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EXPERIMENTAL ARTICLES

Genome Variability of the Yeast *Yarrowia lipolytica*

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Abstract—Using sequence analysis of internal transcribed spacers ITS1 and ITS2, RAPD-PCR, and pulsed-field gel electrophoresis of intact chromosomal DNA, we investigated the molecular and genetic peculiarities of the genomes of 40 *Yarrowia lipolytica* strains. All the strains showed nearly identical ITS-sequences. On the other hand, karyotypic analysis revealed significant differences in the chromosomal patterns of *Y. lipolytica*. The number and order of individual chromosomes vary from strain to strain. Chromosome-length polymorphism of the *Y. lipopytica* strains was pronounced and independent of their geographic origin and the source of isolation. Intraspecific polymorphism of *Y. lipolytica* chromosomes is discussed.

Key words: Yarrowia lipolytica, dimorphic yeasts, 5.8S-ITS, chromosomal DNA, molecular karyotyping, phylogenetic analysis.

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The gene pool of the yeasts used in fundamental and applied studies is constantly expanding. New promising microorganisms with unusual important properties are resorted to; including the dimorphic yeast *Yarrowia lipolytica*, a producer of various physiologically active substances, including hydrolytic enzymes. Full-fledged use of this yeast is impossible without knowledge of its molecular and genetic peculiarities. In its physiological, genetic, and molecular characteristics, *Y. lipolytica* is completely different from the well-studied yeast *Saccharomyces cerevisiae* [1, 2]. Comparative analysis of more than 40 full-sized fungal genomes showed that the yeast *Y. lipolytica* represents a separate phylogenetic lineage and is genetically unrelated to other species of ascomycetous yeasts [3]. Determination of the complete nucleotide sequence of the genome of *Y. lipolytica* based on the genetic line CLIB122 (http://www.ncbi.nlm.nih.gov) formed a good basis for investigating the species and strain characteristics of this yeast.

Modern genetics and molecular taxonomy allow us to unambiguously carry out identification of yeast species and strains on the basis of molecular markers, including individual genes, nucleotide sequences, or whole chromosomes. In order to establish the phylogenetic relationship between the yeasts and determine their species identity, analysis of the ribosomal RNA gene sequences is used. Based on the results of sequencing the 26S rRNA D1/D2 domain of the type cultures of over 600 species of ascomycetous yeasts [4, 5], a computer database was created that is currently used to determine the taxonomic position of new strains. In order to determine the phylogenetic relationship between close species, analysis of the 5.8S-ITS fragment spanning the 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 is additionally used. This rRNA region is characterized by significant interspecies divergence and low level of intraspecific polymorphism. The length of the ITS region is constant in the strains of the same species [6], but its sequence may vary [7, 8].

Essentially important information on the chromosomal composition of yeast genomes can be obtained with pulsed-field gel electrophoresis of native chromosomal DNA. This method representing a sort of molecular karyology is widely used for the studies of the evolution and taxonomy of different yeasts [9, 10].

In this work, the study of molecular and genetic peculiarities of the genome of the yeast *Y. lipolytica* was carried out using 40 strains of different geographic and ecological origin.

MATERIALS AND METHODS

The strains studied and their origin are shown in the table. The yeasts were cultivated at 28°C on complete YPD medium (10 g/l of yeast extract, 20 g/l of peptone, 20 g/l of glucose, and 20 g/l of agar).

Polymerase chain reaction was performed on a Tercyk DNA amplifier (DNK-Technology, Russia) directly on yeast cells with primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG) for the D1/D2 domain and ITS1 (5'-TCCGTAGGTGAACCTGCGG), ITS4 (5'-TCCTCCGCTTATTGATATGC) for the 5.8S-ITS-fragment. A small amount of yeast biomass was suspended with a microbiological loop in 30 μl of the buffer containing 3 mM $MgCl_2$, 0.3 mM of

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Origin of the studied strains of *Yarrowia lipolytica*

