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EXPERIMENTAL ARTICLES

A Genetically Isolated Population of *Saccharomyces cerevisiae* in Malaysia

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Abstract—A divergent population of *Saccharomyces cerevisiae* has been identified in Malaysia by molecular and genetic analysis. It has also demonstrated that the yeast *S. bayanus* may be found in South America. The origin of *S. cerevisiae* is discussed.

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Key words: *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, RFLP analysis, rDNA, genetic analysis.

Genetic studies of over 100 wild and cultured strains of *Saccharomyces cerevisiae*, differing in ecological and geographical origin, led to identifying them as representatives of one and the same biological species [1–15]. In cases where fertile, single-spore, parental strains of *S. cerevisiae* were used, their intraspecific hybrids also exhibited high fertility, with ascospore survival rates in the range 50–100%. Conversely, *S. paradoxus* (the wild counterpart of *S. cerevisiae*) is represented by four geographical populations: European, Far Eastern, North American, and Hawaiian, which are genetically isolated in part [6, 8, 10, 13, 14, 16, 17]. Hybrids of European and Hawaiian strains are practically sterile [6].

In this work, we report on genetic reidentification of certain *Saccharomyces* strains, which were isolated from natural sources in Malaysia and Argentina and deposited in the Culture Collection of the Department of Plant Sciences of the University of Western Ontario (London, Canada).

MATERIALS AND METHODS

Microbiological methods and strains. The strains under study are listed in Table 1, which also contains information on the origin of each strain. The yeasts were cultured and crossed in a complete medium YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 20 g/l agar); sporulation was induced in the acetate medium [7]. The ability to ferment sugars was carried out as described in the same work. Hybridization was performed by two methods: "spore-to-spore" or "spore-to-haploid cell." The membranes of the asci were digested enzymatically, using the gastric juice of the garden snail (*Helix pomatia*); the ascospores were isolated by a micromanipulator. A homothallic culture of *S. bayanus* MCYC 623 (= CBS 7001), marked by the mutation *ura*3, was used as a standard high-fertility tester strain. The biological species *S. cerevisiae* was represented by a haploid genetic line X2180-1A of the genotype MATa *gal*2 *mal mel*. Single-spore type cultures of *S. cariocanus* UFRJ 50816, *S. kudriavzevii* IFO 1802, *S. mikatae* IFO 1815, and *S. paradoxus* CBS 432 were used as controls in PCR-based analyses. Abbreviations designate names of collections: 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); and IFO, Institute for Fermentation, Osaka (Japan).

Molecular methods. Yeast DNA was isolated as described previously [18]. A polymerase chain reaction (PCR) was performed using a Tertsik™ thermocycler. The primers DIR (5'-TTCGCAGATGGGTTGGGACAA-3') and REV (5'-TAAGCTTGCTGGAACAGTTGTGTT-3'), used for amplifying *MEL* genes, made it possible to obtain 1300-bp amplicons. The following conditions were used: initial denaturing at 94°C (3 min); 30 cycles of denaturing at 94°C (30 s), primer annealing at 56°C (30 s), and DNA synthesis at 72°C (60 s); and completion of extensions at 72°C (10 min). Amplification of 5.8S rDNA and of internal transcribed spacers ITS1 and ITS2 (the 5.8S–ITS region) was performed using the primers pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3'). The cycling program comprised the initial denaturing at 94°C (3 min), 30 cycles of denaturing at 94°C (30 s), primer annealing at 56°C (30 s),

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Table 1. Origin of yeast strains of the genus *Saccharomyces*, included into the study

Strain No.	Substrate, location, and year of isolation	Fermentation capacity*				Assigned species identity
		Mel	Mal	Gal	Suc	
UWO 99-807.1.1	Nectar of *Nothofaqus* sp., Patagonia, Argentina (1999)	+	+	+	+	*S. bayanus*
UWO 99-808.3	Nectar of *Nothofaqus* sp., Patagonia, Argentina (1999)	+	+	+	+	*S. bayanus*
UWO 03-429.1	Bertam palm (*Eugeissona* sp.) nectar, Malaysia (2003)	+	+	+	+	*S. cerevisiae*
UWO 03-433.3	Bertam palm (*Eugeissona* sp.) nectar, Malaysia (2003)	+	+	+	+	*S. cerevisiae*
UWO 03-459.1	Bertam palm (*Eugeissona* sp.) nectar, Malaysia (2003)	+	+	+	+	*S. cerevisiae*
UWO 03-461.4	Bertam palm (*Eugeissona* sp.) nectar, Malaysia (2003)	+	+	+	+	*S. cerevisiae*

* Mel, Mal, Gal, and Suc designate, respectively, the capacity for melibiose, maltose, galactose, and sucrose fermentation.

