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Molecular Polymorphism Distribution in Phenotypically Distinct Populations of Wine Yeast Strains

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Electrophoretic karyotyping and mitochondrial DNA restriction analysis were used to analyze natural yeast populations from fermenting musts in El Penedès, Spain. Both analyses revealed a considerable degree of polymorphism, indicating heterogeneous natural populations. By specifically designed genetic selection protocols, strains showing potentially interesting phenotypes, such as a high tolerance to ethanol and temperature or the ability to grow and to ferment in wine-water-sugar mixtures, were isolated from these natural populations. Genetic analysis showed a strong correlation between the selected phenotypes and mitochondrial DNA polymorphisms. Karyotype analysis revealed several genetically similar yeast lineages in the natural yeast microflora, which we interpret as genetically isolated subpopulations of yeast strains with distinct genetic traits, which may correspond to specific microenvironments. Thus, molecular polymorphism analysis may be useful not only to study the geographical distribution of natural yeast strains but also to identify strains with specific phenotypic properties.

El Penedès is the major sparkling-wine-producing region of Spain. For more than a century, wine and sparkling wine have been produced in this area from musts from three traditional grape varieties, Macabeu, Xarel.lo, and Parellada. Although the traditional practice is the use of natural mycoflora to start must fermentation, commercial yeast strains have been used in the last decade as starters to increase the velocity and reproducibility of fermentations. In addition, production of sparkling wine by the traditional method requires the addition of sugar and preconditioned yeast cells (the so-called pied de cup) to young wine and requires the so-called second fermentation to proceed for several months in the characteristic sparkling-wine bottles. During the second fermentation, yeasts must ferment all the sugar available, increasing the alcohol concentration and building a considerable CO_2 pressure in the bottle. Very few yeast strains from the natural mycoflora are able to ferment under such demanding conditions; therefore, we started the analysis and characterization of the mycoflora associated with the three traditional grape varieties from El Penedès so that we could isolate naturally occurring yeast strains with the appropriate genetic makeup to be used through the processes from must to sparkling wine.

Although wine yeast strains have been used for centuries, their unequivocal characterization has been possible only recently, when genomic, karyotypic, and mitochondrial DNA (mtDNA) analyses have permitted us to identify them and to monitor their evolution in spontaneous fermentations (21, 27, 28). The yeast microfloras in different wine-producing regions have been analyzed (26, 28). The data show changes in the composition of the microflora between different vineyards and from one year to another in the same vineyard (26, 28). In addition, a sequential substitution of *Saccharomyces cerevisiae* strains occurs in must fermentations as they progress to higher levels of alcohol (19, 22).

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In this paper, we present a study of the natural yeast microfloras of fermenting musts from the three traditional grape varieties from El Penedès, characterized by both mtDNA and karyotype patterns. We then define a set of selectable and/or screenable phenotypic characteristics indicative of their usefulness in sparkling-wine production. Third, we correlate the genetic patterns of the isolated yeast strains with these selectable characteristics. We conclude that the natural mycofloras are made up of relatively small, genetically isolated yeast subpopulations that can be distinguished by both their genetic markers and their phenotypic characteristics. Several studies have shown a geographic distribution of molecular polymorphisms in different wine-producing areas (4, 11, 19, 26, 28); in this work, we show that these polymorphisms detect not only differences between the different "terroirs" but also the phenotypic variation among strains sharing the same distribution area.

MATERIALS AND METHODS

Sampling of yeast strains. Grapes of the three traditional varieties of Macabeu, Xarel.lo, and Parellada from the vineyards of the firm Nadal, located in El Pla del Penedès, 50 km southwest of Barcelona, Spain, were separately pressed, clarified, and allowed to ferment in 20,000-liter tanks in accordance with the standard procedures of the firm. Samples from the surface, the center, and the bottom of the three tanks were taken at different stages of fermentation, as monitored by the change of the fermenting must density. From a starting density of 1,070 g/liter, samples were taken at 1,025, 1,010, and 995 g/liter, corresponding to alcohol concentrations of 6.5, 8.5, and 10.5% (vol/vol). Yeast cells present in the samples were centrifuged, resuspended in YPD (10 g of peptone per liter, 5 g of yeast extract per liter, 2% glucose) and frozen at −80°C after the addition of glycerol to 50%. Streaks from the starting frozen samples on YPD plates were incubated at 30°C, and several isolated colonies from each plate were picked, restreaked, grown in YPD, and frozen as described above.