Collection number	Source and place of isolation
CLIB122	Genetic line
CBS 599	Rancid margarine, The Netherlands
CBS 2070	Damaged cornea, Italy
CBS 2072	Unknown
CBS 2073	Olives, Italy
CBS 2074	Olives, Italy
CBS 2075	Rancid margarine, The Netherlands
CBS 2078	Soil, The Netherlands
CBS 2787	Human skin, Germany
CBS 5570	Human lungs, Argentina
CBS 5589	Human lungs, Argentina
CBS 5699	Hydrocarbons, France
CBS 5919	Unknown
CBS 6012	Unknown
CBS 6114	Unknown
CBS 6124 (T)	Corn-processing plant, United States
CBS 6124.1	Monospore culture of strain CBS 6124
CBS 6124.2	Monospore culture of strain CBS 6124
CBS 6125	Corn, United States
CBS 6303	Soil, Japan
CBS 6317	Dairy product, United States
CBS 6614	Sewage, France
CBS 6659	Soil, Russia
CBS 6660	Soil, Russia
CBS 7033	Soil, Japan
CBS 7034	Soil
CBS 7504	Soil, France
VKPM Y-2	Bulgaria
VKPM Y-34	Unknown
VKPM Y-35	Unknown
VKPM Y-184	Unknown
VKPM Y-281	Unknown
VKPM Y-1711	Unknown
VKPM Y-1712	Oil
VKPM Y-3153	Salad from delicacies
VKPM Y-3154	Soil, Poland
VKPM Y-3155	Genetic line
VKPM Y-3156	Strain CBS 7504 mutant
VKPM Y-3157	Genetic line
VKPM Y-3195	Unknown

Notes: Abbreviated collection names: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CLIB, Collection de Levure d'Intérêt Biotechnologique, Thiverval-Grignon, France; and VKPM, All-Russian Collection of Industrial Microorganisms, Moscow.

T designates the type culture.

each dNTP, and 50 pmol of each of the primers. The mixture was then incubated at 95°C for 15 min for cell lysis and supplemented with 2.5 units of *Taq*-polymerase (Syntol, Russia). For the D1/D2 domain amplification, the PCR was performed in the following mode: initial denaturation, 1 min at 94°C followed by 36 cycles: DNA denaturation, 94°C for 1 min; primer annealing, 52°C for 1 min; DNA synthesis, 72°C for 1 min; and final elongation, 1 min at 72°C. For amplification of the 5.8S-ITS fragment, initial denaturation was carried out at 94°C for 3 min followed by 30 cycles in the following mode: denaturation at 94°C, 30 s; primer annealing at 56°C, 30 s; DNA synthesis at 72°C, 60 s; and final elongation, 72°C for 10 min.

Sequencing and phylogenetic analysis. The amplified D1/D2 and 5.8S-ITS fragments were eluted from the gel using the GeneClean Kit (Bio 101 Inc., United States) according to the manufacturer's protocol. The nucleotide sequences were determined for two chains by direct sequencing according to the Sanger method on a Beckman-Coulter automatic sequencer (United States). The search for homology with known nucleotide sequences was carried out using the BLAST software package. Multiple alignment of the obtained and already known nucleotide sequences was carried out manually using the BioEdit program. The determination of the phylogenetic relationships and the construction of the phylogenetic tree was carried out with a MEGA 3 Neighbor-Joining tool using the neighbor-joining algorithm [11]. The bootstrap indices determining the statistical significance of the group identification were determined for 1000 pseudoreplicas.

PCR with random primers (RAPD-PCR). Yeast DNA was isolated according to the method described earlier [12]. DNA amplification with RAPD primers OPA-04 (5'-AATCGGGCTG) and OPA-11 (5'-CAATCGCCGT) was carried out in 30 μl of the buffer containing 3 mM $MgCl_2$, 0.3 mM of each dNTP, 50 pmol of each primer, 1.25 U of active *Taq*-polymerase (Syntol, Russia), and 20–200 ng of the genomic DNA analyzed. Forty-five PCR cycles were carried out in the following mode: DNA denaturation, 94°C for 1 min; primer annealing, 36°C for 1 min; and DNA synthesis, 72°C for 2 min.

DNA amplification with the microsatellite primers $(ATG)_5$, $(GAC)_5$, $(GTG)_5$ and the minisatellite primer M13 (5'-GAGGGTGGCGGTTCT) was carried out in 30 μl of the buffer containing 3 mM $MgCl_2$, 0.3 mM of each dNTP, 30 pmol of each primer, 1.25 U of active *Taq*-polymerase (Sintol, Russia), and 20–200 ng of the genomic DNA analyzed. Forty PCR cycles were carried out in the following mode: DNA denaturation at 94°C, 1 min; primer annealing at 52°C, 2 min; and DNA synthesis at 74°C, 3 min.

