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EXPERIMENTAL ARTICLES

Massive Isolation and Identification of *Saccharomyces paradoxus* Yeasts from Plant Phyllosphere

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Abstract—Year-round studies of epiphytic yeast communities revealed that the number of ascosporogenous yeasts of the genus *Saccharomyces* inhabiting living and decaying leaves of some plants increased considerably in certain short periods (at the beginning of summer and in winter). Massive isolation of saccharomycetes was performed from 11 plant species; earlier, these yeasts had been revealed mainly in sugar-rich substrates. The isolates were identified as *Saccharomyces paradoxus* based on their physiological properties and RELP analysis of 5.8S-ITS. Possible reasons for short-term increases in the number of saccharomycetes in plant phyllosphere are discussed.

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Key words: Saccharomyces paradoxus, phyllosphere, PCR-analysis, Moscow region.

Yeasts of the genus *Saccharomyces* are the most studied microorganisms because of their great practical importance. At present, six biological sibling species are distinguished within the genus *Saccharomyces*: *Saccharomyces cerevisiae* Meyen ex Hansen, *S. paradoxus* Bachinskaya, *S. bayanus* Saccardo, *S. cariocanus* Naumov et al., *S. kudriavzevii* Naumov et al., and *S. mikatae* Naumov et al. [1, 2]. The yeasts of this genus are mainly associated with the fermentation industry; from natural habitats, only representatives of the species *S. paradoxus* have regularly been isolated. This species was first isolated in 1914 from elm and oak bark exudates in the St. Petersburg and Poltava regions [3]. Since then, *S. paradoxus* has been repeatedly isolated from various regions of the world. Molecular and genetic studies have revealed the occurrence of at least four geographically divergent natural populations of *S. paradoxus*: European [4, 5], Far Eastern [4, 6], Hawaiian [7], and North American [8, 9]. Since the yeasts *S. paradoxus*, in spite of their broad distribution, are scarce in natural ecotopes, standard inoculation methods usually reveal few strains of this species. The method of enrichment cultures is therefore usually applied for the isolation of saccharomycetes from environmental sources. Yeasts of the genus *Saccharomyces* are usually revealed in sugar-rich natural substrates such as exudates of trees, mainly oak [10–12]; they have seldom been isolated from other habitats such as soils, plant leaves, and decaying plant debris [13, 14]. The occurrence, and especially domination, of *S. paradoxus* in these substrates is extremely rare.

In the 2002–2005 period, we studied the seasonal dynamics of epiphytic yeast communities developed on various plant species in Moscow oblast [15, 16]. For the first time, we succeeded in massive isolation of *S. paradoxus* from the phyllosphere of several plant species. The observed abundance of saccharomycetes on plant leaves in certain seasons indicates that some features of their ecology and distribution in nature are far from being well-understood.

This work contains data on the abundance of saccharomycetes in the plant phyllosphere; possible reasons for this phenomenon are discussed.

MATERIALS AND METHODS

Microbiological methods and strains. The studies were carried out in two regions of the Moscow oblast: a woodland located on the territory of the Losiny Ostrov Reserve, and the area surrounding Burtsevo village, Shakhovskii region. Fifty-four species of plants were tested in two types of biogeocenoses: mixed birch–spruce forest, and a secondary postforest meadow. The leaves were sampled 2–3 times a week during the whole year; from the end of October through the end of March, the leaves were collected from under the snow cover. Samples of buds and flowers were also analyzed. The samples were plated within one to two days after sampling.

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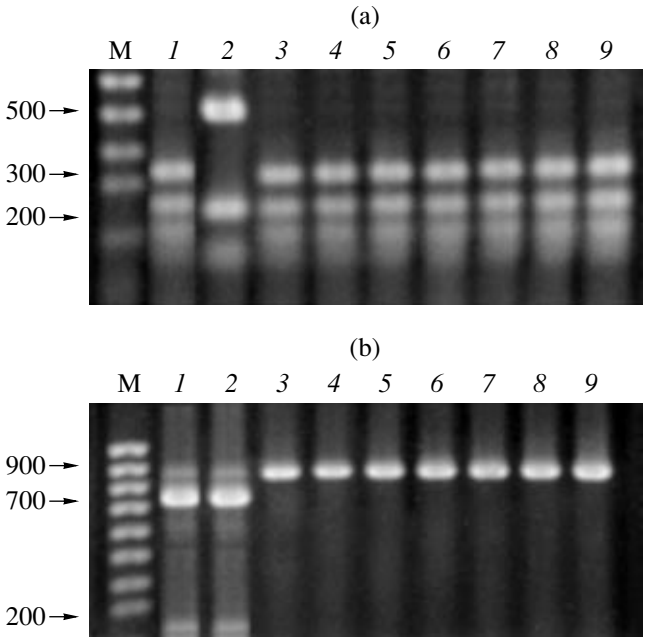

