated from mosses and two were isolated from the surface of bilberries gathered in the coniferous forests of the central part of European Russia (Table 1). The strains slightly differed in the range of utilizable carbon sources (Table 2), but all of them were similar to the type strain of the anamorphous species *C. huempii* in morphology and to representatives (including the type strain of the species *M. frigida* (Fell et al.)) of the genus *Mrakia* Yamada and Komagata in the life cycle.

Figure 1 shows the DNA patterns of yeasts obtained with the universal primer no. 21. The amplified fragments of the DNA of the strains under study varied from 0.5 to 2.5–3 kbp. The distribution of PCR fragments in the molecular mass was almost identical for all seven strains: there was one major DNA fragment about 0.5 kbp in size and 8 to 10 minor DNA fragments with sizes varying from 0.6 to 2.5 kbp. The reference species *C. huempii* and *M. frigida* exhibited entirely different DNA patterns (Fig. 1, lanes 1 and 9).

PCR with the universal primers nos. 45 (Fig. 2) and 15/19 (data not presented) gave the same results as PCR with the primer no. 21. Namely, the DNA patterns of all seven strains were similar to each other and substantially differed from those of the reference species (Fig. 2, lanes 1 and 9). Twelve PCR fragments were from 0.5 to 3 kbp in size.

Description of *M. curviuscula* sp. nov.

In extracto malti post 3 dies cellulae vegetativae sunt ovoideae, elongatae ad curviculae, (2.5–6.5)–(7.0–15.5) μm, singulae vel binae. Post unum mensem sedimentum et anulus formantur. Coloniae in agaro malti post unum mensem (20°C) glabra vel pallidobrunneus, 17 mm in diametro; margo glabro vel undatus.

Pseudomycelium et mycelium formantur.

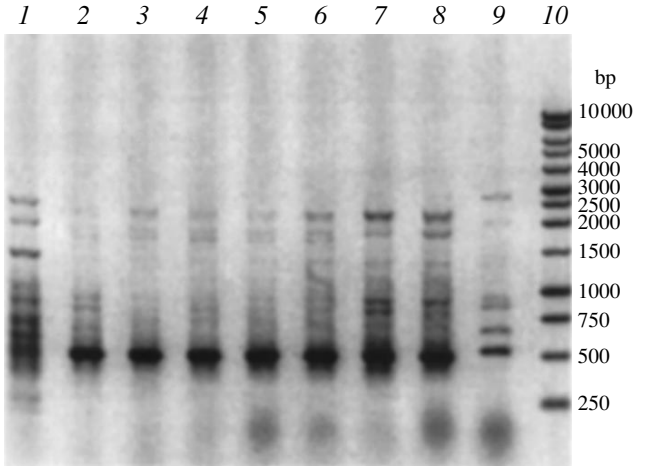

Fig. 1. The UP-PCR patterns (universal primer no. 21) of the seven yeast isolates classified into the new species *M. curviuscula* and the type strains of close yeast species. Lanes: (1) the type strain of *C. huempii*; (2–8) the seven strains of *M. curviuscula*; (9) the type strain of *M. frigida*; and 10, a 1-kb DNA ladder. Electrophoresis in 1% agarose gel.

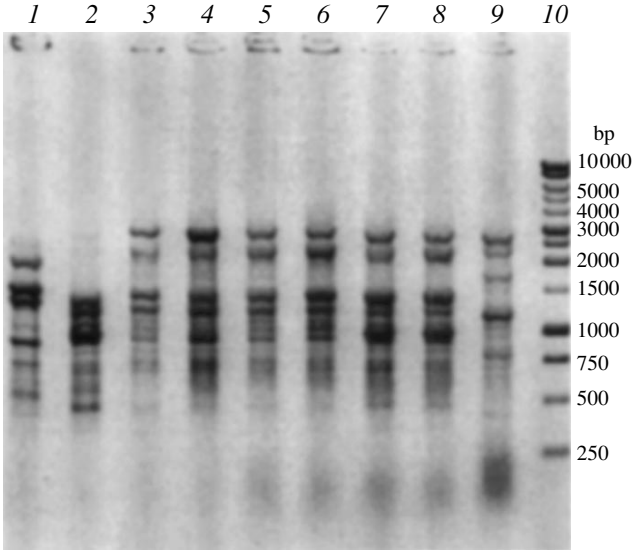

Fig. 2. UP-PCR patterns (universal primer no. 45) of the seven isolates and two type strains of *C. huempii and M. frigida.* The designations of lanes is the same as in Fig. 1. Electrophoresis in 1.2% agarose gel