Pulsed-field gel electrophoresis of chromosomal DNA and Southern hybridization. The chromosomal DNA preparations were obtained as described earlier [12]. The chromosomal DNAs were separated on the

CHEF-DR III apparatus (BioRad, United States). Pulsed-field gel electrophoresis was carried out at 50 V for 48 h at a field-switching time of 40 min followed by 70 h at a field-switching time of 50 min, and after that for 22 h at a field-switching time of 55 min. The buffer used was 0.5× TBE cooled to 12°C. The sizes of individual *Y. lipolytica* chromosomes were determined with the commercial standards of the chromosomal DNA of *Saccharomyces cerevisiae* YNN 295, *Pichia canadensis* YB-4662-VIA, and *Schizosaccharomyces pombe* 972 h^- with known chromosomal sizes and order. After electrophoresis, the gel was stained with ethidium bromide for 2–3 h, then washed in distilled water for 2 h, and photographed under UV illumination.

Chromosomal DNA was transferred to the nitrocellulose membrane with a vacuum mode using a Vacuum blotter apparatus (BioRad, United States). The DNA was fixed on the membrane by annealing at 80°C for 2 h. The PCR-amplified fragments of the *URA3* gene and the *ARS68* and *ARS18* centromere sequences were used as probes. Amplification was carried out with primers URA31(5'-ATGCCCTCCTACGAAGCTC)/URA32(5'-AATCTTCTGGTAAGCCTCCCA), ARS181(5'-CACCCAAGTAGCATGCATAAG)/ARS182(5'-CACTGATTAATTTTCGGGCCA), and ARS681(5'-TCGTC-GTGTTCAGGAACTGTT)/ARS682(5'-GTTTT-GAGCCCATCTTGTTG) in the same mode as the amplification of 5.8S-ITS fragments.

The probes were labeled using the nonradioactive method using dUTP labeled with digoxigenin (dig-II-dUTP) from the DIG High Prime DNA Labeling and Detection Starter Kit I according to the Roche (Germany) instruction. Hybridization and hybridization signal events were also carried out according to the Roche instructions.

RESULTS AND DISCUSSION

To determine the species identity of the strains presented in the table, we sequenced the D1/D2 domain of the 26S rRNA gene. Most of the strains did not differ from the type culture *Y. lipolytica* CBS 6124 in the sequences of this region. In strain CBS 6660, one nucleotide substitution was found, while in eight strains (CBS 2787, CBS 5570, CBS 5589, CBS 2070, CBS 6125, CBS 7504, CBS 6303, and CBS 6317), an insertion of one nucleotide was revealed. Thus, all the 40 strains belonged to the species *Y. lipolytica*. The molecular polymorphism of *Y. lipolytica* was further studied by means of sequencing the rRNA 5.8S-ITS fragments, RAPD-PCR, and molecular karyotyping.

Comparative analysis of the nucleotide sequences of the 5.8S-ITS region suggested that the strains studied were closely related. Strains CBS 6125, CBS 5570, CBS 6124.1, and CBS 6124.2 had ITS sequences identical to those of the type culture CBS 6124. The latter two strains are monospore segregants of the type culture.

In the remaining strains, the differences in the ITS region were from one to six nucleotides. The nucleotide substitutions revealed were independent of the geographic origin of the strains and their source of isolation. Based on the phylogenetic analysis, 40 strains were divided into two main clusters (Fig. 1). In the first cluster, four groups (I–IV) could be identified; in the second, two groups (V and VI). Strains VKPM Y-3155 and VKPM Y-3195 occupied an isolated position in the first and second clusters, respectively. Most strains belonged to group VI. They had five nucleotide substitutions in the ITS region, compared to the type culture CBS 6124.

The genetic line CLIB122 resulted from crossing the monospore segregant CBS 6124.2 with strain CBS 7504 and subsequent reciprocal crossings with it [13]. According to our analysis, CLIB122 had identical ITS sequences with strain CBS 7504 and differed from CBS 6124.2 in one substitution and one nucleotide insert.

