APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1995, p. 3521–3529 Vol. 61, No. 10
0099-2240/95/$04.00+0

Genetic Diversity and Geographical Distribution of Wild *Saccharomyces cerevisiae* Strains from the Wine-Producing Area of Charentes, France

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Received 21 March 1995/Accepted 19 June 1995

Electrophoretic karyotyping, mitochondrial DNA restriction fragment length polymorphism analysis, and PCR amplification of interspersed repeats were used to study the variability, phylogenetic affinities, and biogeographic distribution of wild *Saccharomyces cerevisiae* enological yeasts. The survey concentrated on 42 individual wine cellars in the Charentes area (Cognac region, France). A limited number (35) of predominant *S. cerevisiae* strains responsible for the fermentation process have been identified by the above molecular methods of differentiation. One strain (ACI) was found to be distributed over the entire area surveyed. There seemed to be little correlation between geographic location and genetic affinity.

Several methods based on the analysis of DNA polymorphism have recently been applied to differentiate among the enological strains of *Saccharomyces cerevisiae* (3, 5, 7, 11, 14, 18). They constitute powerful tools, not only for industrial and technological controls, but also for ecological investigations of the intraspecific diversity of the indigenous microflora of wines. Two methods have been frequently used for their ease and reliability: analysis of mitochondrial DNA (mtDNA) restriction profiles and analysis of pulsed-field gel electrophoresis (PFGE) chromosomal patterns (27).

In a previous study (10), we examined the evolution of the native population of *S. cerevisiae* during the course of spontaneous wine fermentation. By comparative mtDNA restriction patterns, it was shown that the fermentation process is carried out and completed by a limited number (one to three) of dominating strains associated with a variable number of secondary strains. These first results have been confirmed by other observations in different wine-producing areas (6, 9, 21, 26). The term "predominant" can be proposed to designate those few strains which display the strongest ability to grow during alcohol production.

Investigations on the natural variability of *S. cerevisiae* strains within specific wine-producing regions have also been made (9, 26, 28). An extensive polymorphism has generally been observed. Examples of strains isolated from the same location over several consecutive years have been related (9, 28). In some instances, such perennial strains appeared to be widespread in the examined area as described for the Champagne region (28).

No extensive geographical survey of the diversity of predominant strains collected during spontaneous fermentation has yet been performed. We present here the results of such a study carried out on the Charentes area (Cognac region, France), chosen because technological practices which can reduce the natural diversity (seeding by dried yeasts, sulfiting of grape musts before fermentation) are generally avoided there. Strain differentiation was performed by using PFGE karyotypes in association with mtDNA restriction analysis and PCR amplification of specific interspersed regions. An attempt to analyze the differences between strains in terms of genetic relatedness has been implemented.

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MATERIALS AND METHODS

Samples. Samples of grape must from the vine variety "Ugni blanc" were taken from fermentation tanks in 42 wineries spread over 36 villages in the Charentes wine-producing area.

The wineries Ga, Aa, and J, which are situated relatively far apart, were chosen for more accurate study so as to monitor the evolution of the population of *S. cerevisiae* during fermentation. These samples, from the 1990 vintage, were taken at three different stages, beginning (BF), middle (MF), and end of fermentation (EF). These stages were determined by measuring the voluminal mass (vm) of the grape must, i.e., the weight in grams of 1 liter of must so that $vm_{BF} \approx 1{,}070$, $vm_{MF} \approx 1{,}030$, and $vm_{EF} \approx 1{,}000$.

For the other wineries studied, the samples of must were taken exclusively at the EF.

The perenniality of the strains was monitored for the sites Ga, Aa, and GDF for several years including 1988, 1990, 1991, and 1992. In total, 46 samples were collected at the EF and analyzed to identify the dominant strains and to show their geographical distribution.

For each sample, dilutions from 10^{-1} to 10^{-4} were prepared for cultivation on YPD agar medium (yeast extract [10 g · liter^{-1}], pancreatic digest of casein [10 g · liter^{-1}], D-glucose [20 g · liter^{-1}], agar [20 g · liter^{-1}]). After incubation at 28°C for 48 h, all the yeast colonies which had grown, from 10^{-1} dilution, on the petri dishes (about 200 to 500 colonies) were collected together for the global analysis of their genetic material. This collection of colonies is considered to be representative of the total yeast biomass present at this step of the fermentation. Higher dilutions (10^{-2} to 10^{-4}) were used to isolate, at random, 10 to 30 separated colonies which were submitted for identification to classic biochemical tests, according to the procedures of Kreger-van Rij (13). All the samples (total yeast biomass and colonies) were preserved at −80°C in cryotubes containing YPD and glycerol at 25%.

For each sample, the PFGE karyotypes of the different total yeast biomasses and colonies were carried out in order to differentiate the predominant and associated strains. These strains were then compared from site to site by using both electrophoretic chromosomal patterns and mtDNA restriction profiles. In some cases, strain identity was confirmed by polymorphism of genomic DNA sequences amplified by PCR.

Killer activity. The killer (K), neutral (N), or sensitive (S) phenotypes of the strains of *S. cerevisiae* were determined by using two reference strains of the killer group K2, NCYC 738 (K) and STV 85 (S), according to the method described by Barre (1).

