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EXPERIMENTAL ARTICLES

The Finding of the Yeast Species *Saccharomyces bayanus* in Far East Asia

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Abstract—The genetic analyses of nine Far East Asian *Saccharomyces* isolates allowed us to identify three species (*S. cerevisiae*, *S. paradoxus*, and *S. bayanus*). The occurrence of the last species in Far East Asia was shown for the first time. A new methodology for the molecular genetic differentiation of *Saccharomyces* sensu stricto species is described. The ecogeographical distribution of *Saccharomyces* yeasts is discussed.

Key words: *Saccharomyces*, hybridization, PCR analysis, Far East.

Although the ecogeographical distribution of *Saccharomyces* sensu stricto yeasts has been studied for more than one hundred years, little is known about the ecogeography of its sibling *S. cerevisiae* species. The cosmopolitan species *S. cerevisiae* comprises mainly cultured strains, while its wild populations are few [1]. The related wild species *S. paradoxus* is widespread over the world and there are European, Far Eastern, Hawaiian, and North American divergent populations [2–5]. Three endemic wild *Saccharomyces* species, namely, *S. cariocanus* from Brazil and *S. kudriavzevii* and *S. mikatae* from Japan are also known [6].

Of particular interest is the cryophilic species *S. bayanus*, which is mainly associated with low-temperature fermentation processes in the production of wines and ciders in Europe [7–9]. The five known natural isolates of *S. bayanus* were recovered from the caddis fly *Mesophylax adopersus* in Spain (strain MCYC 623), the *Amanita citrina* mushroom in Slovenia (strain CCY 21-31-12), the fruit flies *Drosophila persimiles* in Yosemite National Park and *D. pseudoobscura* in the environs of Lake Berryessa, Davis, the United States (strains UCD 51-206 and UCD 61-137, respectively), and the hornbeam *Carpinus betulus* exudate in Hungary (strain NCAIM Y.00789) [1, 10].

The aim of this work was to identify the *Saccharomyces* strains isolated in the Amur oblast and the Primorsky Kray of Russian Far East and in North Korea.

MATERIALS AND METHODS

Microbiological methods and strains. *Saccharomyces* yeasts were isolated from the exudates collected together with bark pieces from broad-leaved trees and from the fruits of relict plants (Table 1). The bark pieces were incubated at 28°C in a malt extract containing 4 ml/l of 40% lactic acid (chemically pure grade, Reakhim, Russia). The acid was added to the medium in order to inhibit bacterial growth. Samples taken from the malt extract in the phase of active fermentation (2 to 4 days of incubation) were plated onto malt extract agar, and the plates were incubated at 28°C for 2–3 days. The colonies grown on the malt extract agar plates were transferred onto YPD agar, a complete solid nutrient medium containing (g/l) yeast extract, 5; peptone, 10; glucose, 20; and agar, 20. After 1 day of incubation, the grown colonies were transferred onto a sporulation medium containing 30 g/l maltose and 20 g/l agar.

Genetic experiments were carried out using the complete YPD medium. Sporulation was induced by incubating the isolates in the acetate-containing medium described earlier [11]. Sugar fermentation was studied as described in the same paper [11]. Hybridization was carried out by the spore-to-spore method or by the mass mating of haploid cells on the complete YPD medium followed by the isolation of zygotes with a micromanipulator. The ascus walls were digested with the *Helix pomatia* snail gastric juice, and the ascospores were isolated with the micromanipulator.

The monosporous homothallic cultures of *S. bayanus* MCYC 623 (= CBS 7001), *S. cerevisiae* VKM Y-502 (= CBS 5287), and *S. paradoxus* CBS 5829 [1], marked by the mutations *ura3*, *ade1*, and *ade2*, respectively, were used as highly fertile standard test cultures. We also used the haploid genetic lines *S. cerevisiae* S288C (*gal2mal*) and X2180-1A (*agal2mal*). PCR analysis was carried out with the monosporous cultures

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Table 1. The origin and some characteristics of *Saccharomyces* sensu stricto strains