RAPD-PCR. First, we selected the primers capable of differentiating between individual strains. For this purpose, seven oligonucleotides were used: OPA-04, OPA-09, OPA-11, $(ATG)_5$, $(GAC)_5$, $(GTG)_5$, and M13. The study was conducted on six *Y. lipolytica* strains of different origin: CBS 6124, CBS 6303, CBS 7034, CBS 2074, VKPM Y-3154, and VKPM Y-3195 (table). Three of the primers used, $(GAC)_5$, $(GTG)_5$, and M13, yielded reproducible PCR profiles with a large number of well-defined bands. The greatest strain variation in the PCR profiles was noted with the microsatellite primer $(GAC)_5$. This primer was selected for molecular comparison of all the 40 *Y. lipolytica* strains. Figure 2 shows the PCR patterns of certain strains. The strains were characterized by major bands of about 350 and 400 bp. The main differences between the strains concerned major bands varying in size between 600 and 1000 bp, as well as individual minor bands. No correlation was found between the PCR profiles and the geographic origin of the strains, as well as the source of their isolation. For example, the monospore segregants of the type culture CBS 6124.1 and CBS 6124.2 had different patterns (Fig. 2, lanes *3* and *4*).

According to the literature data, major bands about 350 and 400 bp are species-specific for *Y. lipolytica* and allow their differentiation from the taxonomically related yeasts *Candida deformans*, *C. galli*, etc. [14].

Karyotypic analysis. Earlier, molecular karyotyping of certain *Y. lipolytica* strains was carried out [13, 15, 16]. However, the pulsed-field gel electrophoresis conditions used in these works did not allow good separation of the chromosomal bands to be achieved.

In this work, to achieve the optimum separation of the chromosomal bands of *Y. lipolytica* strains, we used several electrophoretic programs; the field-switching