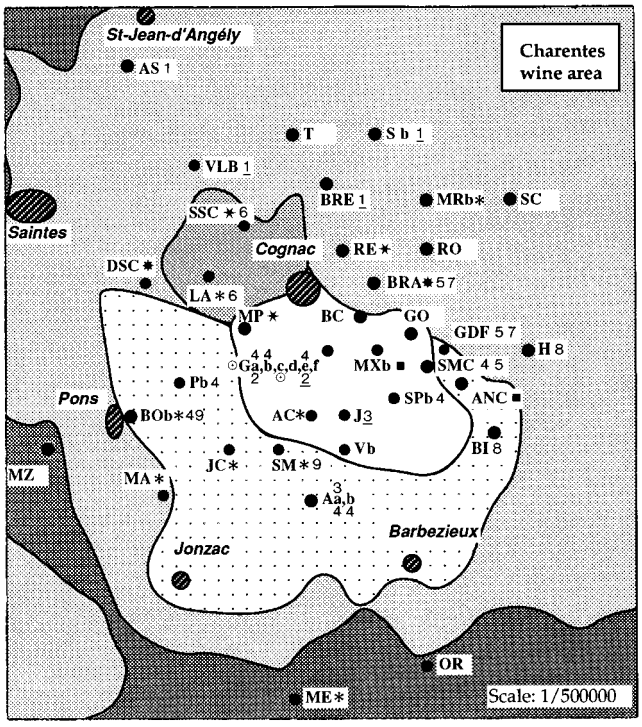

FIG. 1. Location of the 42 wineries examined in the subvintage wine-producing areas of the Charentes region and geographical distribution of the *S. cerevisiae* strains common to several sites. Symbols for the subvintage wine-producing areas: □, Grande Champagne; ◰, Petite Champagne; ▨, Borderies; ▩, Fins Bois; ▩, Bons Bois. Other symbols: ◍, principal towns; ● and capital letters, villages where the examined wineries (lowercase letters for several wineries of the same village) are situated (for example, Ga, -b, -c, -d, -e, and -f are six wineries from the same village, G). Symbols for identical strains: ∗, ACI = BObI = JCI = LAI = MAI = MEI = MRbI = SMI; ■, ANC′I = ANC″I = MXbI; ✱, DSCI = BRAI; ⊕, GaI = GcI; ✶, MPI = REI = SSCI; 1, ASII = BREI = SbI = VLBI; 2, GeI = GaIII; 3, JII = AaII; 4, AaIII = AbII = BObIII = GaII = GbII = GeII = PbII = SMCII = SPbII; 5, BRAIII = GDFII = SMCIII; 6, LAII = SSCII; 7, GDFIV = BRAII; 8, BiII = HII; 9, BObII = SMII, ANC″II = ANC′II, MZ′I = MZ″I, and MZ″II = MZ′II. The different symbols ∗, ■, ✱, ⊕, and ✶ correspond to predominant strains. The strains numbered from 1 to 3 are, depending on their location, predominant (underlined) or minority strains; those numbered 4 to 9 are minority strains only.

Karyotypes. The chromosomal DNA of yeast strains was prepared by a procedure derived from that of Vezinhet et al. (27). At the end of the exponential phase, the cell culture was pelleted by centrifugation (500 × *g*, 5 min), washed in 10 ml of 50 mM Na_2-EDTA (pH 8), and then resuspended in 1 volume of CPES buffer (60 mM citric acid [pH 6], 120 mM Na_2HPO_4 [pH 6], 20 mM Na_2-EDTA, 1.2 M sorbitol, 5 mM dithiothreitol) with added zymolyase 20T (0.2 mg · ml^{-1}) to obtain a final cellular concentration close to 3.5×10^9 cells · ml^{-1}. A volume/volume mixture of cell suspension and 1% agarose at 50°C in CPE buffer (CPES without sorbitol and dithiothreitol) was distributed in a premade mold. After solidification (4°C, 15 min), little pieces about 1 to 1.5 mm in thickness (plugs) were cut up and incubated successively in 3 ml of CPE buffer (37°C, 1 to 2 h) and 3 ml of TESP buffer (10 mM Tris, 0.45 M Na_2-EDTA, 10 g of *N*-lauroylsarcosine · $liter^{-1}$ [pH 8.2]) with an added 0.5 mg of pronase E · ml^{-1} (37°C, 5 h minimum). At this step, plugs may be stored at 4°C in 1 ml of 0.5 M Na_2-EDTA for several months. The separation of chromosomal DNA was obtained by a pulsed-field gel electrophoresis transverse alternating field electrophoresis system (Geneline I; Beckman, Palo Alto, Calif.). Before electrophoresis, plugs were washed several times at room temperature in TE buffer (10 mM Tris, 1 mM Na_2-EDTA [pH 8]) and were loaded in a 1% fast lane (FMC) agarose gel in transverse alternating field electrophoresis buffer (10 mM Tris, 4.35 mM acetic acid, 0.5 mM free acid-EDTA [pH 8.2]). The running conditions were (i) a 30-s pulse time for 4 h and (ii) a 60-s pulse time for 12 h with a constant current at 150 mA. The gels were stained in a solution of 30 μg of ethidium bromide · ml^{-1} for 5 min and then washed in distilled water for approximately 30 min before observation over shortwave UV light.

mtDNA restriction profiles. The mtDNA extraction and purification were carried out according to the rapid miniprep method of Defontaine et al. (4). After purification, the mtDNA was digested by *Eco*RV. This restriction endonuclease was previously selected for the discriminant number of fragments generated (11). The digested mtDNA was separated by electrophoresis in a 0.8% agarose gel with 1× TBE buffer (90 mM Tris-borate, 2 mM Na_2-EDTA [pH 8]) and revealed by ethidium bromide.

Profiles of polymorphic DNA sequences amplified by PCR. The amplification of genomic sequences situated between interspersed repeated elements (Ty yeast transposons) was performed by the method described by Ness et al. (18) with some modifications. Total genomic DNA was prepared according to a rapid method modified from the work of Polaina and Adam (19). The cells from a 10-ml culture in YPD for 24 h at 28°C under shaking were harvested by centrifugation (500 × *g*, 5 min). The pellet was resuspended in 700 μl of TE buffer containing 3% (w/v) of sodium dodecyl sulfate and incubated at room temperature under gentle agitation to cause cell lysis. The genomic DNA was then extracted and purified from cellular lysate, by two successive treatments with 1 volume of phenol-chloroform and one treatment with 1 volume of chloroform-isoamyl. The nucleic acids in the aqueous phase were precipitated by 500 μl of isopropanol. Once washed with 100 μl of 75% ethanol and dried, the DNA pellet was rehydrated with 30 μl of TE buffer containing 2 μl of RNase (10 mg · ml^{-1}) and then quantified by fluorometry at 365 nm in the presence of bisbenzimide (Hoechst 33258) or by spectrophotometry at 260 and 280 nm as described by Maniatis et al. (16). Primers were δ (δ1 and δ2) specific oligomers (18). The amplification reactions were performed with the PCR apparatus Perkin-Elmer Cetus DNA thermal cycler 480 under the following cycler conditions: 30 s at 95°C to denature the DNA, 30 s at 50°C (for the first four cycles) and 30 s at 45°C (for the 30 other cycles) for annealing steps, and 2 min at 72°C for the extension reaction. PCR products were analyzed by electrophoresis in 2% agarose gel with 1× TBE buffer and detected, after ethidium bromide staining, over a UV light source.

Mathematical analysis. Electrophoretic patterns were analyzed by comparison with internal standards. The haploid laboratory strain 288C of *S. cerevisiae* was used as a reference for karyotypes. Bacteriophage lambda (λ) and phi X (ϕ×174) DNA digested by *Hin*dIII and *Hae*III, respectively, was used as size markers for the mtDNA restriction profiles.