Metabolic tests. Utilization of different carbon sources was tested on YEP or SD plates (SD is yeast nitrogen base [GIBCO] plus ammonium sulfate, without amino acids) with 2% of the appropriate carbon source (glucose, maltose, sucrose, lactose, or galactose). Sporulation plates were prepared as described previously (23). Utilization of nitrate and lysine as nitrogen sources was tested with agar plates containing 0.6% glucose, 1.7 g of yeast nitrogen base (without either ammonium sulfate or amino acids) per liter, 5 g of sodium sulfate per liter, and 0.5% either lysine or sodium nitrate. Strains that were sucrose positive, maltose positive, lactose negative, nitrate negative, and lysine negative were considered to be candidates for belonging to the genus *Saccharomyces* (3, 5, 13).

mtDNA analysis. Total DNA extraction and restriction pattern analysis of

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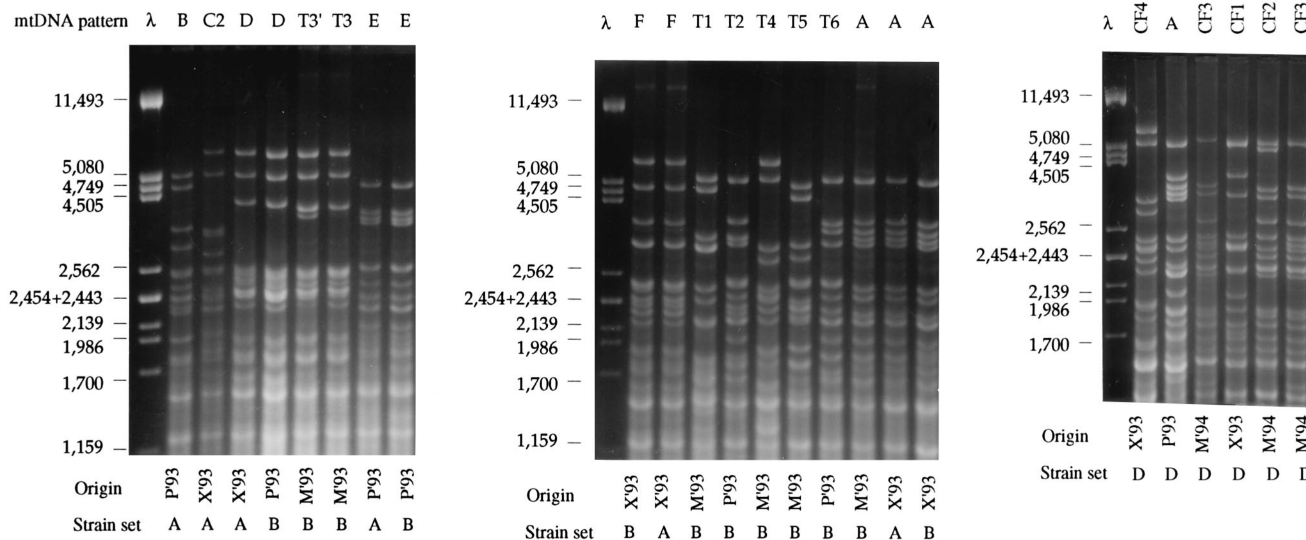

FIG. 1. *Hin*fI restriction patterns of different strains from El Penedès. Lambda DNA digested with *Pst*I was used as the size marker; numbers indicate the sizes in base pairs of the different restriction fragments. The three gels show most mtDNA patterns found at least three times in our different strain sets. Capital letters at the top indicate the code assigned to each mtDNA pattern. At the bottom, M′93, X′93, P′93, and M′94 indicate the original fermentations from which the strains were isolated; the strain sets to which they belong are also indicated (see the text and Table 1). Note the appearance of strains with identical mtDNA restriction profiles in different original fermentations and in different strain sets.

mtDNA were performed by the method of Querol et al. (21), except that lyticase from *Oerskovia xanthoneolytica*, prepared as described previously (25), was used to digest the cell wall. Yeast DNA was digested with either *Hin*fI, *Rsa*I, or *Taq*I, and digestions were analyzed in TBE (100 mM Tris-borate, 5 mM EDTA [pH 8.4])-containing 1% agarose gels.