Fig. 1. The restriction analysis of amplified 5.8S-ITS fragments of rDNA of *Saccharomyces* strains with the aid of endonucleases *Hae*III (a) and *Hpa*II (b). Lanes: (*1*), *S. cerevisiae* CBS 1171; (*2*), *S. bayanus* CBS 380; (*3*), *S. paradoxus* CBS 432; (*4*), no. 11; (*5*), no. 13; (*6*), no. 20; (*7*), no. 25; (*8*), no. 30; (*9*), no. 39. (M) denotes the molecular weight marker "100 bp DNA Ladder" (Fermentas, Lithuania).

To enumerate the yeasts, the leaves were cut with scissors; 5–10 samples of 0.1–0.6 g each were mixed with sterile water (1 : 50) and vortexed for 5 min to desorb the yeast cells. Each sample was plated in duplicate onto wort agar, which was acidified to pH 4–4.5 with lactic acid to suppress bacterial growth. The agar plates were incubated at room temperature for five to seven days. The grown yeast colonies were examined for different colonial morphotypes by using a binocular magnifying glass. For each morphotype, the colonies were enumerated separately; two or three of them were isolated in pure culture and identified to the species level on the basis of morphological and physiological identification criteria. The related physiological tests were performed by routine methods [17]; standard media were purchased from Difco (United States). In each sample, the total number of yeasts expressed in colony-forming units per gram dry weight (CPU/g) and the level of each yeast species in the total yeast population were determined.

Molecular methods. The strains were identified by restriction fragment length polymorphism (RFLP) analysis of the 5.8S-ITS region of rDNA. Isolation of DNA, fragment amplification, and restriction analysis were performed as described earlier [18]. Type cultures of *Saccharomyces cerevisiae* CBS 1171, *S. paradoxus* CBS 432, and *S. bayanus* CBS 380 were used as test cultures.

Polymerase chain reaction (PCR) was carried out with the microsatellite primer $(GTG)_5$ in 30 μl of a reaction mixture containing PCR buffer with 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM of each dNTP, 1.0 μl of primer, 0.5 U *Taq* polymerase (Sintol, Russia), and 20 ng of the analyzed genomic DNA. PCR amplifications were run in a Tercyc DNA amplifier (DNK-Tekhnologiya, Russia) with the initial DNA denaturation step at 94°C for 5 min, followed by 40 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 52°C for 2 min, and DNA synthesis at 74°C for 3 min with the final extension step at 74°C for 10 min. The amplification products were separated by electrophoresis in 1% agarose gel at 60 V in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 10 mM EDTA; pH 8.0) for 3 h. The gel was stained with ethidium bromide and photographed under UV light using a Vilber Lourmat transilluminator (France).

RESULTS AND DISCUSSION

We have isolated 129 strains of ascomycetous yeasts, which reproduced vegetatively by true multilateral budding and abundantly formed azigotic asci with four or, rarely, two–three round ascospores. Based on these features, the strains were preliminary affiliated with the genus *Saccharomyces*. Additional physiological and molecular genetic analyses were performed with 44 strains (Table 1). All the strains fermented galactose, maltose, sucrose, and raffinose, but did not ferment melibiose.

Final identification of the strains was performed on the basis of restriction analysis of the PCR-amplified 5.8S-ITS rDNA fragments. With the aid of *Hae*III endonuclease, species *S. bayanus* can be differentiated from *S. cerevisiae* and *S. paradoxus*, which do not differ in their RFLP profiles. Restriction analysis performed with *Hpa*II endonuclease revealed identical RFLP profiles of *S. cerevisiae* and *S. bayanus*, whereas the corresponding restriction site was absent from the PCR product of *S. paradoxus* [18]. The amplified 5.8S-ITS fragments of 44 strains under study and 3 test strains had the same size, about 850 bp (figure not shown), indicating that all the strains belonged to the genus *Saccharomyces*. The PCR products were analyzed with *Hae*III and *Hpa*II restriction endonucleases (Fig. 1). Type culture *S. cerevisiae* CBS 1171 had four *Hae*III restriction fragments of about 320, 230, 170, and 130 bp and two *Hpa*II fragments of about 730 and 120 bp (Fig. 1, line *1*). Type culture *S. bayanus* had three *Hae*III restriction fragments of about 490, 230, and 130 bp (Fig. 1a, line *2*). All the strains isolated had restriction profiles similar to that of the test culture *S. paradoxus* (Fig. 1, lanes *3–9*). Their PCR profiles were characterized by the absence of *Hpa*II restriction site and had the same *Hae*III restriction profiles as that of *S. cerevisiae*. The RFLP analysis confirmed that all of the isolates belonged to the species *S. paradoxus*.