Evaluation of the genetic relatedness has been presented only in the case of mtDNA. The *Eco*RV restriction patterns gave a data set consisting of 48 objects (strains) and 35 variables (bands). The data set was numerically analyzed with the Jaccard coefficient, and clustering was accomplished by using the average linkage option (23), which is equivalent to the unweighted pair group method analysis (algorithm) of Sneath and Sokal (22). The SAS Statistical Software program (SAS Institute, Cary, N.C.) was used.

RESULTS

Three sites in the Charentes area (GA, Aa, and J [Fig. 1]) were selected so as to study the evolution of the yeast microflora during wine fermentation. The yeast species represented in the population were screened at three successive stages designated BF, MF, and EF (Table 1). A total of 30 colonies were examined for the BF and EF stages; only 10 were examined for the mid-step samples (MF) considered a control with no statistical value. In the three examples analyzed (Ga, Aa, and J [Table 1]), the fermentation was initiated by several species such as *Kloeckera apiculata*, *Candida famata*, a *Rhodotorula* sp., *Metschnikowia pulcherrima*, or *S. cerevisiae*. At this stage (BF [Table 1]), *S. cerevisiae* was always underrepre-

TABLE 1. Analysis of the species composition of the yeast population during a fermentation process from three sites, Ga, Aa, and J, of the Charentes wine-producing area

Yeast sp.	No. of colonies of sp. at site and sampling stage								
	Ga			Aa			J		
	BF	MF	EF	BF	MF	EF	BF	MF	EF
Kloeckera apiculata	25	1		4			6		
Candida famata	1			12			20	2	6
Rhodotorula sp.				6			2		
Metschnikowia pulcherrima							1		
Saccharomyces cerevisiae	4	9	30	8	10	30	1	8	24
Total no. of colonies analyzed	30	10	30	30	10	30	30	10	30

TABLE 2. Analysis of the strain diversity of *S. cerevisiae* during fermentation in three different sites, Ga, Aa, and J, of the Charentes wine-producing area

S. cerevisiae strain[a]	No. of colonies of strain at site and sampling stage								
	Ga			Aa			J		
	BF	MF	EF	BF	MF	EF	BF	MF	EF
GaI	1	5	23						
GaII		2	6						
GaIII	1	2	1						
GaIV	1								
GaV	1								
AaI				3	5	16			
AaII					2	5			
AaIII				3	1	1			
AaIV					2	3			
AaV						1			
AaVI						1			
AaVII				2					
AaVIII						2			
AaIX						1			
JI								3	11
JII								3	6
JIII								1	2
JIV								1	2
JV									1
JVI									2
JVII							1		

[a] Predominant strains are underlined.

sented, while during the following steps (MF and EF [Table 1]) it constituted almost exclusively the totality of the isolated colonies. These results are in agreement with numerous previous observations (8, 12, 20).

The different colonies of *S. cerevisiae* isolated during the fermentation were then analyzed by using karyotypes and mtDNA restriction profiles in order to differentiate the strains. Table 2 presents the results obtained for the three wineries Ga, Aa, and J. In the case of site J (Table 2), for instance, from 41 colonies analyzed seven different strains, numbered from JI to JVII, were identified. Their seven chromosomal and mtDNA restriction fragment length polymorphism patterns are presented in Fig. 2a and b, respectively. Strain JI (Fig. 2a, lane 2, and Fig. 2b, lanes 6 and 7), which corresponded to 11 of 30 of the examined colonies (Table 2, site J, EF), was predominant in the must microflora at the final step. This, along with the less represented JII strain (Table 2, site J, EF, and Fig. 2a, lane 3, and Fig. 2b, lane 10), constituted more than 50% of the fermentative population. The remainder could be considered minority strains. A more marked situation was observed for the two other sites analyzed (Ga and Aa [Table 2]), in which two clearly predominant strains, GaI (23 of 30) and AaI (16 of 30), were identified at the EF (Table 2). These strains were also prevalent at MF (Table 2). At this step, because of the smaller number (10) of colonies examined, not all the minority associated strains isolated from the EF yeast biomass were recorded (Table 2, MF). It could, however, be considered, as demonstrated earlier (10), that the composition of the population remained unchanged from MF to EF. At the BF, because of the weak representation of *S. cerevisiae* in the microflora (Table 1), no valid conclusion on the strain composition of the population could be made.

It appears clearly from these results that the nearly monospecific population of *S. cerevisiae* which developed during fermentation is polyclonal, i.e., constituted of several genetically distinct strains growing simultaneously, one or two being predominant. These results are consistent with recent data obtained for several cases of spontaneous wine fermentation (9, 10, 21, 26).

The ecological survey of the distribution of the *S. cerevisiae* strains in the Charentes wine-producing region was carried out for 42 wineries chosen for their locations in distinct geographical sites (Fig. 1). The wine specimens were collected at the EF, as it could be considered on the basis of Table 2 and previous results (10) that the monospecific biomass is, at this step, representative of the population diversity. Because of the experimental limitations of a large-scale study, we have chosen to examine only 10 colonies for each gathering of must. In these conditions, strains prevalent in the population will be isolated more frequently than associated minority strains. In order to identify the predominant strain(s), the individual karyotypes of the 10 colonies have been compared with the chromosomal pattern established from the yeast total biomass of the sample as highlighted in Fig. 3. For this site (OR), only two different patterns were observed. The first, identified eight times (OR1, OR2, OR4, OR5, OR7, OR8, OR9, and OR10), was very similar to the karyotype of the total biomass and therefore corresponded to the majority strain. The second, observed twice (OR3 and OR6), could be considered an associated strain which differed from the majority strain by one large chromosome (arrows) appearing as a faint band in the total biomass profile. Because of random sampling, some samples revealed a higher number of associated strains, as shown in Fig. 4 for the sites MP and GDF92. They exhibited three and four different karyotypes, respectively, and only one strain (underlined) could be considered predominant. Table 3 presents the different isolated strains designated, in correspondence with Fig. 1, by their respective locations.