Table 2. Fermentation of glucose and assimilation of various carbon sources by the new species *Mrakia curviuscula* and close species

Characteristic	*Mrakia frigida**	*Mrakia curviuscula*	*Cryptococcus huempii* (type strain)
Glucose fermentation	+/p	–	–
Assimilation:			
Glucose	+	+	+
Galactose	+	+	+
L-Sorbose	s	–	s
Sucrose	+	–	–
Maltose	v	+	+
Cellobiose	+	+	+
Trehalose	s	+	+
Lactose	v	+	+
Melibiose	v	–	–
Raffinose	+	–	–
Melezitose	v	+	s/+
Inulin	–	–	–
Starch	v	v	+
D-Xylose	s	+	+
L-Arabinose	+	+	+
D-Arabinose	v	v	–/s
D-Ribose	v	p/s	p/s
L-Rhamnose	v	+	+
Glucosamine	p	+	+
Ethanol	s	v	+
Glycerol	v	–	–
Erythritol	–	–	–
Ribitol	+	s	s
Dulcitol	v	–	–
D-Mannitol	+	+	+
α-Methyl-D-Glucoside	v	–	–
Salicin	+	+	+
2-Ketogluconate	+	+	+
5-Ketogluconate	+	+	+
Glucuronic acid	s	+	+
Inositol	p/–	–	–
Lactic acid	–	p	p
Succinic acid	v	+	+
Citric acid	v	+	+

Note: "–," "p," "s," "v," and "+" stand for "no growth," "poor growth," "slow growth," "strain-variable growth," and "good growth," respectively.
* Data from Kurtzman and Fell [7].

Teliosporae terminales sphaeroides vel pyriformis sunt. Metabasidia ex germinatione teliosporarum unicellulis oriontur.

Vitamina addita necessaria sunt.

Fermentatio nulla.

Assimilatio carbo-compositarum: glucosum, galactosum, glucosaminum, D-xylosum, L-arabinosum, L-rhamnosum, maltosum, trehalosum, cellobiosum, salicinum, arbutinum, lactosum, melezitosum, gluconatum, D-mannitolum, 2-ketognucolatum, 5-ketoglu-