Table 1. The origin of *S. paradoxus* strains studied by molecular methods

Strain no.	Date of isolation	Plant	Substrate	Biogeocenosis	Source of isolation
1	07.05.2004	Cowberry	Greenleaves	Bog	Losiny Ostrov
2	07.05.2004	Cowberry	Greenleaves	Bog	Losiny Ostrov
3	07.12.2004	Maple	Buds	Forest	Losiny Ostrov
4	07.15.2004	Moss	Green parts	Forest	Losiny Ostrov
5	07.08.2004	Hazel	Fruits	Forest	Losiny Ostrov
6	03.27.2004	Ajuga	Green leaves	Forest	Losiny Ostrov
7	03.27.2004	Ajuga	Green leaves	Forest	Losiny Ostrov
8	07.09.2004	Hazel	Fruits	Forest	Losiny Ostrov
9	07.09.2004	Swida	Fruits	Forest	Losiny Ostrov
10	07.04.2004	Dandelion	Green leaves	Bog	Losiny Ostrov
11	03.05.2004	Linden	Forest litter	Forest	Losiny Ostrov
12	02.24.2004	Birch	Forest litter	Forest	Losiny Ostrov
13	07.04.2004	Equisetum	Green parts	Bog	Burtsevo
14	07.05.2004	Equisetum	Green parts	Bog	Burtsevo
15	04.24.2004	Larch	Green needles	Bog	Losiny Ostrov
16	04.24.2004	Larch	Green needles	Forest	Losiny Ostrov
17	04.13.2004	Spruce	Green needles	Forest	Losiny Ostrov
18	04.13.2004	Spruce	Green needles	Forest	Losiny Ostrov
19	04.28.2004	Spruce	Green needles	Forest	Losiny Ostrov
20	07.01.2004	Spruce	Green needles	Forest	Burtsevo
21	07.01.2004	Spruce	Green needles	Forest	Burtsevo
22	07.02.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
23	07.03.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
24	07.04.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
25	07.09.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
26	07.12.2004	Cowberry	Greenleaves	Bog	Losiny Ostrov
27	07.12.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
28	07.12.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
29	07.12.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
30	07.12.2004	Cowberry	Green leaves	Bog	Losiny Ostrov
31	07.03.2004	Spruce	Green needles	Forest	Losiny Ostrov
32	07.05.2004	Spruce	Green needles	Forest	Losiny Ostrov
33	07.15.2004	Spruce	Green needles	Forest	Losiny Ostrov
34	07.21.2004	Spruce	Green needles	Forest	Losiny Ostrov
35	07.21.2004	Spruce	Green needles	Forest	Losiny Ostrov
36	07.15.2004	Pruce	Green needles	Forest	Losiny Ostrov
37	04.24.2004	Larch	Green needles	Forest	Losiny Ostrov
38	07.24.2004	Larch	Green needles	Forest	Losiny Ostrov
39	07.24.2004	Larch	Green needles	Forest	Losiny Ostrov
40	03.27.2004	Ajuga	Green leaves	Forest	Losiny Ostrov
41	07.22.2004	Spruce	Green needles	Forest	Losiny Ostrov
42	07.12.2004	Spruce	Green needles	Forest	Losiny Ostrov
43	07.01.2004	Spruce	Green needles	Forest	Losiny Ostrov
44	07.01.2004	Spruce	Green needles	Forest	Losiny Ostrov