Strain	Source, location, and year of isolation	Fermentation of:				Species affiliation
		Mel	Mal	Gal	Suc	
3.00	Grapes of the vine *Vitis amurensis*, Botanical Garden, Blagoveshchensk, Amur oblast (2000)	–	–	+	+	*S. cerevisiae*
22.00	Fruits of the hawthorn *Grataegus dachurica*, Botanical Garden, Blagoveshchensk, Amur oblast (2000)	–	–	+	+	*S. cerevisiae*
136.01*	Exudate of the elm *Ulmus pumila*, Botanical Garden, Blagoveshchensk, Amur oblast (2001)	+	+	+	+	*S. bayanus*
148.01*	Exudate of the elm *Ulmus pumila*, Botanical Garden, Blagoveshchensk, Amur oblast (2001)	+	+	+	+	*S. bayanus*
159.01**	Exudate of the oak *Quercus mongolica*, Botanical Garden, Blagoveshchensk, Amur oblast (2001)	–	–	+	+	*S. cerevisiae*
163.01**	Exudate of the oak *Quercus mongolica*, Botanical Garden, Blagoveshchensk, Amur oblast (2001)	–	–	+	+	*S. cerevisiae*
61.02	Exudate of the aspen *Populus davidiana*, Marine Experimental Station, Khasan region, Primorsky Kray (2002)	–	–	+	+	*S. paradoxus*
CCY 21-4-89	Fermenting acorn meal, Pionsan, North Korea (1986)	–	+	+	+	*S. cerevisiae*
CCY 21-4-93	Sequoian leaves, Pionsan, North Korea (1986)	–	+	+	+	*S. cerevisiae*

Note: Mel, Mal, Gal, and Suc stand for melibiose, maltose, galactose, and sucrose, respectively.

* These strains were isolated from two trees 500 m apart.

** These strains were isolated from two exudates of the same tree.

of *S. cariocanus* UFRJ 50816, *S. kudriavzevii* IFO 1802, and *S. mikatae* IFO 1815 as the control organisms. Along with the *Saccharomyces* strains that were isolated by us, we also identified two North Korean strains, CCY 21-4-89 and CCY 21-4-93 (Table 1) [12]. The culture collection name abbreviations used in this paper are as follows: MCYC, Departamento de Microbiologia, Escuela Tecnica Superior de Ingenieros Agronomos, Universidad Politecnica de Madrid, Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; VKM, All-Russia Collection of Microorganisms, Moscow, Russia; UFRJ, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brazil; IFO, Institute for Fermentation, Osaka, Japan; and CCY, Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

Molecular genetic methods. DNA was isolated as described earlier [13]. The 5.8S rDNA and internal transcribed ITS1 and ITS2 spacers (5.8S–ITS fragments) were amplified with the primers pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3') [13] 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, 0.3 μM of each primer, 1.25 U *Taq* polymerase (Sintol, Russia), and 20 ng of genomic DNA. PCR amplifications were run in a thermocycler manufactured by DNA Technology (Russia) with the initial DNA denaturation step at 94°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 2 min, primer annealing at 60°C for 1 min, and DNA synthesis at 72°C for 2.5 min, with the final extension step at 72°C for 10 min. The amplification products were electrophoresed in 1% agarose gel at 60–65 V in 0.5× TBE buffer (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) for 2 h. After electrophoresis, the gel was stained with ethidium bromide.

Restriction Fragment Length Polymorphism (RFLP) assay was carried out with *Hpa*II, *Hae*III, and *Taq*I restriction endonucleases purchased from Fermentas (Lithuania). The restriction digests were analyzed by electrophoresis in 1.6% agarose gel at 60–65 V in 0.5× TBE for 3 h. The gel was stained with ethidium bromide and photographed under UV light using a Vilber Lourmat transilluminator (France).

RESULTS AND DISCUSSION

Seven *Saccharomyces* strains were isolated during the 2000–2002 expeditions in the south of Russian Far East. This territory, including the Amur oblast, is a unique ecosystem characterized by a combination of different zonal and exotic landscapes inhabited by endemic and relict plant and animal species. Here, several floral and faunal ecogeographical regions and provinces border. The yeast strains were isolated from the exudates of the oak *Quercus mongolica*, the elm *Ulmus pumila*, and the aspen *Populus davidiana* and from the fruits of relict arborifloral plants, the vine *Vitis amurensis* and the hawthorn *Grataegus dachurica* (Table 1). The genus affiliations of the isolates were

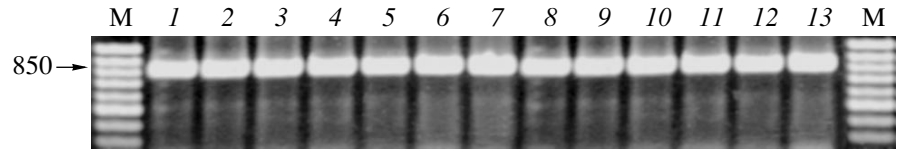

Fig. 1. The size of the amplified 5.8S–ITS rDNA fragments of the *Saccharomyces* sensu stricto strains. *S. cerevisiae*: (*1*) VKM Y-502, (*2*) 159.01, (*3*) 163.01, (*4*) 22.00, (*5*) 3.00; *S. paradoxus*: (*6*) CBS 5829, (*7*) 61.02; *S. bayanus*: (*8*) MCYC623, (*9*) 136.01, (*10*) 148.01; *S. kudriavzevii*: (*11*) IFO 1802; *S. mikatae*: (*12*) IFO 1815; *S. cariocanus*: (*13*) UFRJ 50816; M denotes the molecular weight marker 100-bp DNA Ladder (Fermentas, Lithuania).