Karyotype analysis. Late-logarithmic-phase yeast cultures were embedded in low-melting-point agarose and sequentially digested with lyticase and proteinase K as described previously (10). Yeast chromosomes were separated by pulsed-field gel electrophoresis (PFGE) in a Hula-Gel (Hoeffer) at 180 V, with a pulse ramp from 60 to 120 s, for a total of 40 h in 0.5× TBE buffer at 12°C.

Experimental fermentations. Selection schemes and small-scale fermentations (5 to 500 ml) were carried out with either YEPS (10 g of peptone per liter, 5 g of yeast extract per liter, 2% sucrose) or sterile wine. Wine obtained from the firm Nadal was sterilized by filtration through a 0.42-μm-pore-size filter (Schleicher & Schuell or Corning). Pasteurized or heat-treated wine gave nonreproducible results.

Pied de cup. Small-scale pieds de cup were best performed in 2.5-liter flasks. A 1,200-ml volume of a mixture made from 840 ml of wine, 320 ml of water, 67 g of sucrose, 1.3 g of citric acid, and 360 mg of ENOVIT (AEB Spa, Brescia, Italy) was inoculated with washed cells from 150 ml of a YEPS-grown overnight culture of the appropriate strain. Flasks were kept at 17 to 20°C without agitation. The initial alcohol concentration was 7.5% (vol/vol). Fermentation progress was monitored by measurement of density loss. At an ethanol concentration of 9.5% (vol/vol), carefully measured wine-water-sugar mixtures were added to allow the alcohol concentration to rise to a final level of 11% (vol/vol) as a result of the metabolic activity of the cells. The final cell count was from 4×10^7 to 8×10^7/ml.

Enologic analysis. Fermentations were monitored by measuring the decrease in the medium density (with a standard enologic densimeter), the amount of reducing sugars remaining (with a standard kit from GAB, Moja, Spain [16]), or the alcohol content (with kit 176 290 from Boehringer Mannheim), using the conversion tables and/or instructions provided by the manufacturers in all cases.

RESULTS

Natural population of yeast cells in fermenting musts from El Penedès. Spontaneous fermentations of musts corresponding to the 1993 harvests of Macabeu (M′93), Xarel.lo (X′93), and Parellada (P′93) grapes and the 1994 harvest of Macabeu grapes (M′94) were monitored, and samples were taken at three different stages of the fermentation, as described in Materials and Methods. Yeast cells from these 12 samples were centrifuged, washed, and frozen as described in Materials and Methods. Then 10 to 12 independent clones from each of these 12 original yeast stocks (124 clones in total) were isolated on YPD plates and separately frozen at −80°C. These 124 independent clones, referred to as set A, were tested for utilization of glucose, maltose, sucrose, lactose, and galactose as carbon sources and for utilization of lysine and nitrate as nitrogen sources. From the morphology of vegetative cells and of asci, as well as from metabolic data, we assigned most of the clones (116 of 124) to the genus *Saccharomyces*. Considering their mtDNA restriction patterns (Fig. 1) and chromosomal profiles (Fig. 2), as well as their metabolic behavior, we conclude that most, if not all, of our *Saccharomyces* clones correspond to *S. cerevisiae* (see below) (11). Of the remaining eight clones, many correspond to apiculated yeasts, presumably belonging to the genus *Kloeckera*, except for one or two clones that possibly belong to the genus *Kluyveromyces.*

mtDNA from several *Saccharomyces* clones was analyzed by the 4-base-cutting enzymes *Hin*fI, *Rsa*I, and *Taq*I, giving distinct restriction patterns. *Rsa*I gave somewhat less diversity than either *Taq*I or *Hin*fI (results not shown); therefore, we routinely used *Hin*fI to group the isolated strains into classes according to their mtDNA restriction patterns and occasionally confirmed the homogeneity of these classes by using the other two enzymes. Figure 1 shows several different restriction patterns found in our samples.

The analysis of the 116 *Saccharomyces* clones isolated from spontaneously fermenting musts revealed a large degree of mtDNA polymorphism. At least 24 different mtDNA restriction patterns were observed (Table 1; Fig. 1). Three mtDNA restriction patterns (termed A, B, and C2) account for more than 70% of the whole set, although their relative proportions vary among the different fermentations. The remaining 30% of strains showed mtDNA restriction patterns that were unique or rare (one to five cases).