The killer phenotype was tested for the type K2, which is considered to be prevalent in enological yeasts (25). In the Charentes area, 21 of 35 (60%) predominant strains (Table 3, underlined) were detected as killer while only four were resistant to K2 toxins and 10 were sensitive. The associated strains

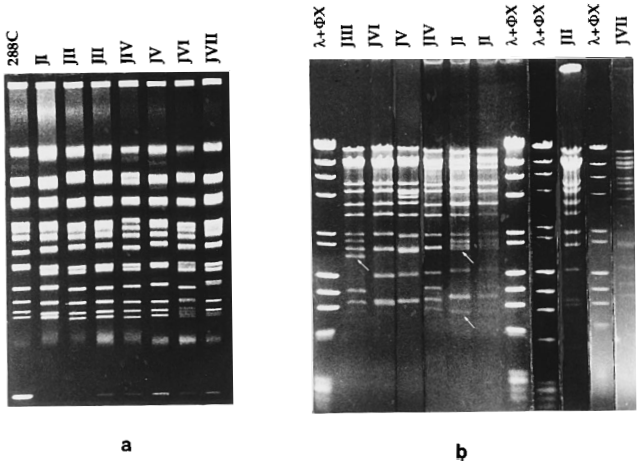

FIG. 2. Transverse alternating field electrophoresis chromosomal patterns (a) and *Eco*RV restriction profiles of mtDNA (b) of the seven different *S. cerevisiae* strains, JI to JVII, isolated from site J in the Charentes wine-producing area. The combination of the two types of genetic patterns is necessary to clearly discriminate some strains. See, for example, the case of JI and JIII, which exhibited very similar karyotypes but had clearly different *Eco*RV restriction fragments highlighted by arrows (b). 288C, haploid laboratory strain of *S. cerevisiae* used as reference; λ+ϕ×, *Hin*dIII-digested λ DNA and *Hae*III-digested ϕ×174 DNA.

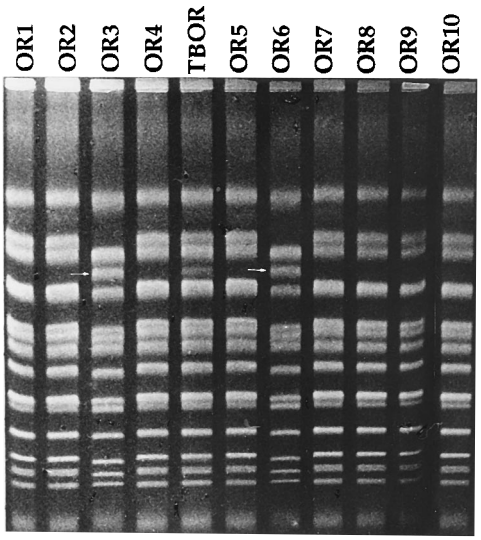

FIG. 3. Example of comparative analysis of the PFGE chromosomal patterns of the total biomass (TBOR) and of 10 colonies (OR1 to OR10) isolated separately from the same sample (OR).

were generally neutral or sensitive. As a whole, the proportion of killer yeasts relative to total yeast population was 49%.

Some dominant strains were found to occur in the same winery, over several years (Table 3). It is, for instance, the case for GaI, which was isolated in 1988, 1990, and 1992 in the same site, Ga (Table 3).

In order to differentiate, from site to site, all the strains isolated, karyotype comparison has been associated with mtDNA restriction profile analysis. In some cases of dubious strain identification, the polymorphic products of genomic DNA amplification by PCR have also been used as illustrated in Fig. 5. The strains MZ′I, GeI, and SPbI for example, showed very similar karyotypes: MZ′I differed from GeI by one single band

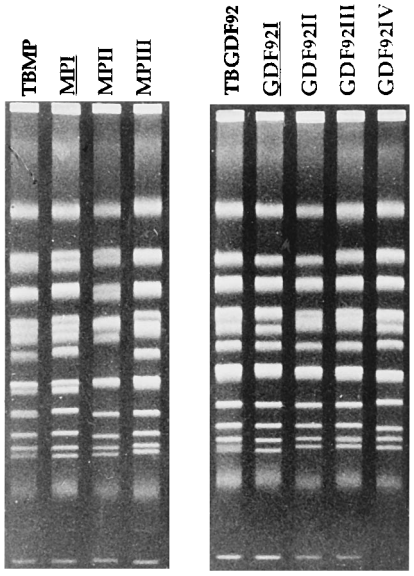

FIG. 4. PFGE chromosomal patterns of the total biomasses (TB) and of the different strains identified in two samples, MP and GDF92, illustrating the polyclonal nature of the population of *S. cerevisiae*. Predominant strains are underlined.

TABLE 3. Presentation of the different strains of *S. cerevisiae* collected in 42 wineries of the Charentes wine-producing area and identified by several methods based on DNA polymorphism[a]

Total biomass	Strains identified	Sampling yr
Aa[b]	AaI (cer N) + AaII (cer K)	1990
Aa[b]	AaI (cer N) + AaIII (cer K)	1992
Ab	AbI (cer K) + AbII (cer K)	1988
AC	ACI (cer K) + ACII (cer K)	1992
ANC′[c]	ANC′I (bay S) + ANC′II (cer S)	1991
ANC″[c]	ANC″I (bay S) + ANC″II (cer S) + ANC″III (cer S)	1991
AS	ASI (che K) + ASII (cer N)	1992
BC	BCI (cer K) + BCII (cer S)	1991
Bi	BiI (cer S) + BiII (cer S)	1992
BRA	BRAI (cer K) + BRAII (cer K) + BRAIII (cer S)	1992
BRE	BREI (cer N) + BREII (cer K) + BREIII (cer K)	1992
BOb	BObI (cer K) + BObII (cer K) + BObIII (cer K)	1992
DSC	DSCI (cer K) + DSCII (cer K)	1992
Ga[b]	GaI (cer K) + GaII (cer K)	1988
Ga[b]	GaI (cer K) + GaII (cer K) + GaIII (cer K)	1990
Ga[b]	GaI (cer K) + GaII (cer K)	1992
Gb	GbI (bay K) + GbII (cer K) + GbIII (cer K)	1988
Gc	GcI (cer K) + GcII (cer K) + GcIII (cer K)	1988
Gd	GdI (cer S) + GdII (cer S) + GdIII (cer K)	1988
Ge	GeI (cer K) + GeII (cer K)	1988
Gf	GfI (cer S) + GfII (cer N)	1988
GDF[b]	GDFI (bay N) + GDFII (cer S)	1991
GDF[b]	GDFI (bay N) + GDFII (cer S) + GDFIII (bay N) + GDFIV (cer K)	1992
GO	GOI (cer K) + GOII (cer S)	1988
H	HI (cer S) + HII (cer S)	1992
J	JI (cer K) + JII (cer K)	1990
JC	JCI (cer K) + JCII (cer S)	1992
LA	LAI (cer K) + LAII (cer S)	1992
MA	MAI (cer K) + MAII (cer S)	1992
ME	MEI (cer K) + MEII (cer S)	1992
MP	MPI (cer K) + MPII (cer K) + MPIII (cer K)	1992
MRb	MRbI (cer K) + MRbII (cer S) + MRbIII (cer S)	1992
MXb	MXbI (bay S) + MXbII (cer S)	1991
MZ′[c]	MZ′I (cer K) + MZ′II (cer N) + MZ′III (bay K)	1991
MZ″[c]	MZ″I (cer K) + MZ″II (cer N) + MZ″III (cer S)	1991
OR	ORI (cer K) + ORII (cer S)	1992
Pb	PbI (bay K) + PbII (cer K)	1992
RE	REI (cer K) + REII (cer S) + REIII (cer S)	1992
RO	ROI (bay K) + ROII (cer S)	1991
Sb	SbI (cer N) + SbII (cer S)	1992
SC	SCI (cer K) + SCII (cer S)	1992
SM	SMI (cer K) + SMII (cer K)	1988
SMC	SMCI (cer K) + SMCII (cer K) + SMCIII (cer S)	1988
SPb	SPbI (cer K) + SPbII (cer K) + SPbIII (cer K)	1992
SSC	SSCI (cer K) + SSCII (cer S)	1992
T	TI (cer S) + TII (cer S)	1991
Vb	VbI (cer S) + VbII (cer S)	1991
VLB	VLBI (cer N) + VLBII (cer S)	1992