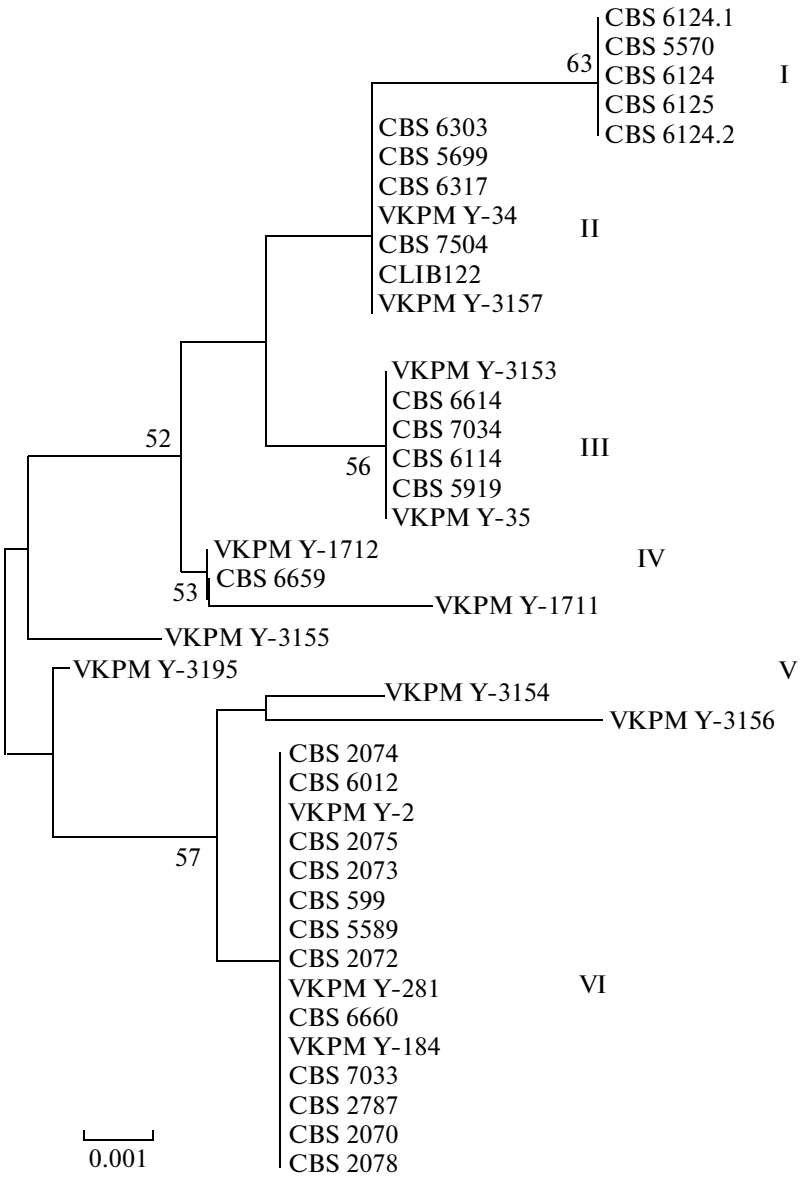

Fig. 1. Phylogenetic analysis of the nucleotide sequences of the 5.8-ITS rRNA region of the yeast *Y. lipolytica*. The bootstrap values >50% are presented. The scale corresponds to one nucleotide substitutions per 1000 nucleotide positions.

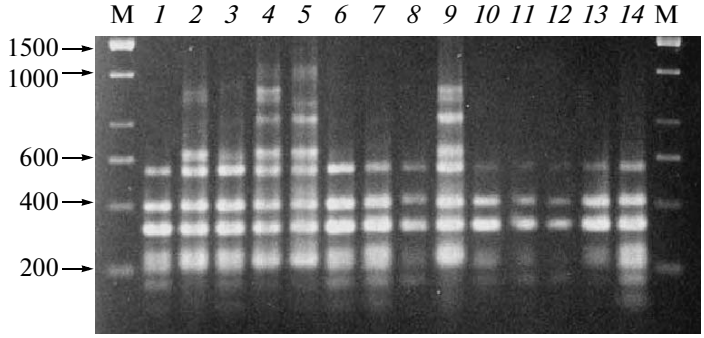

Fig. 2. PCR analysis of *Y. lipolytica* strains using the microsatellite primer $(GAC)_5$: CLIB122 (*1*), CBS 6124 (T) (*2*), CBS 6124.1 (*3*), CBS 6124.2 (*4*), VKPM Y-281 (*5*), CBS 7504 (*6*), VKPM Y-1712 (*7*), VKPM Y-3153 (*8*), VKPM Y-3154 (*9*), VKPM Y-3155 (*10*), VKPM Y-3157 (*11*), VKPM Y-3195 (*12*), VKPM Y-1711 (*13*), and CBS 6303 (*14*). M designates the molecular weight marker (kb). T is the type culture.