Selection of yeast strains for sparkling-wine production. Sparkling wine is produced in El Penedès by the traditional method first developed in La Champagne, France, in the 18th century. This procedure places great demands on yeasts, even for wine-producing yeast strains. It requires yeast cells to start fermentation in a medium that contains 10 to 11% (vol/vol)

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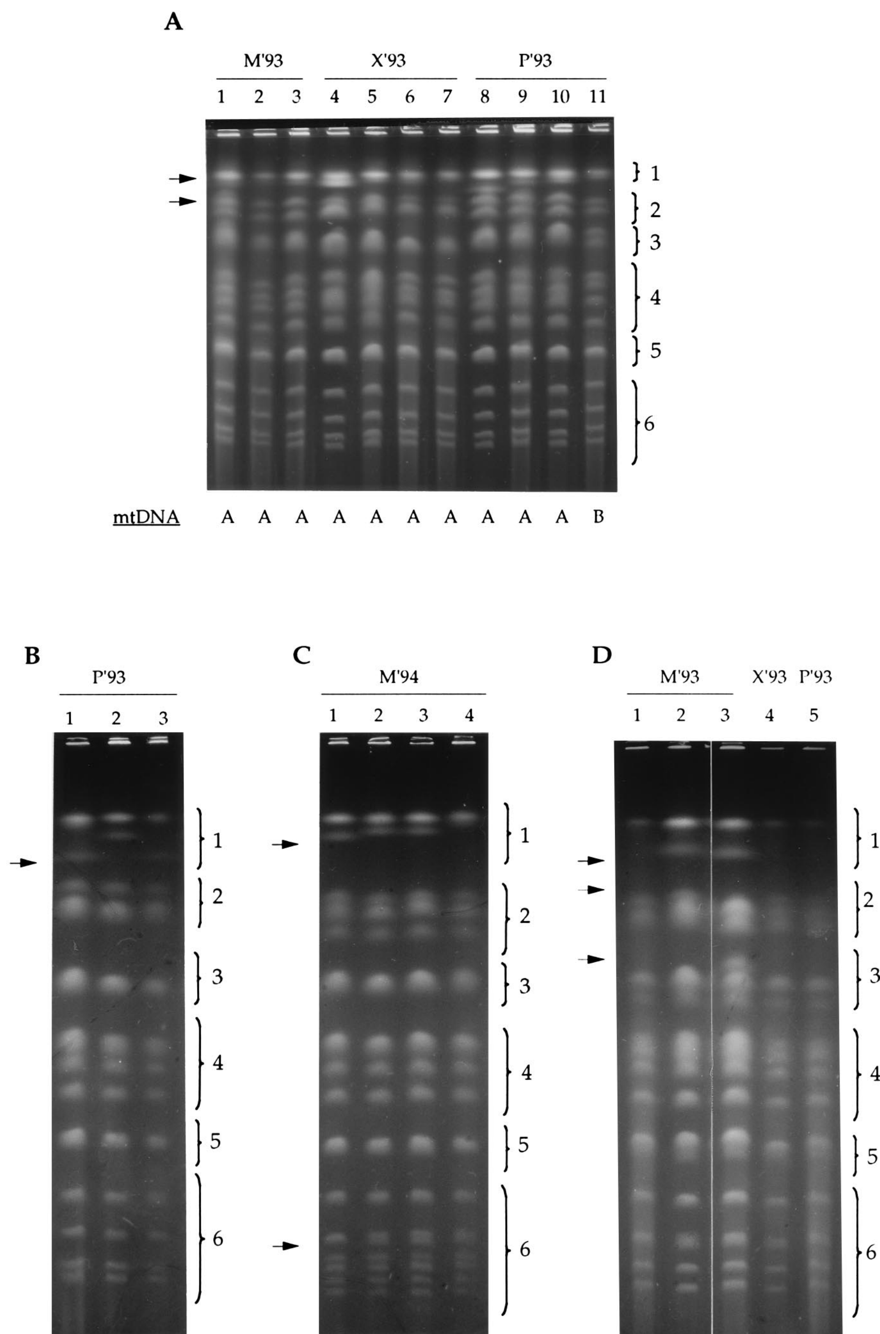