[a] Each strain is designated by letters corresponding to the initial of the village where it was collected (Fig. 1) followed by a number. The strains found to predominate in the biomass are underlined. bay, *S. cerevisiae* var. *bayanus*; cer, *S. cerevisiae* var. *cerevisiae*; che, *S. cerevisiae* var. *chevalieri*; K, N, and S, killer, neutral, and sensitive phenotypes, respectively.

[b] Total biomass sampled from the same winery for two or three different years.

[c] Total biomass collected from two different tanks from the same winery.

between 455 and 590 kb in size (arrow, Fig. 5a). SPbI differed from MZ′I and GeI by the position of its lower band corresponding to chromosome I (arrow, Fig. 5a). Such faint differences have not been observed for their mtDNA restriction profiles and PCR product patterns (Fig. 5b and c). Therefore, these three strains could be considered being closely related. On the other hand, for the two pairs of strains JI and JII and

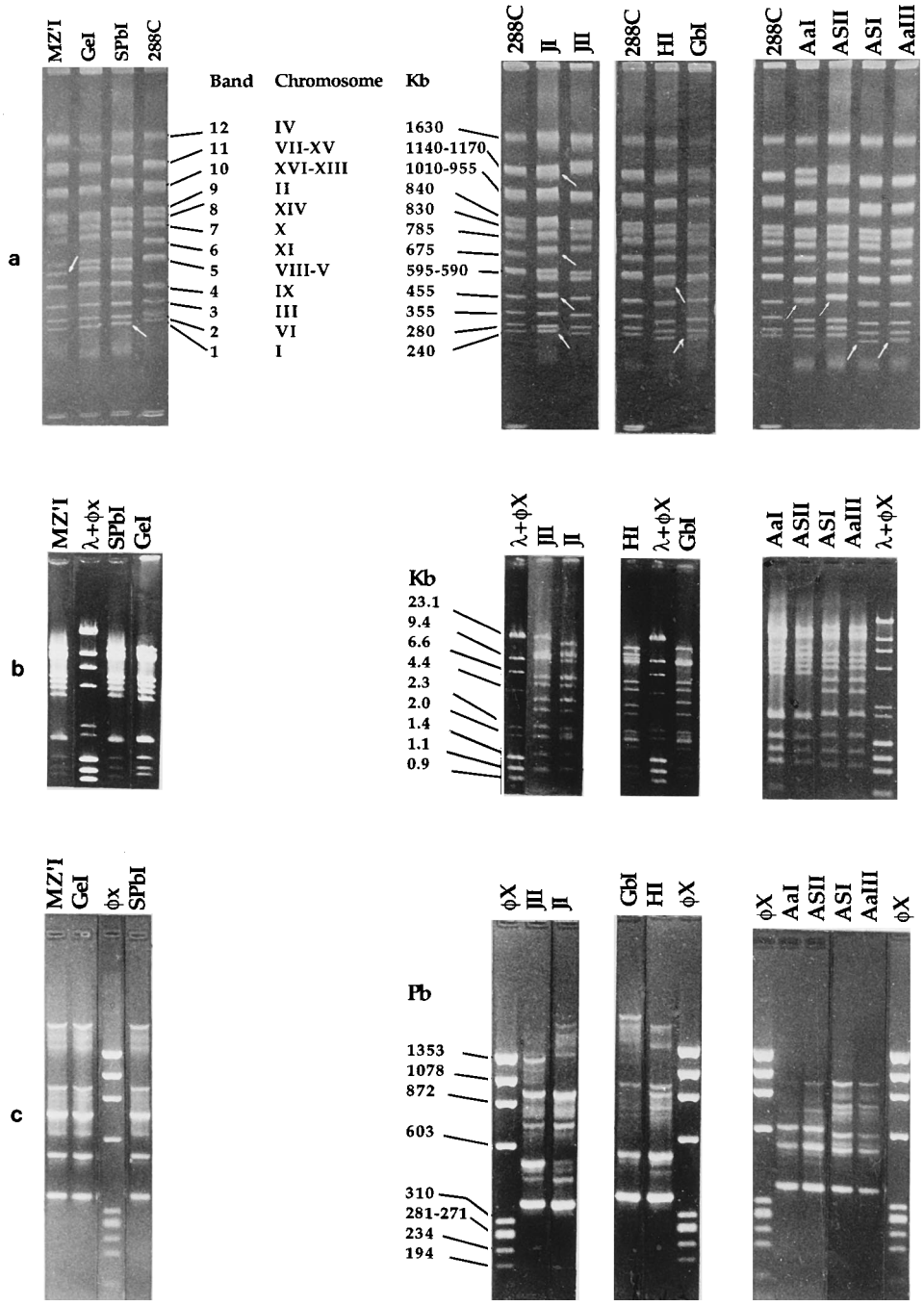

FIG. 5. Electrophoretic patterns of karyotypes (a), *Eco*RV restriction digest of mtDNA (b), and PCR-amplified products of genomic DNA (c), used for the differentiation of the *S. cerevisiae* strains MZ'I, GeI, and SPbI; JI and JII; HI and GbI; and AaI, ASII, ASI, and AaIII. 288C, haploid laboratory strain of *S. cerevisiae*; λ+φ×, *Hin*dIII-digested λ DNA and *Hae*III-digested φ×174 DNA; φ×, *Hae*III-digested φ×174 DNA. Pb, base pairs.

