ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 4, pp. 520–527. © Pleiades Publishing, Ltd., 2010.

EXPERIMENTAL ARTICLES

Mitochondrial DNA Polymorphism of the Yeast *Saccharomyces bayanus* var. *uvarum*[1]

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Received October 15, 2009

Abstract—Genetic relationships among forty-one strains of *Saccharomyces bayanus* var. *uvarum* isolated in different wine regions of Europe and four wild isolates were investigated by restriction analysis (RPLP) of mitochondrial DNA (mtDNA) with four restriction endonucleases, *Alu*I, *Dde*I, *Hinf*I and *Rsa*I. No clear correlation between origin and source of isolation of *S. bayanus* var. *uvarum* strains and their mtDNA restriction profiles was found. On the whole, the mtDNA of *S. bayanus* var. *uvarum* is much less polymorphic than that of *S. cerevisiae*. This observation is in good agreement with results obtained by electrophoretic karyotyping. Unlike wine *S. cerevisiae*, strains of *S. bayanus* var. *uvarum* display a low level of chromosome length polymorphism.

Key words: mtDNA restriction analysis, *S. baynus* var. *uvarum*, wine yeasts, molecular diversity, electrophoretic karyotyping.

DOI: 10.1134/S0026261710040144

The discovery [1–3] of biological species within the genus *Saccharomyces*, viz. *S. cerevisiae, S. arboricolus, S. bayanus, S. cariocanus, S. kudriavzevii, S. mikatae* and *S. paradoxus*, was a breakthrough in evolutionary genetics, taxonomy and breeding of these yeasts. Nowadays, the seven biological species are intensively studied in many laboratories of Western Europe and USA (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed). The gene pool of the cultivated *Saccharomyces* yeasts consists of two biological species: *S. cerevisiae* Hansen and *S. bayanus* Saccardo, and one hybrid taxon *S. pastorianus* Hansen (syn. *S. carlsbergensis* Hansen). For many years, the melibiose-fermenting yeast *S. uvarum* Beijerinck was recognised among wine yeasts. The type cultures of *S. bayanus* and *S. uvarum* were found to be conspecific, having 95% nDNA-nDNA reassociation. Taking into account the priority of the *S. bayanus* description, *S. uvarum* was considered as its synonym [4]. Recently conducted molecular and genetic analyses have shown, however, that the *S. bayanus* species is heterogeneous and can be differentiated into two subgroups, one comprising the type culture of *S. bayanus* and some other, mainly, brewery strains, and the second comprising the type culture of *S. uvarum* and commonly isolated wine strains [2, 5–7]. Based on the partial genetic isolation of strains from both subgroups, two varieties have been established: *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum* [6]. The specific ecological niche of the latter yeasts is found in viticulture and wine-making at low temperatures. *S. cerevisiae* and *S. bayanus* var. *uvarum* can occur in mixed populations in different wines. Moreover, *S. bayanus* var. *uvarum* dominates in certain wine-making processes: some French white wines, Txakoli, Tokaj, Muscat, Amarone wines and cider [8–12]. Identification and molecular characterisation of the *S. bayanus* var. *uvarum* among wine yeasts opened the possibility to use its gene pool in breeding programs. It was shown that interspecific hybridisation *S. cerevisiae* × *S. bayanus* var. *uvarum* led to the creation of highly productive wine yeasts with improved fermentation ability at low temperatures [14, 15]. Hybrids of *S. cerevisiae* × *S. bayanus* have been documented among commercial strains [16–21].

Restriction analysis of mitochondrial DNA (mtDNA) has been successfully used both for delimitation of sibling species in the *Saccharomyces* genus and for characterisation of different wine populations of *S. cerevisiae* [22–24]. So far, only a few *S. bayanus* var. *uvarum* strains were analysed by this method.

In the present study, using the mtDNA restriction analysis we characterised *S. bayanus* var. *uvarum*

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strains isolated from different wine regions of Europe; France, Spain, Italy, Slovakia and Moldavia. All the strains studied have been earlier assigned to *S. bayanus* var. *uvarum* on the basis of genetic hybridisation analysis.