DNA synthesis at 72°C (60 s), and completion of extensions at 72°C (10 min). PCR was run in 30 μl of a buffer that contained 2.5 mM $MgCl_2$, dNTPs (0.1 mM each), primers (50 pM each), 2.5 U *Taq*-polymerase (Sintol, Russia), and 20 to 200 ng DNA.

Restriction fragment length polymorphism (RFPL) analysis of 5.8S–ITS regions of rDNA and *MEL* genes was performed using restriction endonucleases *Hae*III/*Hpa*II and *Hind*III/*Hinc*II, respectively (Fermentas, Lithuania). Restriction fragments were separated in a 2.5% agarose gel at 60–65 V for 4 h; 0.5× TBE was used as a buffer. The gel was stained with ethidium bromide and photographed in UV using a Vilber Lourmat transilluminator (France).

Chromosomal DNA was prepared as described previously [19]. Electrophoresis was performed in a Bio-Rad CHEF-DR III device (United States) at 200 V (for 15 h with a switching time of 60 s and then for 9 h with a switching time of 90 s), using 0.5 × TBE as a buffer. *S. cerevisiae* YNN 295 was used as a karyotyping standard.

RESULTS AND DISCUSSION

For studies of the evolutionary diversity of *Saccharomyces* yeasts, we turned our attention to six new strains isolated in unusual natural sources from different geographical locations (Table 1).

Molecular identification. Initially, the isolates were identified as *Saccharomyces* strains, based on the results of RFLP analysis of 5.8S–ITS regions and molecular karyotyping.

The region spanning the internal transcribed spacers (ITS1 and ITS2) and the gene encoding 5.8S rRNA is known to have a size of about 850 bp, in all representatives of genus *Saccharomyces* [8, 20]. The nucleotide sequences of this region of rDNA are, however, species-specific. Restriction endonucleases *Hae*III and *Hpa*II make it possible to discriminate five out of the six species of *Saccharomyces* [8]. *S. bayanus* and *S. kudriavzevii* are two species that give identical RFLP profiles, when analyzed using the above restriction endonucleases.

We amplified 5.8S–ITS regions in the six isolates under study and the two species-specific reference strains. The size of the amplicons was identical in all the strains, amounting to 850 bp (the figure is not shown). This result confirms that all strains belonged to the genus *Saccharomyces*. Further analysis of amplicons, using restriction endonucleases *Hae*III and *Hpa*II made it possible to identify the strain at the level of species. *Hae*III cleavage patterns separated the strains into two groups (Fig. 1a). The first group comprised reference strains *S. bayanus* MCYC 623, *S. kudriavzevii* IFO 1802, *S. mikatae* IFO 1815, and two strains from Argentina, 99-807.1.1 and 99-808.3 (restriction fragments of about 490, 230, and 130 bp; Fig. 1a, lanes *3, 5–8*). Reference strains *S. cerevisiae* X2180-1A, *S. paradoxus* CBS 432, *S. cariocanus* UFRJ 50816, and the strains from Malaysia, 03-429.1, 03-433.3, 03-459.1, and 03-461.4, formed the second group (restriction fragments of about 320, 230, 170, and 130 bp; Fig. 1a, lanes *1*, *2*, *4*, *9–12*). With *Hpa*II, the six strains under study (99-807.1.1, 99-808.3, 03-429.1, 03-433.3, 03-459.1, and 03-461.4) produced two restriction fragments (of about 730 and 120 bp; Fig. 1b, lanes *7–12*). Reference strains *S. cerevisiae* X2180-1A, *S. bayanus* MCYC 623, and *S. kudriavzevii* IFO 1802 exhibited the same patterns (Fig. 1b, lanes *1, 3,* and *5*). Other control strains did not contain *Hpa*II-restriction sites within the 5.8S–ITS regions (Fig. 1b, lanes *2, 4,* and *6*).

The data obtained indicate that the strains from Malaysia (03-429.1, 03-433.3, 03-459.1, and 03-461.4) belong to the species *S. cerevisiae*. Based on the restriction profiles, the strains from Argentina (99-807.1.1 and 99-808.3) should be classified as *S. bayanus* or *S. kudriavzevii*.