HI and GbI considered separately, although equivalent karyotypes were observed (a few small differences are highlighted with arrows [Fig. 5a]), they displayed distinct mtDNA restriction and PCR patterns (Fig. 5b and c). Four other strains, AaI, ASII, ASI, and AaIII, which presented similar karyotypes (small variations of some band positions are marked with arrows in Fig. 5a) could in fact be subdivided from their mtDNA and PCR product profile comparison (Fig. 5b and c) into two pairs: AaI and ASII and ASI and AaIII. It was finally decided that all specimens having the same genetic patterns (karyotypes, mtDNA, and PCR products) could be considered the same strain even if collected at sites far apart, as illustrated in Fig. 6 for the strain BRAI = DSCI. The different cases of identity observed between isolates are presented in the legend to Fig. 1. Finally, 70 different strains have been clearly identified in the Charentes area: 35 were predominant and 35 were minority strains.

Considering now the geographical distribution of the strains

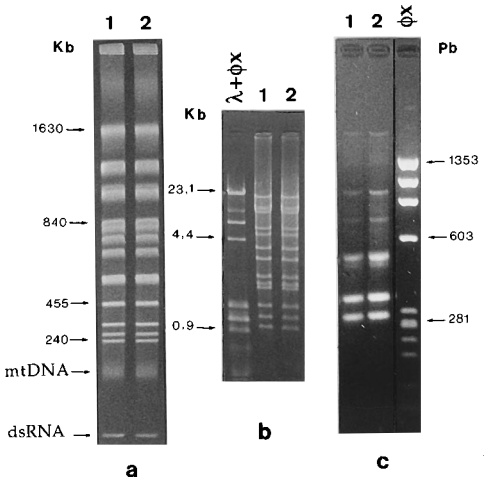

FIG. 6. Example of identical electrophoretic patterns obtained for strains isolated from different locations: strain BRAI (lanes 1) and strain DSCI (lanes 2). λ+ϕ×, *Hin*dIII-digested λ DNA and *Hae*III-digested ϕ×174 DNA; ϕ×, *Hae*III-digested ϕ×174 DNA. (a) Electrophoretic patterns of karyotypes; (b) *Eco*RV restriction digest of mtDNA; (c) PCR-amplified products of genomic DNA. dsRNA, double-stranded DNA. Pb, base pairs.

within the Charentes wine-producing area (Fig. 1), several situations have been observed. Two strains which have been identified at several dispersed sites could be considered widely distributed. Such was the case for the strain ACI (*, Fig. 1), which was found in eight different wineries spread over five subvintage wine areas. This strain was predominant in every sample analyzed. The minority strain AaIII (no. 4, Fig. 1) was found in wineries spread over two subvintage wine areas. A few strains exhibited a more limited distribution, such as the strain BREI (no. 1, Fig. 1), which was isolated four times from different wineries of the same subvintage area ("Fin Bois," Fig. 1). In some instances, the same strain was found in two different wineries situated in the same village, for example, GaI and GcI, or in two or three close villages, as GDFII and SMCIII. Some strains have been found to be dominant in one place and in the minority in others (GeI = GaIII or ASII = BREI = SbI = VLBI). Thus, if it is clear from our observations that no specific dominant strain of a subvintage wine area exists, one strain (ACI) was found to be scattered throughout the geographical area analyzed. This strain can be considered representative of the Charentes wine-producing area.

We have attempted to analyze the extent of the genetic variability between strains with the Jaccard coefficient as a mathematical method. In the case of karyotypes, it was not possible to avoid the overestimation of some faint variations in band position. This resulted in aberrant distances between some strains (results not shown), and the use of chromosomal polymorphism was judged, by this analysis, unfit for estimation of relatedness. Consequently, the genetic proximity between strains has been presented exclusively from mtDNA restriction fragment analysis, with *Eco*RV as endonuclease. Figure 7 shows the normalized mean-distance matrix tree calculated with the Jaccard coefficient for the majority of the isolated strains. At a normalized mean distance of 1.1, the analyzed strains could be separated into two groups. The first group (A, Fig. 7) was represented only by 9 different strains while the second (B, Fig. 7) corresponded to 34 distinct ones. According