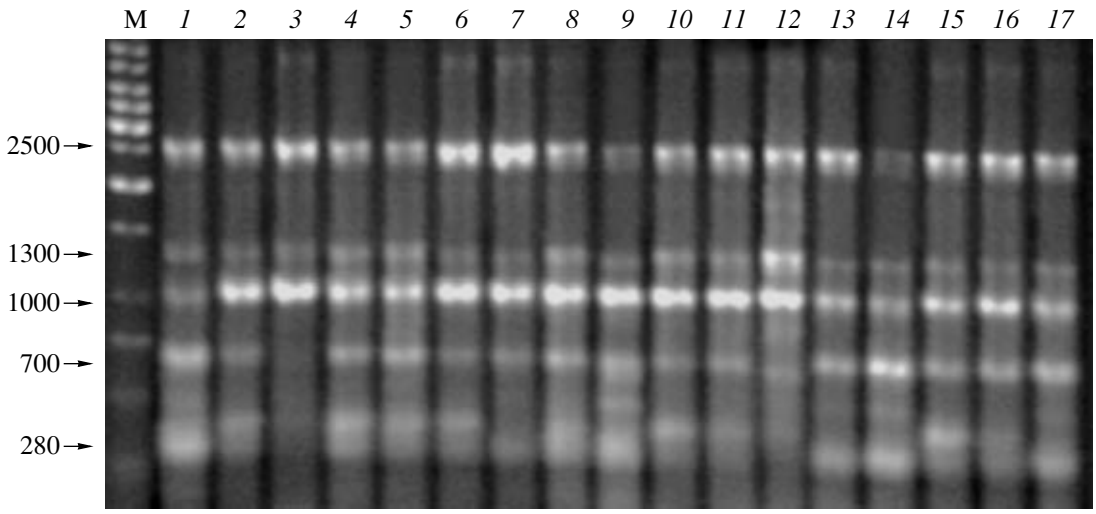

Fig. 2. PCR analysis of yeasts *S. paradoxus* with the use of the microsatellite primer $(GTG)_5$. Lanes: (*1*), no. 1; (*2*), no. 25; (*3*), no. 30; (*4*), no. 6; (*5*), no. 7; (*6*), no. 13; (*7*), no. 14; (*8*), no. 15; (*9*), no. 39; (*10*), no. 17; (*11*), no. 20; (*12*), no. 41; (*13*), no. 3; (*14*), no. 8; (*15*), no. 9; (*16*), no. 11; (*17*), no. 12. (M) denotes the molecular weight marker "1 kb DNA Ladder" (Fermentas, Lithuania).

Earlier, we revealed that individual strains of the European population of yeasts *S. paradoxus* could be differentiated with the aid of the microsatellite primer $(GTG)_5$; strain patterns were found to correlate with the habitat of isolates [5]. Thus, phylogenetic analysis revealed that strains of *S. paradoxus* isolated from the central regions of European Russia and Ukraine, from Northern Europe, and from regions with hot climate (Spain, Southern Crimea, Uzbekistan, and Azerbaijan) formed separate clusters.

We compared the $(GTG)_5$ profiles of 17 strains of *S. paradoxus* isolated from various plant species throughout a year (Fig. 2). All strains had similar patterns with major fragments of about 2500, 1300, 1000, 700, and 280 bp. Insignificant polymorphism of the PCR products was revealed only in the presence or absence of minor bandsd with the size from 280 to 700 bp. Thus, all the strains under study had virtually identical molecular markers (PCR profiles) regardless of the season and the source of their isolation (Table 1) and formed genetically homogeneous populations. Out of the 54 plant species studied, 11 species were most abundantly populated by yeasts *S. paradoxus* (Table 2). Yeasts were isolated mainly from young leaves of these plants and fallen leaves in the forest litter.

The average annual population of *S. paradoxus* did not exceed 0.4% of the total yeast density; however, in certain seasons, the number of this species increased sharply (Fig. 3). The relative abundance of this species reached about 6%, and in several samples up to 100%

Table 2. The average monthly occurrence of *S. paradoxus* on various plants (%)

Species	May	June	July	August	September	October	November	December	January	February	March
Cowberry *Vaccinium vitis-idaea*	0	0	46.7	0	0	0	0	0	0	0	0
Equisetum *Equisetum sylvaticum*	0	0	16.7	0	0	0	0	0	0	0	0
Dandelion *Taraxacum officinale*	0	0	8	0	0	0	0	0	0	0	0
Hazel *Corylus avellana*	0	0	12.5	0	0	0	0	0	0	0	0
Elder *Sambucus racemosa*	0	0	0	60	0	0	0	0	0	0	0
Linden *Tilia cordata*	0	0	0	0	0	0	2.6	10.0	1.2	3.2	3.0
Orchard Grass *Dactylis glomerata*	0	0	0	0	0	0	0	0	15.6	0	0
Oak *Quercus robur*	0	0	0	0	0	0	0	0	8.3	0	0
Sedge *Carex pilosa*	0	0	0	0	0	0	0	0	0	9.5	0
Birch *Betula verrucosa*	0	0	0	0	0	0	0	0	0	5.9	0
Ajuga *Ajuga reptans*	0	0	0	0	0	0	0	0	0	6.7	17.6

in winter and at the onset of spring, under snow cover. In these periods, *S. paradoxus* was revealed on six plant species, both on the leaves of evergreen plants, such as *Aiuga reptans*, *Carex pilosa*, *Dactylis glomerata* and *Dactylis glomerata*, and on fallen leaves. Moreover, the population density of *S. paradoxus* somewhat increased at the onset of summer on young leaves of cowberry and on unripe hazelnut and elderberry. In other seasons, *S. paradoxus* was not observed on these plants or on the other phyllosphere-associated substrates; only a few strains were isolated from buds and flowers.