MATERIALS AND METHODS

Yeast strains. Forty-one strains used in this study (table) were isolated from different types of wine, grape juice, grape berries or vineyard in different regions of Europe: France (13 strains), Spain (7), Italy (6), Slovakia (7), Moldavia (6) and Russia (2). Besides, four wild isolates from *Mesophylax adoperus* (1), fruit body of mushroom (1) and *Drosophila* (2) were analysed. All are monosporic homothallic cultures, which were earlier assigned to the *S. bayanus* var. *uvarum* on the basis of genetic hybridisation analysis. Three *S. bayanus* var. *bayanus* strains, including the type culture of *S. bayanus* CBS 380, strains CBS 424 and CBS 1546, were used for comparison.

Mitochondrial DNA restriction analysis. Mitochondrial DNA was isolated according to Querol et al. [25]. The mtDNA was digested with four restriction endonucleases, *Alu*I, *Dde*I, *Hinf*I and *Rsa*I (Boehringer Mannheim, Germany). Restriction fragments were separated on 0.8% agarose gel by electrophoresis in 1× TAE buffer.

Pulsed field gel electrophoresis. Preparation of chromosomal DNA was described earlier [26]. A CHEF DRII apparatus (Bio-Rad, Richmond, Calif.) was used to separate chromosomal DNAs. The electrophoresis buffer (0.5× TBE) was circulated around the gel and cooled to 14°C. Electrophoresis was carried out 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. A standard set of *S. cerevisiae* YNN 295 chromosomes was obtained commercially (Bio-Rad) and used for comparison.

Phylogenetic analysis. The distances between pairs of strains based on their composite restriction patterns were determined from the matrix of presence/absence of restriction fragments according to the fraction of shared fragments (F_{xy}) from Nei and Li [27] defined as:

$$D_{xy} = 1 - F_{xy} = 1 - (2n_{xy}/n_x + n_y) = 1/2[(n_x - n_{xy}) + (n_y - n_{xy})]/1/2\,(n_x + n_y),$$

where n_{xy} is the number of restriction fragments shared by strains x and y, and n_x and n_y are the restriction fragments exhibited by strains x and y, respectively. This measure of dissimilarity corresponds to the fraction of average restriction fragment differences exhibited between two strains. These dissimilarity values were used to infer a phylogenetic tree according to the Neighbor-Joining method [28], and its corresponding bootstrap analysis (based on 2000 matrix pseudoreplicates). All these procedures were performed with the TreeCon program for Windows [29].

RESULTS

Mitochondrial DNA restriction analysis. Mitochondrial DNAs from 45 *S. bayanus* var. *uvarum* strains were analysed by the 4- and 5-base-cutting enzymes *Alu*I, *Dde*I, *Hinf*I and *Rsa*I. Polymorphism was observed both in number and size of restriction fragments. According to the restriction patterns, *S. bayanus* var. *uvarum* strains were classified into four (for *Hinf*I) and five (for *Dde*I) distinct groups. Three strains (IFI 371, SCU 197 and VKM Y-508) had individual patterns with *Hinf*I, and five strains (VKM Y-508, 17el, M 300, VKM Y-363 and UCD 61-137) showed unique restriction profiles with *Dde*I. For the enzyme *Rsa*I, five distinct restriction patterns were recognised. On the contrary, when mtDNAs were digested with *Alu*I, all strains showed nearly identical patterns with slight variation for the bands smaller than 3000 bp. Two large bands between the standard molecular markers of 5148 and 4361 bp are likely to be specific for *S. bayanus* var. *uvarum*. From the restriction profiles with the four endonucleases, 29 strains can be separated into six distinct groups, while 16 strains present characteristic fingerprints. Each unique fingerprint, produced by an endonuclease, was designated by a letter (table). Figure 1 shows the restriction patterns of some strains.