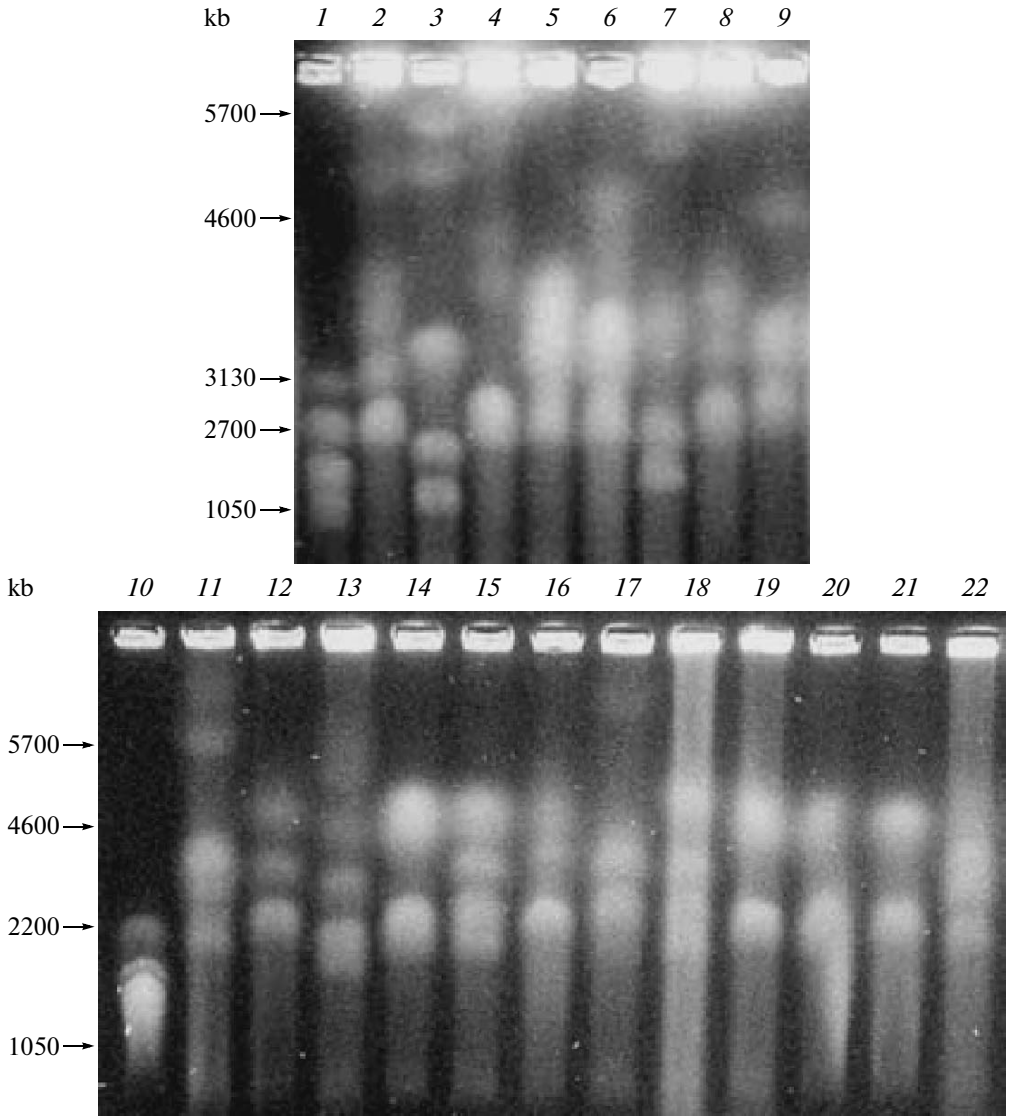

Fig. 3. Pulsed-field gel electrophoresis of the chromosomal DNA of the yeast *Y. lipolytica*: CBS 6124 (T) (*2*), CBS 6124.1 (*3*), CBS 6124.2 (*4*), CBS 7504 (*5*), CLIB122 (*6*), CBS 6660 (*7*), CBS 5589 (*8*), VKPM Y-3154 (*9*), CLIB122 (*11*), CBS 6125 (*12*), CBS 2070 (*13*), CBS 2075 (*14*), CBS 2787 (*15*), CBS 6317 (*16*), CBS 6614 (*17*), CBS 6659 (*18*), CBS 7034 (*19*), CBS 2074 (*20*), VKPM Y-2 (*21*), and CBS 5570 (*22*). The chromosomal order and sizes (kb) are given according to the standards of *P. canadensis* YB-4662-VIA and *S. cerevisiae* YNN 295 (lanes *1* and *10*, respectively).

time, the current, and the duration of electrophoresis were varied. The best results were obtained when the following three-step mode was used (at 50 V): (1) for 48 h with the field-switching time 40 min, (2) for 70 h with the field-switching time 50 min, and (3) for 22 h with the field-switching time 55 min. The order and sizes of the chromosome bands of *Y. lipolytica* strains were determined according to the karyotypic standards of *S. cerevisiae, P. canadensis*, and *Sch. pombe* strains.

The karyotypes of certain strains are shown in Fig. 3. Significant polymorphism of the chromosome sizes was revealed. The chromosomal DNA of different strains was separated into from two to seven electrophoretic bands. Based on the fluorescence intensity, some of the bands possibly contained more than one chromosome. All the strains had two chromosome bands 2300–2800 kb in size, these bands migrating in the doublet in a number of the strains. For example, the DNA of strains CBS 7034, CBS 2074, and VKPM Y-2 formed two bands, which, according to the fluorescence intensity, contained several chromosomes (Fig. 3, lanes *19–21*). Using different karyotyping conditions, we succeeded in separating the intensely fluorescent bands into two and more chromosomes in a number of strains. As a result, the chromosomal DNA of different strains was separated into from five to seven electrophoretic bands. Figure 4 shows the