Both Argentinean strains are capable of fermenting melibiose (Table 1). This trait is characteristic of the yeast *S. bayanus*, whereas all *S. kudriavzevii* strains known to date have the phenotype Mel^-. The four Malaysian strains are also capable of fermenting melibiose. Amplification of a 1300-bp portion of the α-galactosidase gene *MEL* in the six strains under study, followed by the RFLP analysis, demonstrated that the Argentinean and Malaysian strains differed in

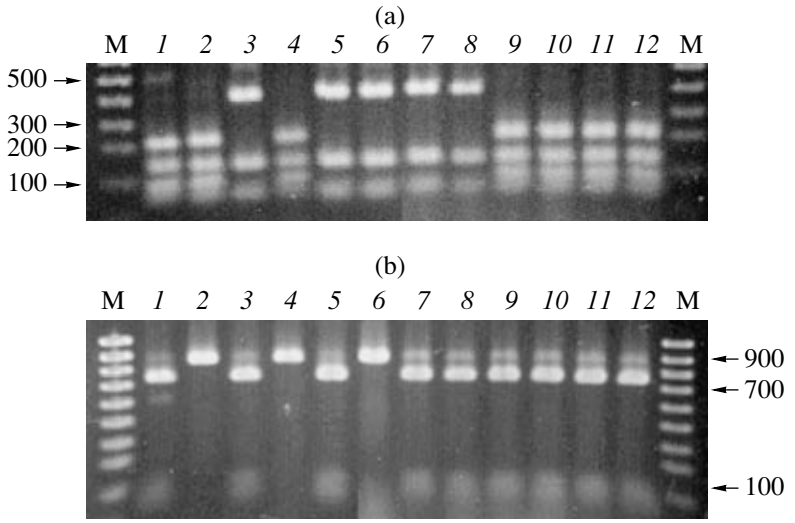

Fig. 1. Restriction analysis of amplified 5.8S–ITS rDNA regions of *Saccharomyces* strains using endonucleases *Hae*III (a) and *Hpa*II (b): *S. cerevisiae* (*1*, X2180-1A; *9*, UWO 03-429.1; *10*, UWO 03-433.3; *11*, UWO 03-459.1; and *12*, UWO 03-461.4); *S. bayanus* (*3*, MCYC 623; *7*, UWO 99-807.1.1; and *8*, UWO 99-808.3); *S. paradoxus* (*2*, CBS 432); *S. cariocanus* (*4*, UFRJ 50816); *S. kudriavzevii* (*5*, IFO 1802); and *S. mikatae* (*6*, IFO 1815). M, molecular weight (bp) marker (100bp DNA Ladder, Fermentas, Lithuania).

their restriction patterns of *MEL* genes. PCR-amplified regions of *MEL* in the Argentinean strains 99-807.1.1 and 99-808.3 resisted cleavage by *Hinc*II and formed fragments of about 850 and 450 bp (the figure is not shown), as a result of *Hind*III restriction. Conversely, the Malaysian strains 03-433.3 and 03-459.1 did not contained *Hind*III-restriction sites within *MEL* genes, and *Hinc*II restriction of the PCR-amplified regions produced three fragments, of about 200, 300, and 800 bp (the figure is not shown). The two restriction patterns obtained are characteristic of *MEL*-genes of *S. bayanus* and *S. cerevisiae*, respectively [18].

Molecular karyotyping provided convincing evidence that the Argentinean strains 99-807.1.1 and 99-808.3 belong to *S. bayanus*. All yeasts of the genus *Saccharomyces* have identical sets of haploid chromosomes (n = 16) with maximum sizes in the range 250–2200 kbp. The sizes and order of individual chromosomes may vary between species [12]. In particular, *S. bayanus* strains have a species-specific karyotype that is readily discernable from other *Saccharomyces* species. Molecular karyotypes of the strains under study are shown in Fig. 2. *S. cerevisiae* YNN 295, a strain with known chromosome sizes and order, was used as a karyotyping standard; the strain *S. cerevisiae* VKPM Y-61 served as an additional control. The Malaysian strains 03-429.1, 03-433.3, 03-459.1, and 03-461.4 did not differ in their karyotypes from strain YNN 295 (Fig. 2, lanes *3–6* and *1*, respectively). This confirms their identification as belonging to the species *S. cerevisiae*. The Argentinean yeasts 99-807.1.1 and 99-808.3 had karyotypes that were practically identical to that of the tester strain *S. bayanus* MCYC 623, which was characterized electrophoretically by the appearance of three chromosomal bands, one corresponding to the size of 1300 bp and two of 245–370 kbp each (Fig. 2, lanes *7–9*). Thus, based on the molecular data,