To determine the relationships among wine strains from different origins, a Neighbor-Joining tree was obtained (Fig. 2). However, no significant correlation was found between the restriction patterns exhibited by the strains and the origin of strains or source of their isolation. For example, two strains VKM Y-361 and VKM Y-362 isolated from Tokaj wine in Slovakia are clustered together with Spanish wine strain IFI 369. Other four Tokaj wine strains were distributed randomly in the tree. Four wild *S. bayanus* var. *uvarum* strains appear at different positions, as well. Only six strains isolated from grape berries and soil under vineyard in Moldavia (M 471, M 472, M 477, M 478, M 488 and M 489) were grouped together.

Molecular karyotyping. The two wine yeasts *S. cerevisiae* and *S. bayanus* are known to have clearly different karyotype patterns, i.e. distribution and sizes of homologous chromosomes [26]. The karyotype of *S. cerevisiae* is characterised by the presence of a large chromosomal band of 1600 kb and by three or more small chromosomes in the region of 245–370 kb. Compared to *S. cerevisiae, S. bayanus* possesses a persistent chromosomal band of about 1300 kb. Within the *S. bayanus* species there are two types of karyotype profiles corresponding to two varieties *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum*. The latter variety is characterised by only two invariably small chromosomes in the region of 245–370 kb instead of three or more in *S. bayanus* var. *bayanus* [7, 8, 11]. All 41 wine strains and four wild isolates strains displayed a karyotype pattern typical of *S. bayanus* var. *uvarum* with the characteristic pair of small chromosomes. However,

List of *Saccharomyces* strains used in this study

Strain designation[a]		Source of isolation	Country	mtDNA restriction patterns			
Original	CBS			*Alu*I	*Rsa*I	*Hinf*I	*Dde*I
S. cerevisiae							
YNN 295	–	Genetic line	–	–	–	–	–
S. bayanus var. *bayanus*							
–	380 (T)	Turbid beer	Unknown	–	–	–	–
–	1546	Beer	Netherlands	–	–	–	–
–	424	Pear juice	Switzerland	–	–	–	–
S. bayanus var. *uvarum*							
58 I	–	Grape juice	France	C	C	A	A
17 el	–	Grape juice	France	C	A	B	E
SCU 11	–	Muscadet wine	France	C	C	A	A
SCU 13	–	Muscadet wine	France	C	C	A	A
SCU 74	–	Muscadet wine	France	C	C	A	A
SCU 197	–	Muscadet wine	France	B	E	E	C
SCU 299	–	Muscadet wine	France	C	D	A	D
SCU 374	–	Muscadet wine	France	C	A	B	D
SCU 397	–	Muscadet wine	France	C	D	A	D
L 19	8711	White wine	France	C	C	A	A
L 99	8712	White wine	France	C	C	A	A
L 490	8713	White wine	France	C	C	A	A
L 1708	8714	White wine	France	C	C	A	A
DBVPG 1640	–	Grape berries	Italy	C	A	B	B
DBVPG 1642	–	Grape berries	Italy	A	C	A	A
DBVPG 1643	–	Grape berries	Italy	A	C	A	A
DBVPG 1689	–	Grape berries	Italy	A	A	B	B
DBVPG 1690	–	Grape berries	Italy	A	C	A	A
DBVPG 1693	–	Grape berries	Italy	C	A	B	B
CECT 1369	–	White wine	Spain	C	C	A	A
CECT 10560	–	Wine	Spain	A	C	A	C
IFI 362	8715	Wine	Spain	A	C	A	A
IFI 369	8716	Wine	Spain	A	B	D	B
IFI 371	8717	Wine	Spain	C	C	F	A
IFI 373	8718	Wine	Spain	C	B	B	B
IFI 391	8719	Wine	Spain	C	A	B	B
M 369	8695	Wine	Slovakia	B	C	A	A
M 471	8689	Grape berries	Moldavia	C	B	C	D
M 472	8690	Grape berries	Moldavia	C	B	C	D
M 477	8691	Grape berries	Moldavia	C	B	C	D
M 478	8692	Grape juice	Moldavia	C	B	C	D
M 488	8693	Vineyard	Moldavia	C	B	C	D
M 489	8694	Soil under vineyard	Moldavia	C	B	C	D
M 300	–	Red sparkling wine	Russia	B	A	B	F
VKM Y-1146	8687	Grape berries	Russia	C	C	A	A
VKM Y-361	–	Tokaj wine	Slovakia	C	B	D	B
VKM Y-362	–	Tokaj wine	Slovakia	C	B	D	B
VKM Y-363	–	Tokaj wine	Slovakia	A	D	A	G
VKM Y-364	–	Tokaj wine	Slovakia	A	C	A	A
VKM Y-508	–	Tokaj wine	Slovakia	A	A	E	I
VKM Y-509	–	Tokaj wine	Slovakia	B	E	B	A
MCYC 623	7001	*Mesophylax adoperus*	Spain	C	D	D	B
CCY 21-31-12	8698	*Amanita citrina*	Slovakia	C	C	A	A
UCD 51-206	8697	*Drosophila pseudoobscura*	USA	B	C	A	C
UCD 61-137	8696	*D. pseudoobscura*	USA	A	B	A	H