determined by analyzing the morphology of their colonies, vegetative cells, and ascospores, as well as by the ability to ferment glucose [14]. The yeast species *S. bayanus* was differentiated based on the ability to ferment sugars, first of all, melibiose. Our studies confirmed the inability of *S. paradoxus* and the wild *Saccharomyces cerevisiae* strains to ferment maltose (Table 1).

The molecular genetic differentiation of the yeast isolates. Our earlier studies showed that six sibling species of the *Saccharomyces* sensu stricto complex differ in the sequence of the internal transcribed ITS1 and ITS2 rDNA spacers [6] and can be differentiated based on the analysis of the restriction fragment lengths of this region [13]. The species *S. cerevisiae, S. bayanus, S. paradoxus*, and *S. mikatae* can be differentiated with the aid of the *Hae*III and *Hpa*II restriction endonucleases, whereas the species *S. kudriavzevii* has a unique *Taq*I restriction profile. Two species, *S. cariocanus* and *S. paradoxus*, do not differ in their RFLP profiles. The amplification of the 5.8S–ITS fragments of the seven Far Eastern isolates and six test cultures showed that their PCR products had the same size, 850 bp (Fig. 1), indicating that all of them belong to the *Saccharomyces* sensu stricto complex.

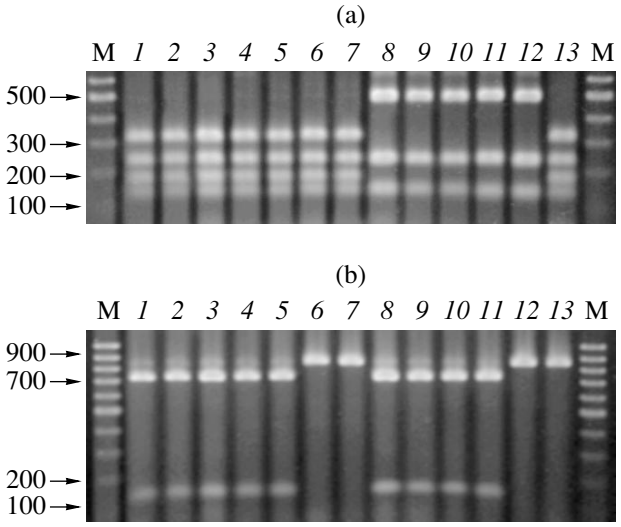

Fig. 2. The analysis of the amplified 5.8S–ITS rDNA fragments of the *Saccharomyces* sensu stricto strains with (a) *Hae*III and (b) *Hpa*II restriction endonucleases. *S. cerevisiae*: (*1*) VKM Y-502, (*2*) 159.01, (*3*) 163.01, (*4*) 22.00, (*5*) 3.00; *S. paradoxus*: (*6*) CBS 5829, (*7*) 61.02; *S. bayanus*: (*8*) MCYC623, (*9*) 136.01, (*10*) 148.01; *S. kudriavzevii*: (*11*) IFO 1802; *S. mikatae*: (*12*) IFO 1815; *S. cariocanus*: (*13*) UFRJ 50816; M denotes the molecular weight marker 100-bp DNA Lad.