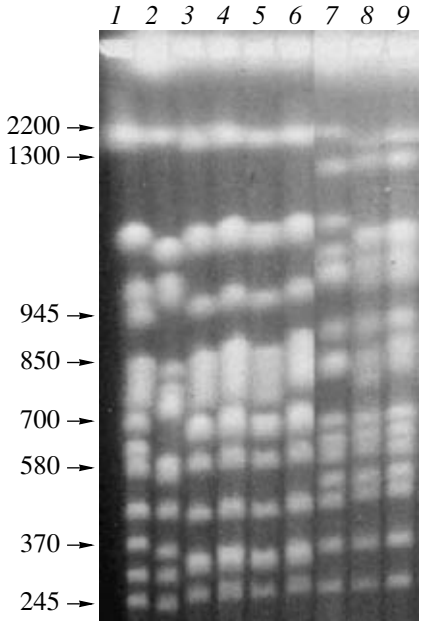

Fig. 2. Pulsed electrophoresis of chromosomal DNAs of *Saccharomyces* strains. *S. cerevisiae*: *1*, YNN 295 (chromosomal standard); *2*, VKPM Y-61 (control strain); *3*, UWO 03-429.1; *4*, UWO 03-433.3; *5*, UWO 03-459.1; and *6*, UWO 03-461.4. *S. bayanus*: *7*, MCYC 623 (control strain); *8*, UWO 99-807.1.1; and *9*, UWO 99-808.3. The order and size (kbp) of chromosomes follow the standard (YNN 295).

Table 2. Analysis of hybrids of yeast species *S. bayanus* (MCYC 623, UWO 99-807.1.1, and UWO 99-808.3) and *S. cerevisiae* (X2180-1A, UWO 03-429.1, UWO 03-433.3, UWO 03-459.1, and UWO 03-461.4)

Hybrid origin	Number of spore-to-spore or spore-to-cell crosses	Number of zygotes formed	Number of tetrads isolated	Ascospore survival rate, %	Meiotic marker segregation*
99-807.1.1 × 623	40	12	24	98	2*URA* : 2*ura* (11)
99-808.3 × 623	44	18	24	98	2*URA* : 2*ura* (14)
03-429.1 × X2180-1A	36	9	26	7	14*MEL* : 18*mel* 18*GAL* : 14*gal* 16*MAL* : 16*mal*
03-433.3 × X2180-1A	35	2	50	8	
03-459.1 × X2180-1A	40	7	25	7	
03-461.4 × X2180-1A	38	3	25	5	

* The number of tetrads analyzed is given in parentheses.

the strains 99-807.1.1 and 99-808.3 were identified as belonging to the strain *S. bayanus.*

Genetic identification of the strains. Tetrad analysis of the Argentinean and Malaysian strains demonstrated a high fertility. The yeasts sporulated well and exhibited a 100% ascospore survival. For each strain, 6–11 tetrads were analyzed. Sporulation of single-spore clones indicated that all six strains were homothallic. Single-spore high-fertility homozygous clones were used for final genetic determination of the strains. Based on the results of molecular identification, the yeasts under study were crossed with high-fertility reference strains of the corresponding species, *S. bayanus* MCYC 623 and *S. cerevisiae* X280-1A. All the hybrids obtained were capable of sporulating and could be used in tetrad analysis. The results of the experiments on obtaining and studying the hybrids are summed up in Table 2. Normal monogenic segregation of the control marker *ura* and the high-survival rate of hybrids with the tester strain MCYC 623 provide unequivocal evidence that the Argentinean strains 99-807.1.1 and 99-808.3 belong to the species *S. bayanus*. Conversely, the Malaysian strains 03-429.1, 03-433.3, 03-459.1, and 03-461.4 formed low-fertility hybrids, when crossed with the reference strain X280-1A. In these crosses, however, we observed regular meiotic segregation and recombination of the three control markers *mel*, *gal*, and *mal*. Although indicative of the Malaysian strains belonging to the species *S. cerevisiae*, the data obtained testified to relatively high genetic divergence. In analyzing ribosomal and *MEL* markers, we did not run preliminary tests that would find these yeasts to be considerably different from each other at the molecular level. It is conceivable, therefore, that divergence is primarily related to chromosomal rearrangements (translocations, inversions, duplications, deletions, etc.).

As indicated in the introduction, the biological species *S. cerevisiae* comprises strains that differ in ecological and geographical origin. In our prior experiments, crosses between reference *S. cerevisiae* strains and cultured or natural strains of this species from diverse world locations (Western-, Central-, and Eastern Europe; Near East, Far East, and Central-, South-, and Southeast Asia; Central- and Southern Africa; North- and South America; and the Hawaiian Islands) always resulted in high-fertility hybrids. It is, therefore, doubtless that the Malaysian population is genetically isolated from the commonly encountered cosmopolitan strains of *S. cerevisiae*. It is known that the spread of the yeast *S. cerevisiae* is due to fermenting activities of humans. The possibility that the Malaysian population is the most ancient representative of the species cannot be ruled out; should that be the case, conventional cultured and ordinary wild strains of *S. cerevisiae* would constitute a species *in statu nascendi*. A more detailed study of Malaysian and other Asian strains of *S. cerevisiae*, involving experiments at the molecular and chromosomal levels, is needed for clarifying this possibility.

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