[a] The acronyms for culture collections are: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CCY = Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; CECT = Coleccion Española de Cultivos Tipo, University of Valencia, Spain; DBVPG = Departimento di Biologia Vegetale, Università di Perugia, Italy; IFI = Instituto de Fermentaciones Industriales, Madrid, Spain; M = Magarach Scientific Research Institute of Viticulture and Wine Making, Yalta, Crimea, Ukraine; MCYC = Microbiology Collection of Yeast Cultures, Departamento de Microbiologia, Universidad Politecnica de Madrid, Madrid, Spain; SCU = Institute Technique de la Vigne et du Vin, Centre de Experimentation de Nantes, France; VKM = All-Russian Collection of Microorganisms, Moscow, Russia; UCD = Herman J. Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, USA. T – type culture.

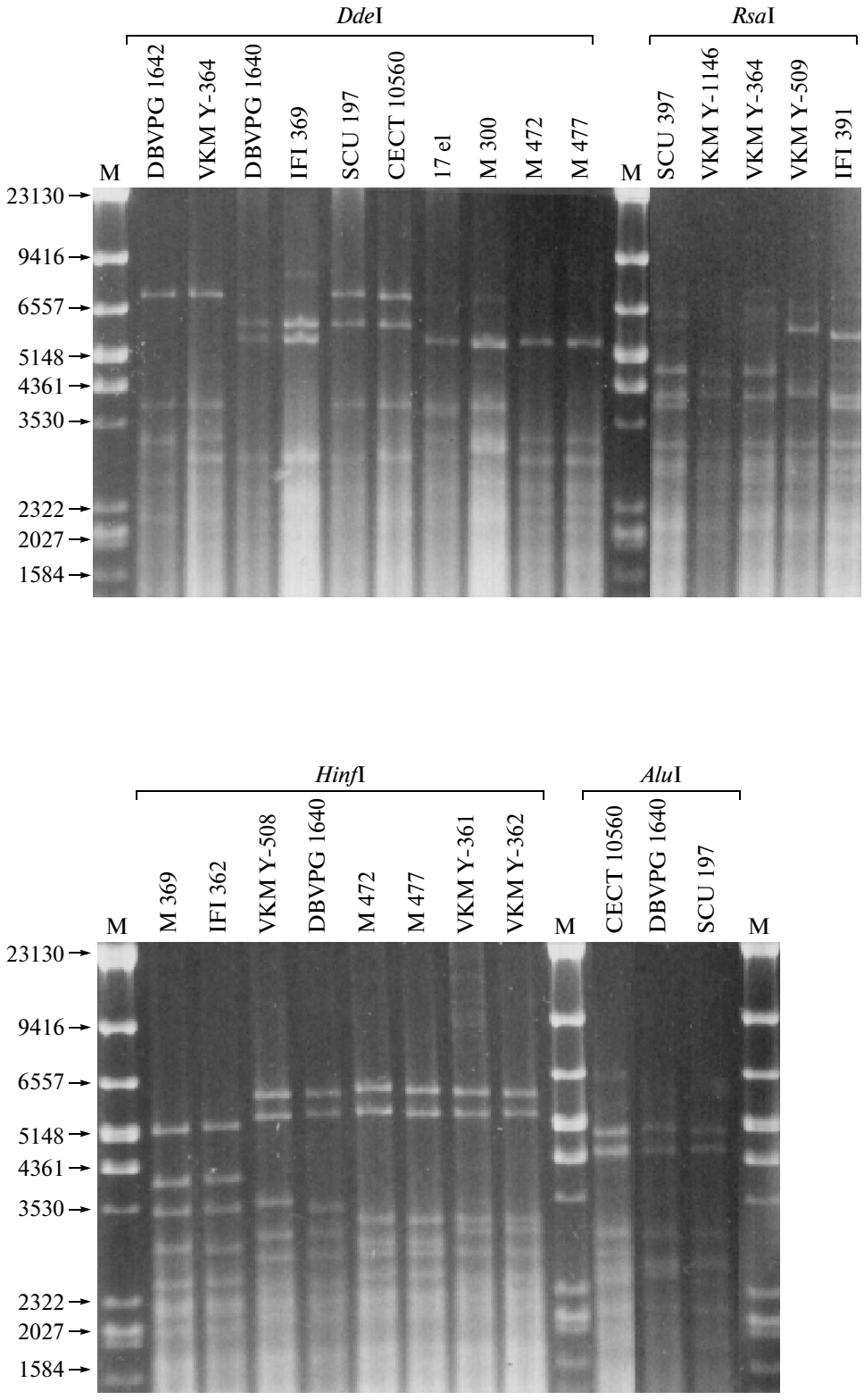

Fig. 1. Mitochondrial DNA restriction analysis of *Saccharomyces bayanus* var. *uvarum* strains with four restriction endonucleases *Alu*I, *Hinf*I, *Rsa*I and *Dde*I. Lanes M correspond to a mixture of lambda DNA digested with *Hind*III and with *Hind*III and *Eco*RI used as size markers.