FIG. 2. PFGE of different *Saccharomyces* clones. M′93, X′93, P′93, and M′94 indicate the original fermentations from which the different clones were derived (Table 1). Brackets and numbers on the right show the chromosome groups as defined in the text and in Fig. 3. Arrows point to bands that show some heterogeneity among clones with the same mtDNA pattern. (A) Strains from set A; capital letters at the bottom indicate the mtDNA restriction pattern corresponding to each clone. Note that all lanes but lane 11 have mtDNA pattern A. (B) mtDNA restriction pattern D (flocculent) strains. (C) mtDNA restriction pattern CF3 (highly fermentative) strains. (D) mtDNA restriction pattern T4 (heat-resistant) strains.

alcohol, which is inhibitory for most wine yeast strains. As a consequence of the high alcohol and low oxygen concentrations, yeast growth, which in musts occurs naturally during the first stages of the fermentation up to high cell counts, is much more difficult in the second fermentation. From our results, it seems very important for the success of the fermentation that yeast cells divide three to four times in the sparkling-wine bottle (not shown).

During the second fermentation of sparkling wine, toxicity due to the rise in alcohol concentration is exacerbated by the parallel consumption of nutrients, mainly sugar and ammonium sources (see reference 7 and references therein). To isolate strains resistant to ethanol under these conditions, we inoculated wine plus 20 g of glucose per liter with the frozen yeast stocks and incubated these cultures at 30°C, taking advantage of the cumulative toxic effect of temperature and alcohol (7). A supplement of mineral salts (2.5 g of ammonium sulfate per liter, 1 g of potassium phosphate per liter, 0.5 g of

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TABLE 1. Distribution of mtDNA patterns in the different spontaneous must fermentations

mtDNA	No. (%) of clones with mtDNA pattern				
	M′93	X′93	P′93	M′94	Total
A	11 (44.0)	5 (20.8)	11 (39.3)	17 (43.6)	44 (37.9)
B	5 (20.0)	1 (4.2)	11 (39.3)	3 (7.7)	20 (17.2)
C	3 (12.0)	0	0	1 (2.6)	4 (3.4)
C2	0	12 (50.0)	1 (3.6)	5 (12.8)	18 (15.5)
D	0	5 (20.8)	0	0	5 (4.3)
E	0	0	3 (10.7)	1 (2.6)	4 (3.4)
H	0	0	0	3 (7.7)	3 (2.6)
Other[a]	6 (24.0)	1 (4.2)	2 (7.1)	9 (23.1)	18 (15.5)
Total	25 (100.0)	24 (100.0)	28 (100.0)	39 (100.0)	116 (100.0)

[a] mtDNA restriction patterns found only once or twice in all four samples.

TABLE 2. Distribution of the different mtDNA patterns in all four sets of independent clones

mtDNA	No. (%) of clones with mtDNA pattern			
	Set A	Set B	Set C	Set D
A	44 (37.9)	15 (31.9)	1 (5.9)	1 (11.1)
B	20 (17.2)	0	0	0
C	4 (3.4)	0	0	0
C2	18 (15.5)	0	0	0
D	5 (4.3)	9 (19.1)	0	0
E	4 (3.4)	1 (2.1)	0	0
F	1 (0.9)	2 (4.3)	0	0
H	3 (2.6)	0	0	0
T1	0	10 (21.3)	0	0
T2	0	3 (6.4)	0	0
T3	0	3 (6.4)	0	0
T4	0	2 (4.3)	16 (94.1)	0
CF3	0	0	0	5 (55.6)
Other[a]	17 (14.7)	2 (4.3)	0	3 (33.3)
Total	116 (100.0)	47 (100.0)	17 (100.0)	9 (100.0)
Other genus[b]	8	0	0	0

[a] mtDNA restriction patterns found less than three times in all four sets.
[b] Non-*Saccharomyces* clones.

magnesium sulfate per liter, 0.5 mg of zinc chloride per liter) was added to facilitate yeast growth. It must be pointed out that both the pied de cup and the sparkling-wine second fermentation are carried out in temperature-controlled environments, between 14 and 20°C; therefore, our test represents a considerable temperature stress. A total of 48 independent clones were isolated and characterized from this selection. This group of clones will be referred to below as set B.