The analysis of PCR products with the *Hae*III and *Hpa*II restriction endonucleases (Figs. 2a, 2b) showed that the yeasts studied can be divided into three groups according to their restriction fragment profiles. Group I included the test strain *S. cerevisiae* VKM Y-502 and four Far Eastern strains (3.00, 22.00, 159.01, and 163.01), which had four *Hae*III restriction fragments of about 320, 230, 170, and 130 kb in size (Fig. 2a, lanes *1–5*) and two *Hpa*II restriction fragments of about 730 and 120 kb in size (Fig. 2b, lanes *1–5*. Group II included the test strain *S. paradoxus* CBS 5829, the Far Eastern strain 61.02, and *S. cariocanus* UFRJ 50816 (Figs. 2a, 2b, lanes *6*, *7*, and *13*, respectively). The strains of group II were distinguished from the strains of group I by the absence of the *Hpa*II restriction site, but had the same *Hae*III restriction profiles. Group III included the test strains *S. bayanus* MCYC 623 and *S. kudriavzevii* IFO 1802 and two Far Eastern strains, 136.01 and 148.01 (Figs. 2a, 2b, lanes *8–11*). The *Hae*III restriction fragments of the members of this group had identical sizes (490, 230, and 130 kb). The *Hpa*III restriction profiles of this group were identical to those of the members of group I. The test strain *S. mikatae* IFO 1815 was similar to the strains of group III in the *Hae*III restriction profile and to the strains of group II in the *Hpa*II restriction profile (Figs. 2a, 2b, lane *12*). The *Taq*I restriction profile of the test strain *S. kudriavzevii* IFO 1802 differed from the respective profiles of all other 12 strains (whose profiles were identical), suggesting that strains 136.01 and 148.01 belong to the species *S. bayanus* rather than to the species *S. kudriavzevii*. The results of RFLP assay showed that strain 61.02 belongs either to *S. paradoxus* or to *S. cariocanus*. In view of the fact that there are only two known strains of *S. cariocanus*, which are endemic to Brazil, while the species *S. paradoxus* is ubiquitous, the strain 61.02 was assigned to *S. paradoxus*.

Thus, the restriction enzyme analysis of the seven Far Eastern isolates showed that strains 3.00, 22.00, 159.01, and 163.01 belong to the species *S. cerevisiae*, strain 61.02 belongs to *S. paradoxus*, and strains 136.01 and 148.01 are referred to *S. bayanus*.

Table 2. Analysis of the hybrids of *S. cerevisiae* (strains VKM Y-502, 3.00, 22.00, 159.01, and 163.01), *S. paradoxus* (strains CBS 5829 and 61.02), and *S. bayanus* (strains MCYC 623, 136.01, and 148.01)

Hybrid	Number of spore pairs crossed	Number of zygotes produced	Number of tetrads isolated	Viability of ascospores, %	Number of the *2ADE : 2ade* tetrads analyzed
3.00 × 502	30	4	25	61	10
22.00 × 502	30	2	25	80	12
159.01 × 502	30	5	24	66	12
163.01 × 502	30	9	24	65	8
136.01 × 502	30	16	25	0	–
148.01 × 502	30	8	25	0	–
61.02 × 502	30	5	25	0	–
3.00 × 5829	30	9	25	0	–
22.00 × 5829	30	11	25	0	–
159.01 × 5829	30	9	25	0	–
163.01 × 5829	30	4	24	0	–
61.02 × 5829	30	11	25	53	6
136.01 × 623	30	14	25	99	18*
148.01 × 623	30	14	25	99	18*

Note: The asterisk marks the segregation *2URA : 2ura.*

The genetic identification of the strains. Such studies require highly fertile monosporous yeast cultures. The analysis of the six to ten tetrads of the Far Eastern strains showed that they are homothallic and that their ascospores are highly viable (89–100%). The preliminary molecular genetic differentiation of the strains under study allowed us to minimize the number of hybridization experiments: each of these strains was crossed only with the suspected species and one of the control test cultures (either *S. cerevisiae* or *S. paradoxus*).

The ability of the Far Eastern strains to cross with the test cultures *S. cerevisiae*, *S. paradoxus*, and *S. bayanus* (Table 2) confirmed that they belong to the *Saccharomyces* sensu stricto complex. The high viability of hybrid ascospores and the regular meiotic segregation of the control markers *ade* and *ura* indicated a genetic homology of the parent strains and their affiliation to one species, whereas the sterility of hybrids suggested that their parents belong to different *Saccharomyces* species. The results of hybridization analysis confirmed the presumptive identification of the Far Eastern strains, namely, that strains 3.00, 22.00, 159.01, and 163.01 belong to the species *S. cerevisiae*, strain 61.02 belongs to *S. paradoxus*, and strains 136.01 and 148.01 belong to *S. bayanus* (Table 2).