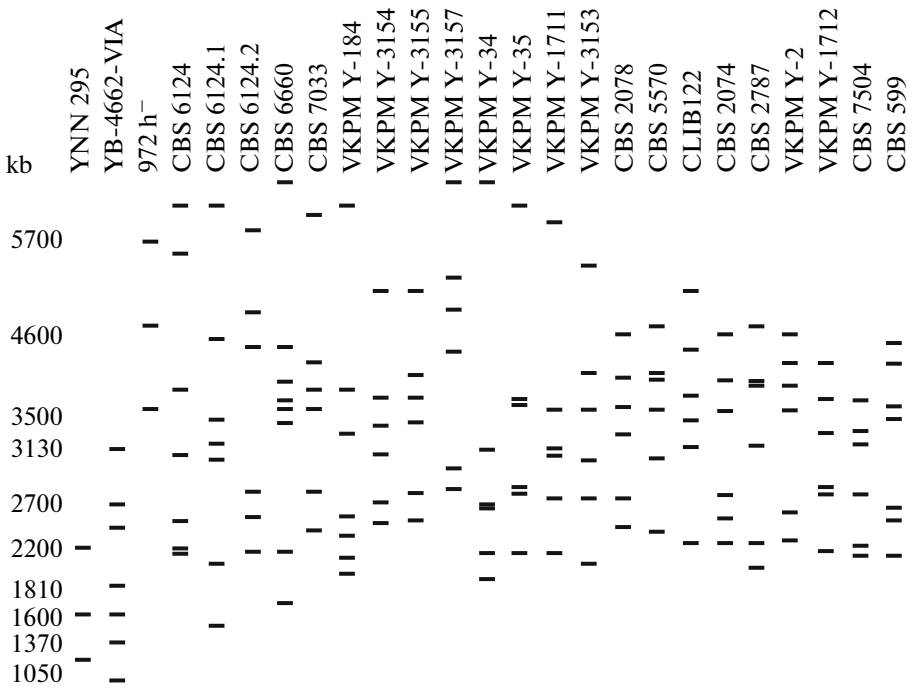

Fig. 4. Summary scheme of the molecular karyotypes of the yeast *Y. lipolytica*. The chromosomal band sizes (kb) are given according to the standard strains *S. cerevisiae* YNN 295, *P. canadensis* YB-4662-VIA, and *Sch. pombe* 972 h⁻. Different karyotyping modes were used.

scheme of the molecular karyotypes of *Y. lipolytica* strains developed on the basis of the data from several electrophoretic gels. Summing up the pulsed-field gel electrophoresis data on the chromosomal DNA, we can identify three groups of strains with similar karyotypes: (*1*) CBS 2072, CBS 2073, CBS 2074, CBS 2075, CBS 6317, CBS 6614, CBS 7034; (*2*) CBS 5589, CBS 2787, CBS 5699, CBS 5919, CBS 6125; and (*3*) CBS 7033, CBS 2070, CBS 6012, CBS 6114, CBS 6303, CBS 6659, VKPM Y-281, VKPM Y-3195. The remaining strains had unique karyotype profiles.

Since the largest chromosomal band in some of the strains exceeds in size chromosome I of the *Sch. pombe* 972 h⁻ karyotypic standard (5700 kb), we only succeeded in making a rough estimate of the size of all chromosomal bands of *Y. lipolytica* strains. The widest range of the chromosomal band sizes (from 1400 to 6200 kb) was noted in strains CBS 6124.1, CBS 6660, and VKPM Y-34; the least range, in strains CBS 599, CBS 7504, and CBS 5570 (from 2000 to 4000 kb). It should be noted that the monospore cultures CBS 6124.1 and CBS 6124.2 differed significantly in chromosomal band size. This is indicative of the heterozygous nature and, probably, of the hybrid origin of the type culture CBS 6124. We succeeded in separating the chromosomal DNA of the genetic line CLIB122 into six electrophoretic bands. The nuclear DNA of this strain is known to be represented by six chromosomes [17]. According to the karyotypic analysis, most strains also have a haploid number of chromosomes (six) and the strains with a large number of chromosomal bands are likely to be aneuploid.