A second absolute requirement for industrial sparkling-wine production is the ability to support the standard pied de cup protocol. This traditional practice ensures that yeast cells become gradually adapted to high levels of alcohol, so that they can serve as starters for the second fermentation. Small-scale pieds de cup were performed in 50-ml flasks containing a wine-water-sugar mixture with 740 ml of wine per liter and 65 g of sucrose per liter, designed to start from 8% ethanol and to reach 12% ethanol when all the sugar was consumed. Combinations of the three original frozen yeast stocks from each of the M′93, X′93, P′93, and M′94 fermentations were used to inoculate four flasks (one flask for each original fermentation), which were incubated at 20°C until most of the sugar was consumed. Then these cultures were used to inoculate a second set of flasks with a similar water-wine-sugar mixture but designed in this case to start at 9% ethanol and to finish at 12% ethanol. These flasks were again incubated at 20°C until all the available sugar had been consumed. Samples from this second round were kept frozen for further selection protocols (see below). A third round was performed, this time with wine plus 24 g of sugar as the medium per liter—the same conditions as in the second fermentation. A total of 17 clones were isolated and characterized from these last cultures (set C).

Finally, we developed a screening protocol to isolate yeast strains with high fermentation capacities. Test tubes containing wine-water-sugar mixtures (740 ml of wine per liter, 65 g of sucrose per liter) to raise the alcohol content from 8 to 12% (vol/vol) upon fermentation were separately inoculated with 90 independent clones from the second step of the pied de cup selection and incubated at 17°C. The fermentation capacity was monitored by using inverted Durham tubes and checking for the appearance of gas bubbles. The nine faster-fermenting clones (set D) were reisolated, rescreened, and stored as described above.

Genetic and mtDNA analysis of phenotypically selected yeast strains. Our next step was to analyze the different sets of yeast strains from the three different selection schemes (sets B, C, and D) and to compare their compositions with the random sample taken from the original yeast stocks (set A). On the basis of their morphological and metabolic characteristics, as well as their mtDNA and karyotype patterns, we assigned all of them to *S. cerevisiae*. We presume that the selection schemes we imposed excluded clones from other species present in set A (Table 2). The mtDNA restriction analysis of these strains revealed 10 new restriction patterns that were not found in set A (Tables 1 and 2; Fig. 1). Apart from that, the composition of mtDNA patterns varied widely among the different sets. For example, mtDNA patterns B and C2 were absent from yeast strain sets B, C, and D, whereas they accounted for more than one-quarter of the clones in set A when combined. Conversely, mtDNA patterns T1 (21% of set B), T4 (94% of set C), and CF3 (56% of set D) were absent in set A, indicating that their combined proportion in the initial stocks was equal to or lower than 1%. The difference in composition between sets B, C, and D is also remarkable. These three sets had very few mtDNA patterns in common, and when a given mtDNA pattern was found in more than one set (like pattern T4 in sets B and C), its proportion was very different in the two sets (Table 2). mtDNA pattern A was present in all four sets, probably representing a major lineage in El Penedès natural populations. However, whereas it was found in a similar, high proportion in sets A and B, it was almost residual in sets C and D (Table 2).

We conclude from our data that our selection schemes have selected not only strains with determined phenotypic traits but also strains with different mtDNA patterns. To assess whether this correlation between the phenotype and mtDNA pattern is observed for different phenotypes, we screened our four sets of yeast strains for two completely independent phenotypes that we had not selected for: galactose utilization and flocculation.

The ability to grow on galactose was once used as a systematic key to distinguish between the species *S. cerevisiae* and *S. bayanus* (13). According to the latest systematic tendencies, the species *S. bayanus* (*S. uvarum*) should be reserved for a much better defined set of yeasts, regardless of whether they use galactose or melibiose (3, 15). Nevertheless, galactose utilization is a very easy test that can be used to trace specific genetic makeups. All tested strains of mtDNA restriction patterns A, B, and T4 were able to grow in galactose, whereas only one of

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TABLE 3. Appearance of galactose utilization and flocculation phenotypes in clones with different mtDNA patterns

mtDNA	No. of clones tested	No. of clones showing:	
		Growth in galactose	Flocculation
A	16	16	0
B	6	6	0
D	8	1	8
T4	8	8	0
CF3	5	0	0

eight mtDNA restriction pattern D strains and none of the mtDNA restriction pattern CF3 strains showed this phenotype (Table 3). Thus, our data show a clear correlation between mtDNA pattern and galactose utilization.

Flocculation is a very desirable property for many applications, because it facilitates the removal of cells from the medium (24). In sparkling-wine production, it could facilitate the process of removal of yeast cells and cell debris immediately before commercial corking of the bottles, a very time-consuming step called riddling or rémuage. In our strains, we observed a very strong correlation between flocculation capacity and mtDNA pattern D; all clones showing this mtDNA pattern were flocculent, and all flocculent clones found up to now showed this particular mtDNA pattern (Table 3).