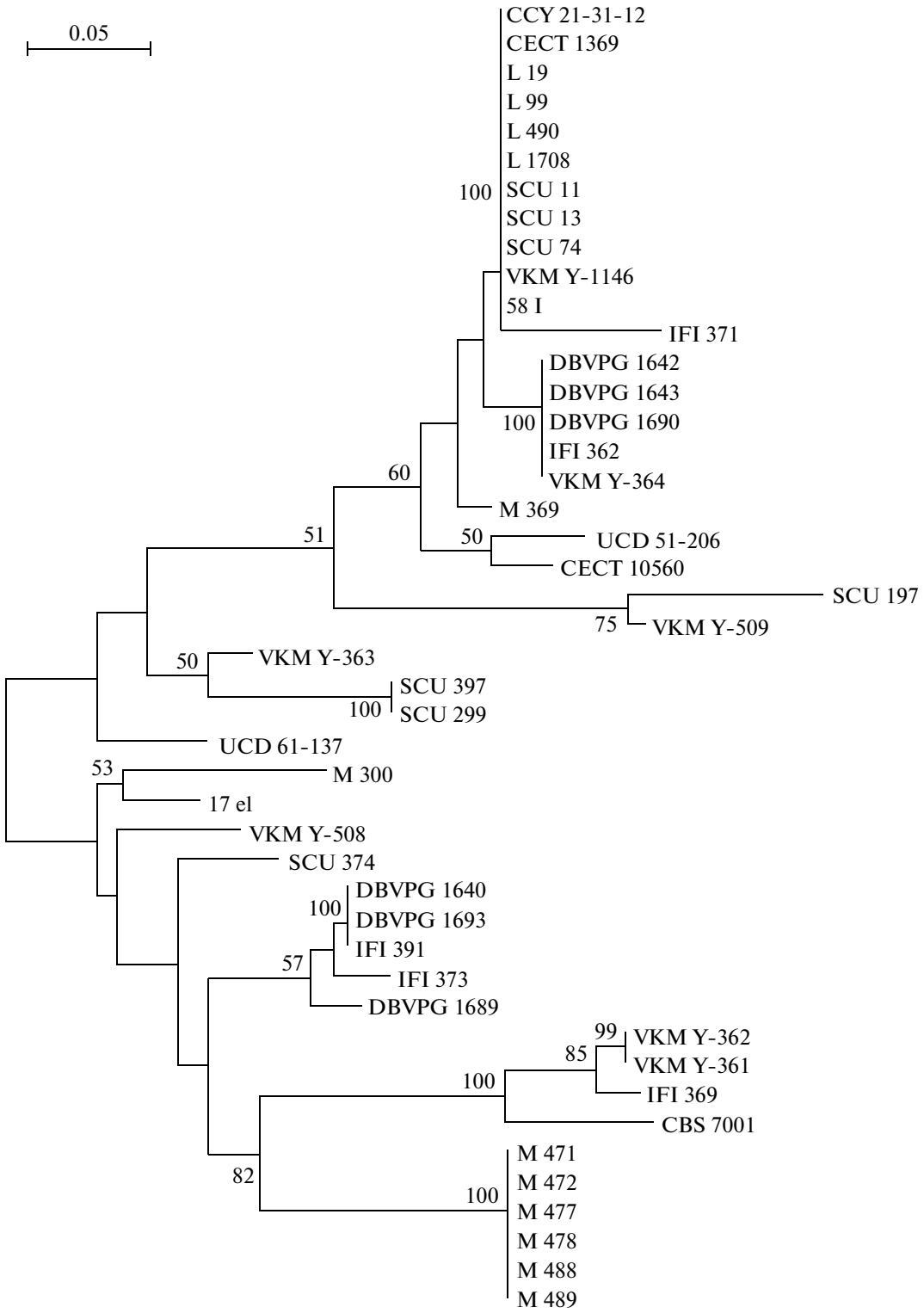

Fig. 2. Neighbor-joining tree obtained from the Nei and Li dissimilarities between pairs of strains according to their restriction patterns. The Nei and Li dissimilarity corresponds to the fraction of average restriction fragment differences between strains. The numbers at the nodes are the percentages of bootstrap replicates in which the clusters were found when values were ~ 50%.