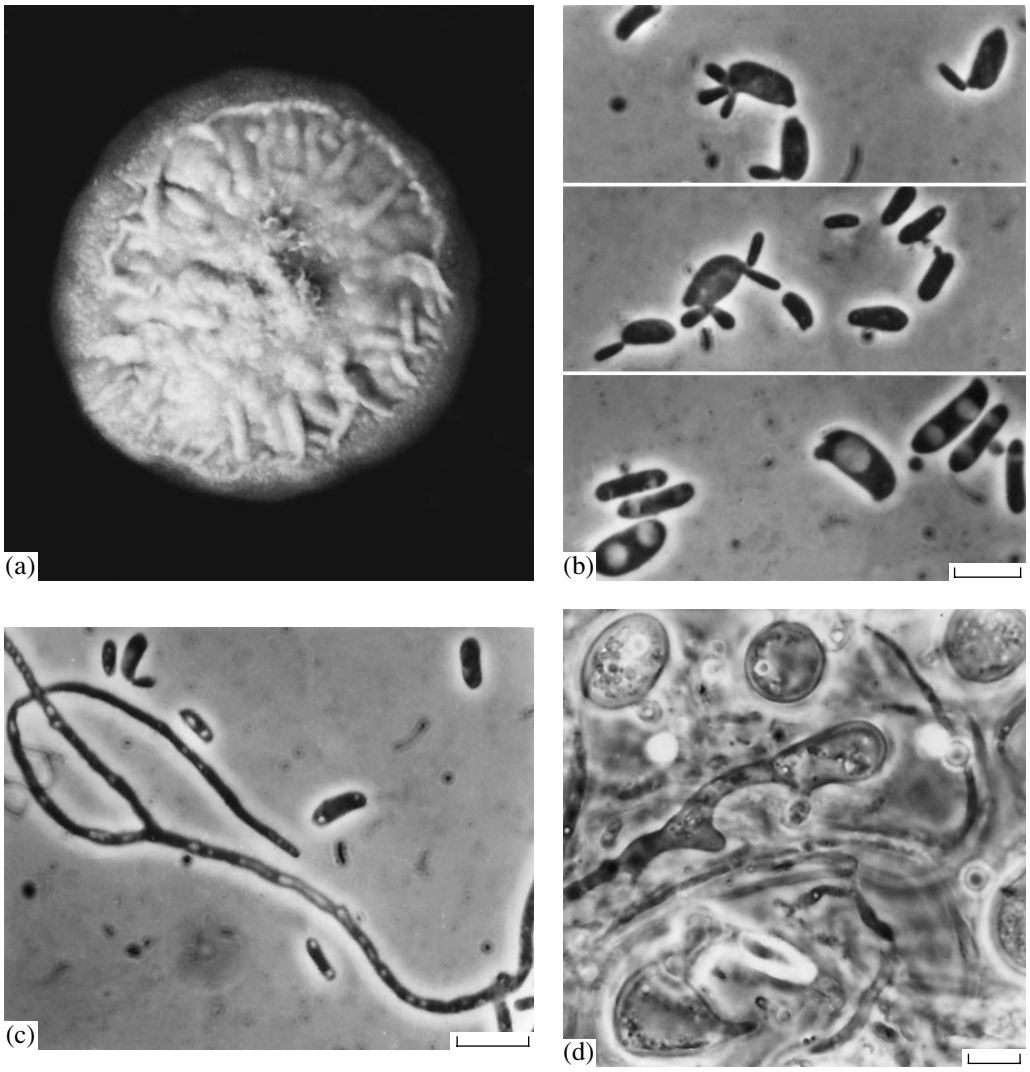

Fig. 3. (a) A 30-day-old giant colony of *M. curviuscula* sp. nov. grown at 11°C on malt agar (magnification, 2.5×); (b) 3-day-old budding cells grown at 21°C on malt agar; (c) 10-day-old mycelium grown at 21°C on malt agar; and (d) teliospores in a 30-day-old mycelium grown at 21°C on malt agar. Scale bars for (b, c, d) represent 10 μm.

conatum, acidum glucuronicum, acidum succinicum, acidum citricum assimilantur at non L-sorbosum, sucrosum, α-methyl-D-glucosidum, melibiosum, inulinum, glycerolum, *i*-erythritolum, galactitolum, nec *i*-inositolum. Assimilatio D-ribosum, D-arabinosum, amylum solubile, ribitolum, D,L-acidum lacticum, ethanolum variat.

Kalii nitras assimilantur.

Materia amyloideae formantur.

Temperatura maxima 25°C.

Ureum hydrolysatur.

Diazonium caeruleum B: positivum.

Proportio molaris gianini+cytosini in acido deoxyribonucleico: 49.3–50.0%, typus 49.6%.

Typus: KBP Y-3618 (Oz-358) isolatus ex muscus in collectione zymotica Moskva, Rossia.

One-month colonies grown on malt agar are from smooth to folded and wrinkled, 15–20 mm in diameter, dark-brown, flat, sometimes umbonate, even-edged, without mycelial border (Fig. 3a).

Cells grown in liquid malt for 3 days are bean-shaped.

Cells are from 2.5 to 6.6 μm in diameter and from 7 to 15.5 μm long.

Buds (from 1 to 3 at one locus) are extrapolar and often situated perpendicular to the concave side of the mother cell (Fig. 3b). After 7 days of growth, the mycelium forms a ring. The mycelium is branched, slightly septate, and lacks buckles (Fig. 3c). After 30 days of growth, the mycelium ring and precipitate are well pronounced. Film is not formed. After 7 days of growth on corn agar at 18–20°C, a pseudomycelium is produced. Some cells

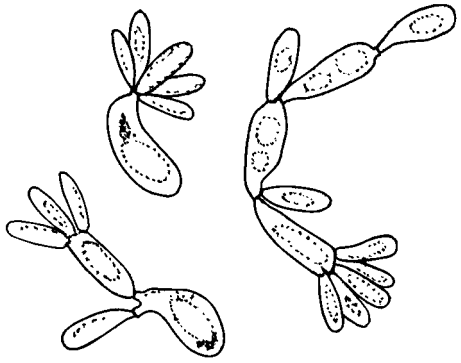

Fig. 4. A 6-day-old pseudomycelium grown at 11°C on corn agar.

form small chains of 3–4 cells. Buds are rod-shaped and often occur as extrapolar bundles (Fig. 4).

The species is homothallic, spore-forming. Vegetative cells produce mycelium without buckles. Teliospores are formed at the hyphal ends (Figs. 3d and 3) and germinate with the formation of non septate tubular metabasidia each with 1–2 sporidia (Fig. 6). Some strains are characterized by the formation of chlamidospores in vegetative oval cells and on the mycelium (in the latter case, hooks are not formed, indicating the absence of diploidization).

Glucose is not fermented.

The species assimilates glucose, galactose, glucosamine (very actively), ribose (slowly), xylose, L-arabinose, rhamnose, maltose, trehalose, cellobiose, salicin, arbutin, lactose (actively), melezitose, 2- and 5-ketogluconates, glucuronic acid, lactic acid (slowly), succinic and citric acids (Table 2).