Karyotypic analysis of wine strains from El Penedès. Karyotypic analysis has been widely used to characterize wine yeast strains (2, 4, 8). We used different strains from all four yeast strain sets (Fig. 2) to analyze the karyotypic diversity of the natural populations and to compare it with the diversity found by the mtDNA restriction pattern analysis. To this end, we analyzed by PFGE several independent clones of each of the patterns A, D, CF3, and T4 (Fig. 2), choosing clones isolated from different original musts when possible. It must be pointed out that these four subgroups of clones show not only different mtDNA patterns but also different phenotypic traits. mtDNA pattern A is the major mtDNA pattern in most fermenting musts. Strains showing mtDNA pattern D are the only ones that are flocculent, whereas CF3 strains are strongly fermentative and T4 strains are resistant to heat stress.

Interpretation of PFGE gels is not easy, partially because of the large variability in size of yeast chromosomes (14). Therefore, we consider it not practical to assign specific chromosome numbers to each band from the wild-type strains, as we can do for laboratory strains (Fig. 3) (10). Instead, and for simplicity of discussion, we arbitrarily define six groups of chromosomes that are easy to identify under our PFGE conditions (Fig. 2 and 3). These groups can also be identified in haploid laboratory strains, in which specific chromosomes can be assigned to each band or group of bands, as shown in Fig. 3. It is tempting to assign corresponding bands in wild strains to each chromosome in the same way, but we consider that it would be too risky without appropriate gene-mapping experiments, which we have not yet performed.

From the gels in Fig. 2 and the scheme in Fig. 3, several conclusions can be drawn. First, all karyotypes are relatively similar, with similar numbers of chromosomal bands running in similar positions in the gel. Second, strains with different mtDNA restriction patterns also show different karyotype profiles, as can be observed by comparing chromosome group 3 from lanes 10 (mtDNA pattern A) and 11 (mtDNA pattern B) in Fig. 2A or chromosome groups 2, 3, and 6 from strains showing mtDNA patterns D, CF3, and T4 in Fig. 2B to D. These differences are schematically summarized in Fig. 3.

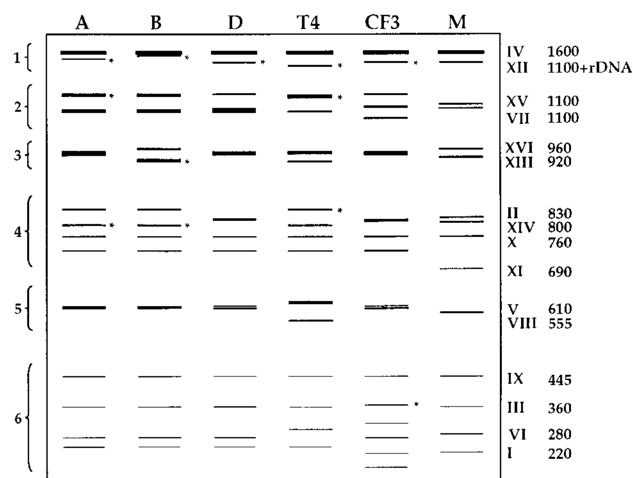

FIG. 3. Schematic representation of different karyotypes from different clones showing mtDNA patterns A, B, D, T4, and CF3 (indicated at the top). Asterisks indicate bands that show some variability among clones with the same mtDNA restriction pattern (data from many different gels). M corresponds to a laboratory haploid strain, W303a (from the Yeast Genetic Stock Center, Berkeley, Calif.). Roman numerals on the right mark the positions of the different yeast chromosomes of the laboratory strain; arabic numbers next to them indicate the chromosome sizes in kilobases (from reference 10). For simplicity, we have subdivided the karyotypes into six different chromosome groups, indicated on the left.