The genetic study of the North Korean strains CCY 21-4-89 and CCY 21-4-93 was hampered by the low viability of their ascospores and the heterogeneous meiotic segregation of the strains with respect to the growth rate of colonies (including respiration-deficient microcolonies). The microscopic examination of monosporous cultures showed that they contain aggregates of haploid cells. The mating types of monosporous clones were determined by the formation of zygotes in mixtures with the mating type testers S288C and X2180-1A. The strains CCY 21-4-89 and CCY 21-4-93 exhibited the meiotic segregations 2α:2**a**:3D and 5α:3**a**:1D, where α and *a* denote haploid yeasts with the α and **a** mating types and D denotes diploid sporulating yeasts. Fertile monosporous haploid clones were selected by analyzing their hybrids with the genetic lines *S. cerevisiae* S288C and X2180-1A (Table 3). Most of the hybrids were highly fertile but almost all of them, like the parent strains, exhibited heterogeneous segregation with respect to the growth rate of colonies. Based on the last characteristic, the two best monosporous cultures, 24-4-89:7A and 24-4-93:6B, were chosen for further analysis. The analysis of the ten tetrads of the hybrids of these cultures with strain S288C showed that these hybrids are characterized by the regular segregation 2 : 2 with respect to the control marker *gal2*. The hybrid of the strain 24-4-89:7A showed a monogenic segregation with respect to the marker *mal*. The other strain, 24-4-93:6B, likely had more polymeric *MAL* genes, as is evident from the analysis of the ten tetrads of its hybrid, eight of which exhibited the segregation $4Mal^+$:$0Mal^-$ and two of which exhibited the segregation $3Mal^+$:$1Mal^-$. The results of this analysis showed that both North Korean strains belong to the species *S. cerevisiae.*

It should be noted that the ascomycetous yeasts of Russian Far East, including *Saccharomyces* sensu stricto, are far from being well studied [15], in contrast to the yeasts isolated in Japan [16–18]. Unfortunately,

Table 3. The viability of the ascospores of the North Korean strains CCY 21-4-89 and CCY 21-4-93 and their hybrids with the genetic lines *S. cerevisiae* S288C and X2180-1A

Strain	Number of tetrads analyzed	Viability of ascospores, %	Strain	Number of tetrads analyzed	Viability of ascospores, %
21-4-89	9	44	24-4-93	8	44
21-4-89 : 7A × S288C	31	90	24-4-93 : 6B × S288C	28	96
21-4-89 : 7B × S288C	4	94	24-4-93 : 7B × S288C	6	88
21-4-89 : 8A × S288C	5	50	24-4-93 : 73 × S288C	7	96
21-4-89 : 9A × X2180-1A	5	100	21-4-93 : 8A × X2180-1A	7	96

the ecogeographical studies of the Japanese isolates had been performed before the sibling species of *S. cerevisiae* were discovered. Our analysis of six wild Japanese *Saccharomyces* strains showed that four of them belong to the species *S. cerevisiae* and two strains belong to the species *S. paradoxus.* Four other strains were earlier described as new species *S. kudriavzevii* and *S. mikatae* [6].

Among the 17 wild *Saccharomyces* strains from Russian Far East, 5 were found to belong to the species *S. cerevisiae* and 12 were assigned to the species *S. paradoxus* [2, 20]. Four other wild *Saccharomyces* strains isolated from bordering areas (three Siberian strains and one Middle Asian strain) were also identified as *S. cerevisiae* strains.

Thus, in this work, we succeeded in the identification among the Far Eastern natural *Saccharomyces* isolates of three species, *S. cerevisiae*, *S. paradoxus*, and *S. bayanus* (the last species was detected in Far East for the first time). Noteworthy is the fact that both of the studied *S. bayanus* strains were isolated from the exudate of the elm *Ulmus pumila* (Table 1). Another known *S. bayanus* strain was isolated from the exudate of the hornbeam *Carpinus betulus* in Hungary [10]. At the same time, among the 50 *Saccharomyces* strains isolated from the exudates of the oaks *Quercus robur, Q. mongolica*, *Q. rubra*, and *Q. alba* in East Europe, the Caucasus, Far East, and North America, we were able to identify only the species *S. paradoxus* (46 isolates) and *S. cerevisiae* (4 isolates). These data suggest that the species *S. bayanus* is likely associated with the exudates of particular broad-leaved trees. Seasonal and climatic temperature factors likely play an important part in the ecogeographical distribution of cryophilic *S. bayanus* yeasts. The incubation of *S. bayanus*-containing enrichment cultures at low temperatures (below 20°C) may facilitate the isolation of this species. The active *MEL*-encoded α]-galactosidase of *S. bayanus* likely plays an adaptive role. It should be noted in this regard that the *S. paradoxus* strain isolated from the exudate of the elm *Ulmus carpinifolia* in the United States [3] is also able to ferment melibiose, which is a very rare ability for this species.

Based on the Vavilov's theory of geographical centers of biodiversity and taking into account that most of the *Saccharomyces* sensu stricto species (*S. bayanus, S. cerevisiae, S. kudriavzevii, S. mikatae*, and *S. paradoxus*) were found in Far Eastern Asia, we can suggest that it is expedient to search for new *Saccharomyces* species in this geographical region. The gene pool of the Far Eastern *Saccharomyces* isolates is of great importance for fundamental studies.

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