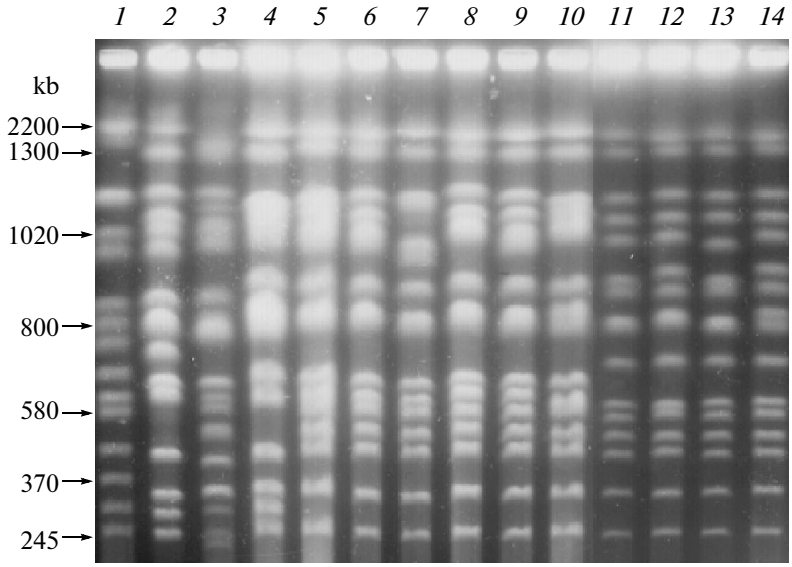

Fig. 3. Chromosomal patterns of *Saccharomyces bayanus* strains. *S. bayanus* var. *bayanus*, lanes: *2*—CBS 380, *3*—CBS 1546, *4*—CBS 424; *S bayanus* var. *uvarum*, lanes: *5*—VKM Y-1146, *6*—M 471, *7*—M 472, *8*—DBVPG 1642, *9*—IFI 362, *10*—CECT 1369, *11*—SCU 13, *12*—SCU 74, *13*—SCU 367, *14*—L 99. The chromosome sizes refer to the chromosomes of the standard strain of *S. cerevisiae* YNN295 (lane *1*).