The species does not assimilate sorbose, sucrose, inositol, melibiose, raffinose, inulin, glycerol, erythritol, dulcitol, methanol, creatin, and creatinin.

Some strains can grow on D-arabinose, starch, and ethanol.

Good growth is observed between 5 and 25°C.

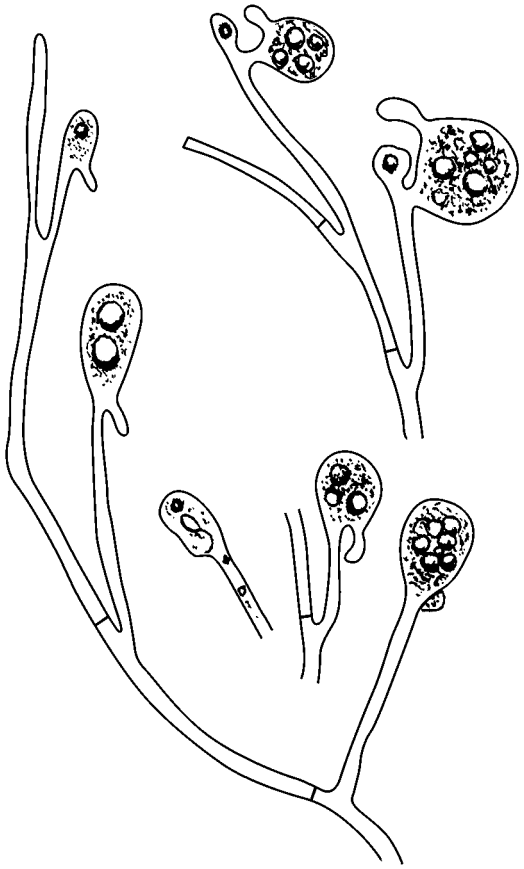

Fig. 5. Diagram illustrating the formation of teliospores.

Nitrates are assimilated.

Vitamins are necessary.

Reaction for the formation of starch is positive.

Urea is hydrolyzed.

The species cannot grow in the presence of 0.1% cycloheximide.

Growth on 50% glucose is very poor, if at all.

The type strain KBP Y-3618 (= VKM Y-3618(t), original designation Oz-358) is stored in the collection of yeasts at the Department of Soil Biology, Faculty of Soil Science, Moscow State University.

Table 3. Differentiating characteristics of close basidiomycetous yeasts of the order *Cystofilobasidiales*

Species	Basidium	Pigmentation	G+C, mol %	T_{max}, °C	Fermentation	Assimilation of inositol/glucuronic acid
Cystofilobasidium spp.	Tubular holometabasidium	+	56.6–66.3	37	–	+/+
Cyst. lari-marini	The same	–	ND*	37	+	+/+
Mrakia frigida	Unicellular metabasidium, rarely with one septum	–	52.9–56.1	<20	+ weak	v**/+
Mrakia curviuscula	Unicellular metabasidium	–	49.3–50.0	25	–	–/+
Cryptococcus huempii	Metabasidium is absent	–	52.7	25	–	–/+

* ND stands for not determined; ** "v" stands for ""strain-variable growth."

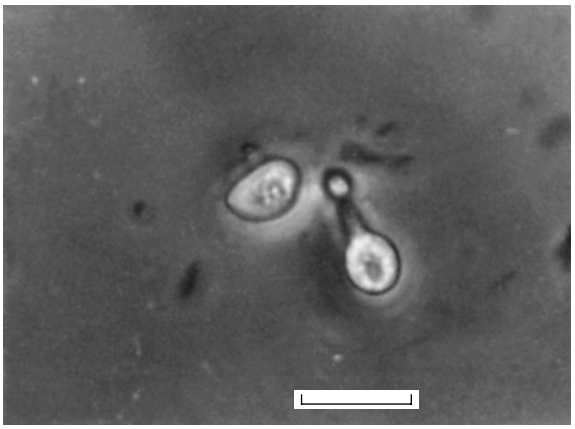

Fig. 6. The germination of a teliospore. Scale bar represents 10 μm.

DISCUSSION

The seven strains under study are very similar to the anamorphous yeast species *Cryptococcus huempii* (Ramirez and Gonzalez) Roejmans et al. in morphology and the range of utilizable carbon and nitrogen sources but differ from the type strain of this species in the sexual cycle. On the other hand, the seven strains are close to the homothallic yeasts of the genus *Mrakia* in their life cycles, characterized by the formation of teliospores and holobasidia, but differ from the type species of this genus, *M. frigida*, in some molecular biological and biochemical characteristics (Table 3).