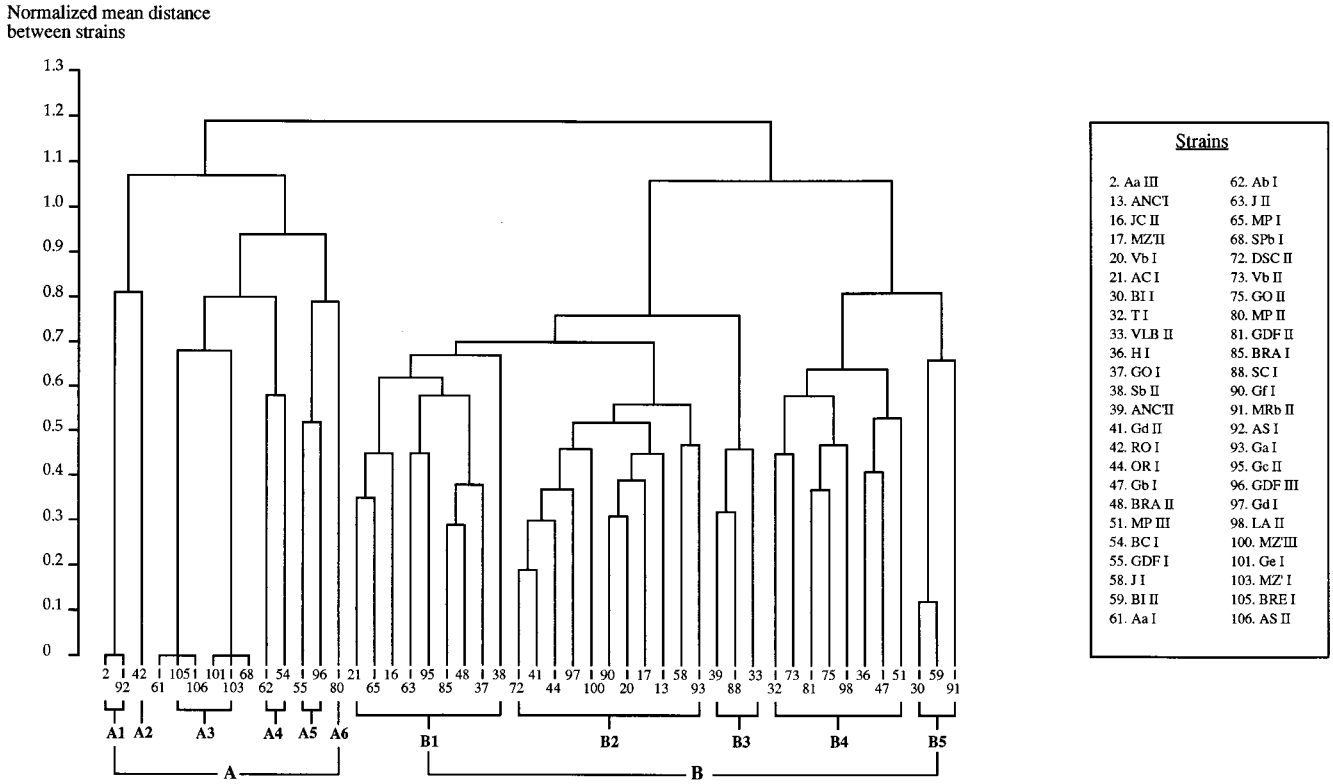

FIG. 7. Distance matrix dendrogram derived from Jaccard coefficient analysis of mtDNA *Eco*RV restriction patterns of 48 *S. cerevisiae* strains from the Charentes wine-producing area.

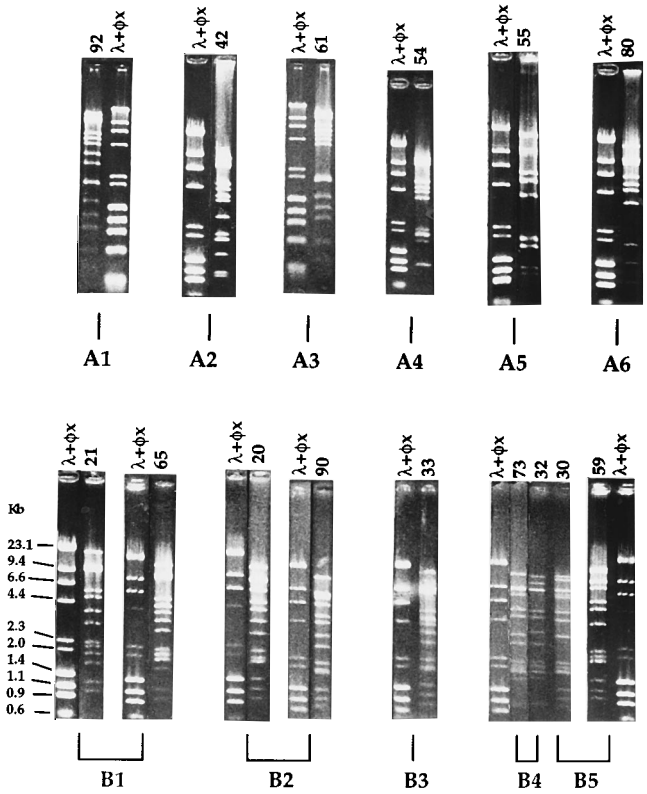

FIG. 8. Examples of mtDNA restriction profiles of *S. cerevisiae* strains representative of 11 clusters of relatedness, A1 to B5, obtained at a genetic distance of 0.7 according to the Jaccard coefficient. Sets of strains belonging to the same group of proximity, for instance, B2 (strains 20 and 90) or B4 (strains 32 and 73), exhibited strong similarities in the number and size of *Eco*RV fragments. In contrast, strains from far-apart clusters have completely different profiles, as exemplified by strains 92 (A1) and 55 (A5). λ+φ×, *Hin*dIII-digested λ DNA and *Hae*III-digested φ×174 DNA.

to the distance measurements, these two groups could themselves be subdivided in a more defined fashion. In order to correlate genetic distances with geographical distribution of strains, we have chosen to distinguish five subclusters within group B at a normalized distance of 0.7. Group A has been considered as a whole as it was subdivided into six subclusters which were represented by only one or two strains. As illustrated by Fig. 8, definable mtDNA restriction patterns were obtained for each subgroup at this genetic distance. The different strains from an identical group were generally found to be widespread over the examined area as illustrated by group B1 (Fig. 9a). This is consistent with the fact that one strain of this group, ACI, was widely dispersed over the vineyard (Fig. 1). Only one group (B4, Fig. 7) seems to have been isolated in a more restricted area, i.e., the northern part of the vineyard (Fig. 9b). Adding the three strains of the B5 group, which is the most directly related to B4, would not result in any change. However, no direct correlation of the level of relatedness with the degree of geographical proximity can be demonstrated.

DISCUSSION

In Charentes as in other wine-producing regions (see, for example, references 8, 12, and 20), wine fermentation is conventionally carried out by the species *S. cerevisiae*. The monospecific population, which develops from mid- to final fermentation stages, was shown to be polyclonal with one or two strains representing more than 50% of the biomass associated with a higher number of minority strains. These results are in agreement with previous observations (6, 9, 21, 26), and the predominant strains can be considered the most adapted to conditions of wine production. This criterion has been recently used in programs of enological strain selection (9, 26).

In fact, the majority of the predominant *S. cerevisiae* strains isolated in Charentes were K2 killer, while the associated strains had generally a neutral or sensitive phenotype. Contrasted situations have been observed in other wine regions. In a restricted area of the Bordeaux region (Graves), all the strains which dominated fermentation were identified as killer (Frezier and Dubourdieu [9]). On the other hand, observations made in Tuscany, Italy (24) have shown that in frequent cases the majority of the must fermenting population was not composed of killer strains. In several cases of fermentation analyzed in this work, some sensitive strains have been found to coexist with killer predominant strains. This phenomenon has already been described in the literature (12, 24, 25). According to Heard and Fleet (12), who studied the incidence of killer yeasts in Australian wineries, the killer effect depends strongly on the ratio of killer to sensitive cells at the beginning of fermentation. No noticeable effect was obtained for ratios as high as 1:7 while pronounced killer activity occurred when the ratio was approximately 1:1. Experiments on killer-sensitive association carried out in low volume in controlled conditions could result in different conclusions (25).

The fact that some Charentes predominant strains have been found over several consecutive years in the same cellar can be interpreted as a consequence of their prevalence in the local microflora and/or of their technological fitness. It could also be due to some autofeeding of musts by dominant strains which have survived in cellars as resting cells during one or several years. Notwithstanding the fact that vats and other equipment are carefully washed after each vintage, some previous observations made in Champagne (28) tend to refute this second hypothesis. In this vineyard for which cases of strain perenniality have also been described, the strain sampling was done directly at the sites where grapes were crushed and pressed. These places are clearly separated from the cellars. As a consequence, it can be assumed that the samples could not be contaminated by yeasts developing in cellars and were representative of the natural microflora present on grapes.