the level of polymorphism was not high enough to differentiate individual strains. A certain level of chromosome length polymorphism was observed only for the middle-sized bands. To illustrate these results, karyotypes of some strains are shown in Fig. 3 (lanes *5–15*). The type culture CBS 380 and two other *S. bayanus* var. *baynus* strains, CBS 1546 and CBS 424, showed notably different karyotype patterns with three or four small chromosomes (Fig. 3, lanes *2–4*). A UPGMA cluster analysis revealed that the chromosome length polymorphism observed for some *S. bayanus* var. *uvarum* strains again did not correlate with their geographic origin or source of isolation (data not shown).

DISCUSSION

Cultured *S. cerevisiae* strains are characterised by extensive restriction fragment length polymorphism in mtDNA [24, 25]. Guillamón et al. [24] documented a high level of intraspecific variability within wine *S. cerevisiae* populations and demonstrated a correlation between mtDNA patterns and ecological or geographic origin of strains.

This study presented results of mtDNA restriction analysis of 41 wine and four wild strains, previously assigned to *S. bayanus* var. *uvarum* on the basis of genetic analysis. These results confirm the usefulness of mtDNA restriction patterns obtained with *Alu*I to differentiate between wine *S. bayanus* var. *uvarum* and wine *S. cerevisiae* strains as previously proposed by Guillamón et al. [23]. All 45 *S. bayanus* var. *uvarum* strains and the type culture of *S. bayanus* CBS 380 exhibited two persistent bands between 4361 and 5148 bp. With four restriction endonucleases, *Hinf*I, *Dde*I, *Rsa*I and *Alu*I, the strains analysed were separated into six groups and 16 strains showed unique fingerprints. However, the phylogenetic analysis of these results by the Neighbor-Joining method did not reveal a clear correspondence between the restriction patterns exhibited by the strains and their sources of isolation or geographic origins. For example, four wild *S. bayanus* var. *uvarum* strains were intermixed in the dendrogram with strains isolated from wine sources in different regions. We found that wine *S. bayanus* var. *uvarum* strains are less polymorphic than *S. cerevisiae* strains. This observation is in good agreement with the data obtained by molecular karyotyping and isoenzyme electrophoresis. Unlike *S. cerevisiae* strains showing pronounced chromosome length polymorphism [26], karyotype patterns of *S. bayanus* var. *uvarum* are rather similar. Wine *S. cerevisiae* strains are also characterised by variable allozyme patterns. For example, among 18 wine *S. cerevisiae* isolates no two yeasts had identical electrophoretic patterns for five enzymes studied [30]. *S. bayanus* var. *uvarum* strains are less heterogeneous on their isoenzyme profiles than *S. cerevisiae* strains [31, 32]. It is worthy to note that *S. bayanus* var. *uvarum* strains are homothallic and show, as a rule, good sporulation and high ascospore viability. These characteristics are important for the survival of yeasts in nature and may lead to the elimination of deleterious mutant alleles and chromosome rearrangements. This may explain the intraspecific homogeneity of *S. bayanus* var. *uvarum* compared to wine *S. cerevisiae* strains, which are very heterogeneous on sporulation properties and exhibit a higher genetic polymorphism.

ACKNOWLEDGMENTS

The study is dedicated to the memory of Professor Federico Uruburu (1934–2003), the organizer of molecular genetic studies of yeasts at the Spanish Type Culture Collection. E.S.N. and G.I.N. were supported by grants from Generalitat Valenciana (GV01-268) and in part by a grant from the Russian Fund for Basic Research (no. 09-04-00664).

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