We studied in detail the karyotypes of 10 *Y. lipolytica* strains by means of Southern hybridization. Representatives differing in the ITS-sequence, as well as in the number and size of electrophoretic bands, were selected. Three molecular markers located on different chromosomes in strain CLIB122—namely, *URA3* (chromosome E, 4220 kb), *ARS68* (chromosome A, 2300 kb), and *ARS18* (chromosome C, 3270 kb)—were chosen as probes. The overall results of Southern hybridization with these molecular markers are shown in Fig. 5. It was established that the localization of these molecular markers is not the same in different strains. For example, probe *ARS68* was hybridized in eight strains with the least-size chromosome. However, in strains CBS 6660 and CBS 5570, the hybridization signal was revealed in the chromosome ranking second in size. The chromosomal localization of the marker *URA3* located at the genetic line CLIB122 in the greatest-size chromosome was also not the same in different strains. Characteristically, two hybridization signals with probe *URA3* were revealed for strains CBS 599 and CBS 6660. The DNA of these strains was separated into seven electrophoretic bands (Figs. 4, 5), which may be indicative of their aneuploidy. The analysis showed that the monospore segregants CBS 6124.1 and CBS 6124.2 differed not only in the sizes of the chromosomes, but also in their order. It should be noted that, when different genetic lines obtained in a

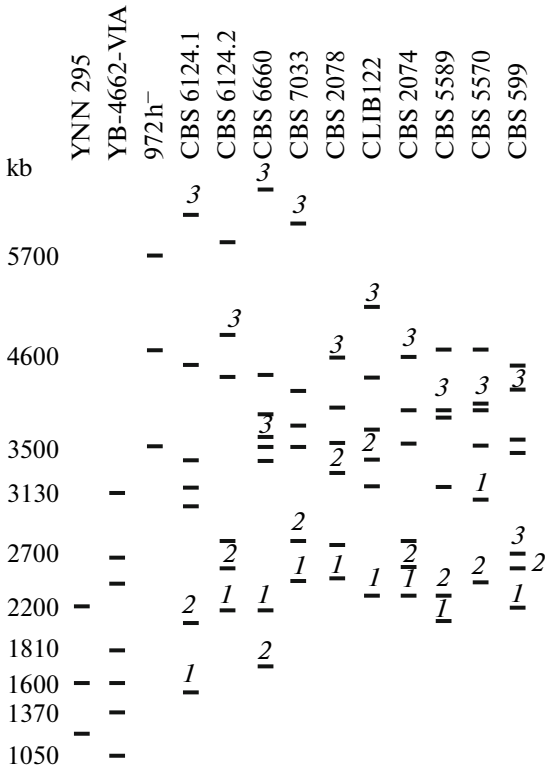

Fig. 5. Scheme of Southern hybridization of the chromosomal DNA of the yeast *Yarrowia lipolytica* with probes: *ARS68* (*1*), *ARS18* (*2*), and *URA3* (*3*). The chromosomal band sizes (kb) are given according to the standard strains *S. cerevisiae* YNN 295, *P. canadensis* YB-4662-VIA, and *Sch. pombe* 972 h$^-$.

number of laboratories based on CBS 6124.1 or CBS 6124.2 were hybridized, numerous genetic anomalies occurred, e.g., low hybrid formation rate, low fertility or sterility of the hybrids, and irregular meiotic segregations of the control markers [18–20].

The strains of *Y. lipolytica* utilizing different fats as a source of carbon are often isolated from substrates with a high lipid content. These yeasts are widely used as lipase producers [21–23]. In strain CBS 7504, the gene *LIP2* controlling up to 97% of the secreted lipase (Lip2) was identified. The genes *LIP7* and *LIP8* are responsible for the remaining 3% of lipase activity [22]. In the genetic line CLIB122, the *LIP2* gene is localized in chromosome A. Taking into account significant polymorphism of molecular karyotypes of the yeast *Y. lipolytica*, including aneuploidy, it would be expected that, in different strains, specific genes, including the main lipase gene, may have different chromosomal localization. The already started study of the molecular divergence of *Y. lipolytica* lipases based on the strains of different origin will allow us to purposefully use the natural gene pool of this yeast for gene-engineered selection developments.

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