The new species *Mrakia curviuscula*, into which the seven strains were classified, has much in common with yeasts of the genus *Mrakia* (homothallism, the formation of mycelium without buckles, teliospores, and 1-2-cell-metabasidia, the production of starch, the assimilation of glucuronic acid and nitrates, and psychrophily) but differs from the type species of this genus, *Mrakia frigida* Yamada et Romagata, by inability to ferment glucose and grow-on sucrose, raffinose, and inositol, as well as in the maximal growth temperature and in some molecular biological and biochemical characteristics.

The comparative PCR analysis of the seven strains and the reference species *C. huempii* and *M. frigida* with three universal primers showed that all of these strains belong to one species, which differs from both reference species. The generic affiliation of the new species was established from the comparison of its characteristics with those of the related basidiomycetous yeasts of the order *Cystofilobasidiales.*

In the modern yeast systematics, the genera *Mrakia* and *Cystofilobasidium* and some species of the anamorphous genus *Cryptococcus* are included into the new order *Cystofilobasidiales* Boekhout et Fell, which also comprises, at an 87% similarity level, a monophyletic cluster of taxa belonging to *Tremellomycetidae* of the class *Hymenomycetes* among *Basidiomycota* [13]. Unlike other hymenomycetous yeasts, the teleomorphous genera of this cluster, *Mrakia* and *Cystofilobasidium*, produce teliospores. On account of their characteristics, the anamorphous species *Cryptococcus aquaticus* and *C. huempii* should be included in one cluster with the genus *Mrakia.*

REFERENCES

1. Bab'eva, I.P., Golubev, V.I., Kartintsev, A.V., Gorin, S.E., and Zaslavskaya, P.L., Yeasts in Forest and Meadow Biogeocenoses, *Vestn. Mosk. Univ., Ser. Biol. Pochvoved.*, 1973, no. 6, pp. 67–73.
2. Bab'eva, I.P., Yeasts in the Biogeocenoses of Different Natural Zones, *Pochvennye organizmy kak komponenty biogeotsenoza* (Soil Organisms as Components of Biogeocenoses), Moscow, 1984, pp. 131–141.
3. Bab'eva, I.P., Kartintseva, A.A., Maksimova, I.A., and Chernov, I.Yu., Yeasts in the Spruce Forests of the Central State Forest Biosphere Reserve, *Vestn. Mosk. Univ., Ser. 17: Pochvoved.*, 1999, no. 4, pp. 45–49.
4. Bab'eva, I.P., Lisichkina, G.A., Maksimova, I.A., Reshetova, I.S., Terenina, E.E., and Chernov, I.Yu., A New Yeast Species, *Candida anutae* sp. nov., from the Fruiting Bodies of Agarics, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 276–279.
5. Bab'eva, I.P. and Lisichkina, G.A., A New Species of Psychrophilic Basidiomycetous Yeasts *Leucosporidium fasciculatum* sp. nov., *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 801–804.
6. Maximova, I.A., Yeasts Biodiversity in Forest Ecosystems of European Russia, *The rising power of Yeasts in Science and Industry*, 10th Int. Symp. on Yeasts, 2000, p. 138.
7. Kurtzman, C.P. and Fell, J.W., *The Yeasts: A Taxonomic Study*, New York: Elsevier, 1998.
8. Marmur, J.A., A Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms, *J. Mol. Biol.*, 1961, vol. 3, pp. 208–214.
9. Owen, R.J., Hill, R.L., and Lapage, S.P., Determination of DNA Base Composition from Melting Profiles in Dilute Buffers, *Biopolymers*, 1969, vol. 7, pp. 503–516.
10. Danilevich, V.N. and Grishin, E.V., A New Approach to Isolation of Genomic DNA from Yeast and Fungi: Obtaining of DNA-Containing CM Envelopes and Their Direct Use in PCR, *Bioorg. Khim.*, 2002, vol. 28, no. 2, pp. 156–167.
11. Bulat, S.A., Mironenko, N.V., and Zholkevich, Yu.G., The Genetic Structure of the Soil Population of the Fungus *Fusarium oxysporum* Schlechtend FR: The Molecular Reidentification of the Species and the Genetic Differentiation of Isolates by the Polymerase Chain Reaction Technique with Universal Primers (UP-PCR), *Genetika*, 1995, vol. 31, no. 3, pp. 315–323.
12. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Lab., 1989.
13. Fell, J.W., Roejmans, H., and Boekhout, T., *Cystofilobasidiales*, a New Order of Basidiomycetous Yeasts, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 907–913.