The data obtained indicate that *Saccharomyces* yeasts, contrary to the existing ideas about their predilection for sugar-rich substrates, are typical epiphytic species and can form a major part of the plant-associated yeast population. However, the development of these yeasts on leaves was transient and had pronounced seasonal dynamics; this is why their occurrence in epiphytic yeast communities can be revealed only in the course of continuous analysis throughout a year. In most earlier studies, epiphytic yeasts were counted mainly in the autumn, when their population and species diversity were maximal [19] or, rarely, three or four times a year [20].

Earlier, we revealed that the populations of some other species of ascomycetous yeasts in plant phyllosphere showed temporary peaks, whereas basidiomycetous species dominated throughout the whole period of plant ontogeny. For instance, ascosporous yeasts *Torulaspora delbrueckii* were abundant on young leaves of *Impatiens nolitangere* for a short time period in the middle of June and were not revealed in the other periods of plant ontogeny up to the complete plant death at the end of October [16]. Some other species of ascomycetous yeasts exhibited similar seasonal dynamics in the plant phyllosphere.

Such temporary increase in the population of ascomycetous yeasts and their predominance in epiphytic communities appeared to be associated with the physiological features of the yeasts, as well as with certain peculiarities of plant ontogeny, in particular, with variations in the amount and composition of plant exudates. Most representatives of the order *Saccharomycetales* are characterized by restricted assimilation capabilities and can assimilate only a few simple sugars of plant exudates. The *S. paradoxus* isolates grew on only 7 of the 44 test compounds which are usually applied for yeast identification. To compare, basidiomycetous yeasts that constantly prevailed in the phyllosphere utilized about 75% of the test compounds.

The restricted capability of nutrient assimilation by *S. paradoxus*, as well as its lacking such morphological adaptive properties typical of epiphytic basidiomycetes as the formation of polysaccharide capsules, carotenoid pigmentation, and the ability to form chlamidospores and ballistospores, do not enable this species to form the epiphytic community of the phyllosphere on equal terms with the dominating basidiomycetous species.

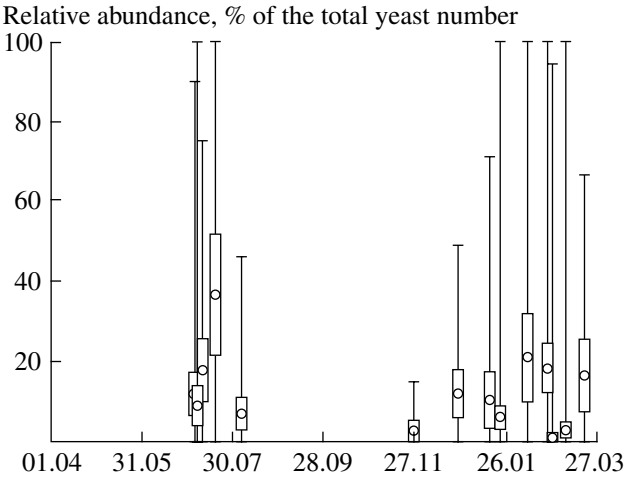

Fig. 3. Annual variations in the relative abundance of yeasts *S. paradoxus* in the plant phyllosphere. Circles represent average values at the time of analysis; rectangles denote the error in mean; vertical line segments stand for deviation limits.

We assume that all these features of *S. paradoxus* determine its position in the succession of epiphytic yeasts in phyllosphere; this species is abundant either at the initial stage of leaf development or at the period of leaf decay in the forest litter. At the initial phase, the leaves are protected by a thick cuticle, which decreases surface exudation of plants; this results in insufficient supply of nutrients for the development of the basidiomycetous community. In this period, local accumulation of plant exudates was observed [21, 22]; in several zones, the concentration of simple sugars increased considerably; such conditions were favorable for the development of saccharomycetes and other copiotrophic ascomycetes. A similar pattern was observed at the stage of leaf decay in the forest litter; nutrient accumulation occurred in only a few zones, where the relative number of minor yeast species, such as *S. paradoxus*, in the phyllosphere epiphytic complex temporarily increased.

It cannot be excluded that the population density of *S. paradoxus* in the winter is associated with high survival of their cells and ascospores at low temperatures.

Based on the results obtained, it can be concluded that yeasts *S. paradoxus* of the plant phyllosphere are of great importance for gaining deeper insight into the problem of the population genetics of yeasts.

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