The method proposed in this paper to isolate the majority strain(s) from a must sample, by comparison of PFGE karyotypes of the total biomass with those of a limited number (10) of individual colonies, has been shown to be very efficient. From 42 distinct wineries, 35 predominant and 35 secondary strains have been isolated. A marked polymorphism of both electrophoretic chromosomal and mtDNA restriction profiles has generally been observed. This is consistent with previous observations made for enological strains of *S. cerevisiae* (7, 11, 27, 28). However, several Charentes strains exhibited very similar karyotypes differing only by faint variations of band position or the presence of doublets. As enological yeasts are at least diploid (2, 17), some of these differences could be attributed to structural heterozygosis, i.e., presence of differently sized homologous chromosomes. As demonstrated by Bidenne et al. (2), the chromosomal length polymorphism can be partly explained by structural reorganizations. These modifications have been observed only for homologous chromosomes of diploid or polyploid strains and could occur at a relatively high frequency during mitosis (15). However, the time scale of these variations in nature is still unknown.

In a recent paper, Mortimer et al. (17) have carried out a detailed genetic study of 43 different enological strains of *S.*

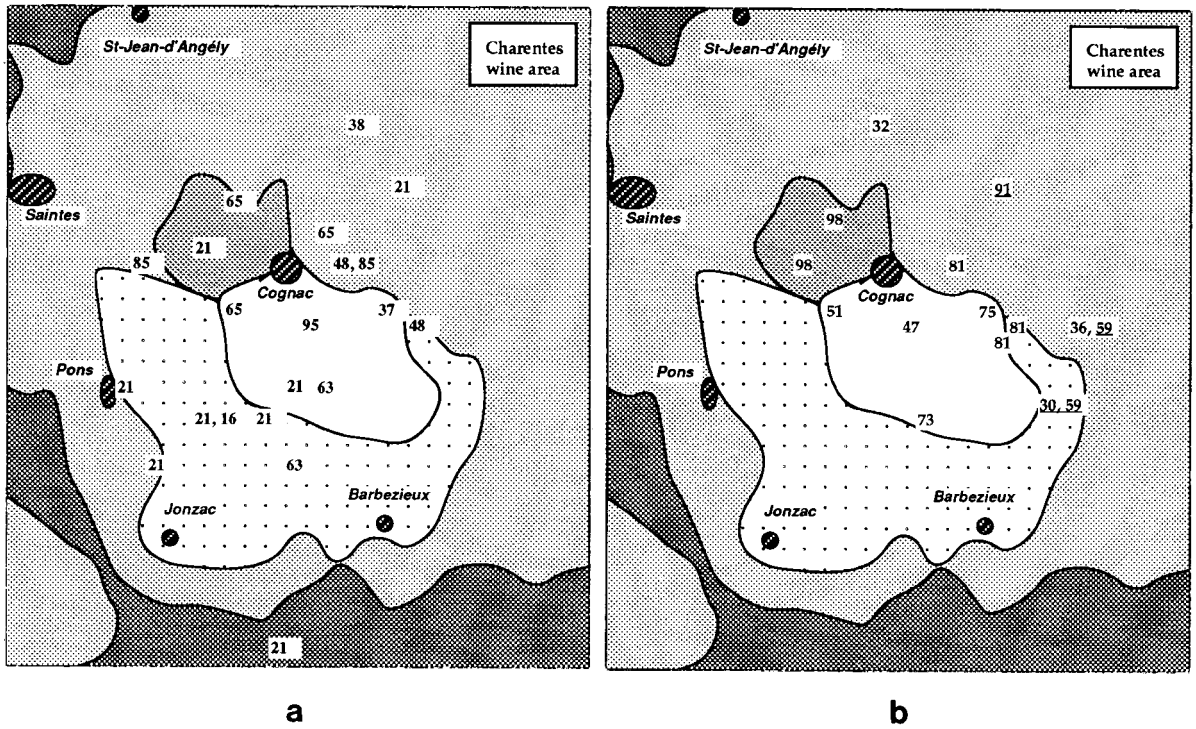

FIG. 9. Geographical distribution of the strains clustered by their genetic relatedness in group B1 (a) and in groups B4 and B5 (b). 16, 21 (ACI), 37, 38, 48, 63, 65, 85, and 95, strains from group B1; 32, 36, 47, 51, 73, 75, 81, and 98, strains from group B4; 30, 59, and 91, strains from group B5. Subvintage wine-producing areas: □, Grande Champagne; ⊡, Petite Champagne; ▤, Borderies; ▨, Fins Bois; ▩, Bons Bois. ●, principal towns.

cerevisiae collected during fermentation of Italian wines. From their observations, they have developed a model called "genome renewal" to explain the rapid evolution of wine yeast strains. They have proposed that new genotypes could arise from diploid homothallic strains, changing multiple heterozygotes into completely homozygous diploids. Some of these new diploids may exhibit greater fitness than their siblings or parents and will replace the original strain. According to this attractive theory, the dominant enological strains will presumably be found more frequently as homozygous than as secondary strains, and it would be relevant to proceed to their genetic analysis.

Considering the geographical distribution of *S. cerevisiae* strains throughout the Charentes vineyards, only two of them, ACI and AaIII, have been found to be widespread in the analyzed area. Such strains could be considered as representative of an enological region or "terroir." This concept of native specific strains is supported by preliminary results obtained in the Champagne vineyard (28). For this area, one strain has also been encountered in samples originating from several places over 6 years.

The analysis of strain relatedness from mtDNA restriction fragments allowed us to distinguish several subclusters characterized by strong similarities of electrophoretic patterns. However, no correlation between the degree of genetic relatedness and the geographical vicinity has been evidenced. It was not possible to decide, from our results, whether the occurrence of the same group of related strains over a large area was the result of dispersion by humans or natural agents or the result of parallel modifications from an original common ancestor. More accurate studies will require employment of molecular probes and/or genome sequence analyses to obtain the next level of resolution.

ACKNOWLEDGMENTS

This work has been supported by the French Ministry of Research and Technology (convention CIFRE no. 309/91) and by grants of Lallemand S.A. (Toulouse, France) and of the National Interprofessional Office of Cognac (BNIC).

We thank Luc Lurton for his interest in the work.

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