A third aspect, shown in Fig. 2, is a certain karyotype heterogeneity among strains with the same mtDNA pattern. This heterogeneity is increased in the fastest band on chromosome group 1. For example, in Fig. 2A, all lanes but lane 11 correspond to strains showing mtDNA restriction pattern A; nevertheless, comparison of this band from different lanes reveals a clear heterogeneity in both the position and intensity of this band. The same can be said for strains showing mtDNA patterns D, CF3, and, perhaps less clearly, T4 (Fig. 2B to D). Apart from that, differences are observed in other groups of bands. For example, in Fig. 2A, lanes 4 to 7 (X′93 strains) show a different pattern of the relative intensities of the two bands on chromosome group 2 compared with the rest of mtDNA pattern A strains (either M′93 or P′93 strains). Densitometric analysis of this region of the gel gives an upper-to-lower band ratio of 0.34 ± 0.04 for X′93 strains and 1.16 ± 0.09 for M′93 and P′93 strains, suggesting heterozygosis in the X′93 strains. Karyotypes from mtDNA pattern D strains (Fig. 2B) show differences only in the hypervariable band. Strains with pattern CF3 are somewhat less homogeneous, because some minor differences can be seen not only in the hypervariable band but also in the second-slowest band of chromosome group 6 (Fig. 2C, lower arrow). Similarly, mtDNA pattern T4 strains (Fig. 2C) show differences in chromosome groups 1, 2, and 3; strains showing larger differences (like, for example, lanes 3 and 4 in Fig. 2D) come from different original fermenting musts.

Taken together, the results of our karyotype analysis show differences between strains with different mtDNA patterns, a certain heterogeneity among strains showing the same mtDNA pattern, and a hypervariable, low-mobility PFGE band. As a general trait, strains from the same grape variety tend to be more similar to each other than to strains with the same mtDNA restriction pattern from other grape varieties, suggesting that the karyotype pattern is a good indicator of the degree of genetic proximity between strains.

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DISCUSSION

Our study of the yeast microflora in a particular vineyard in El Penedès has some interesting peculiarities. Our samples come from a relatively small vineyard of about 120 ha where the three traditional grape varieties are grown and harvested. It is also an ancient vineyard, as it appears in reports from the 16th century. Finally, the standard procedures followed routinely by the firm Nadal allowed us first to take samples of the three separated fermentations corresponding to the three grape varieties and, second, to test isolated clones for sparkling-wine production under conditions very similar to the regular commercial procedures.

The analysis of the natural mycoflora of fermenting musts from El Penedès shows a large variability in the molecular polymorphisms, revealed by both mtDNA pattern and karyotype data. Some degree of diversity has also been reported in similar studies from different French (4, 26, 28) and Spanish (11, 19) vineyards, but in our case it is probably increased by the circumstance that three different grape varieties are separately cultivated and processed. The differences in mtDNA pattern composition seen among grape varieties harvested in the same year can arise from either differences in the initial mycoflora present on the grapes, differences in the composition of nutrients of the resulting musts, or differences in climatic conditions during the harvests. Macabeu is harvested between the last week of August and the first 2 weeks of September, Xarel.lo is harvested in 2 or 3 weeks in the middle of September, and Parellada is harvested at the end of September and the beginning of October.

mtDNA restriction patterns have been widely used to identify wine yeast strains (11, 18–20, 28). However, the meaning of the heterogeneity in mtDNA restriction pattern is not clear. It has been reported recently that mtDNA structure can be used as a systematic test to distinguish between very similar *Saccharomyces* species (6, 11), so that the overall structure of the mitochondrial genome is probably rather homogeneous among *S. cerevisiae* strains. The observed polymorphisms most probably arise from either point mutations or small deletions rather than from larger rearrangements of the mitochondrial genome. The mitochondrial genome of *S. cerevisiae* has been reported to recombine with high frequency, since markers separated by only 1.5 kb behave as though they are genetically unlinked (9, 17). Thus, mtDNA lineages are independently maintained and accumulate genetic variations during vegetative growth; in sporulation/conjugation cycles, recombination between the two parental mtDNA genomes results in a further increase in mtDNA heterogeneity. From this point of view, mtDNA patterns reflect the genetic pedigree of the clone, because zygotes inherit significant proportions of the mtDNA genome from both parental strains.

Karyotype analysis shows a clear heterogeneity between the different sets of strains. Although most of this heterogeneity occurs between clones with different mtDNA patterns (Fig. 3), some variation is observed among clones with the same mtDNA pattern, especially for clones isolated from different fermentations (Fig. 2 and 3). In this regard, the observed hypervariability of a low-mobility band, which can be used as an indication of clonality, is particularly interesting. In laboratory strains, this band corresponds to chromosome XII, which includes the repetitive rDNA genes (10). We consider it likely that the hypervariability of this band in wine strains corresponds to differential amplifications of rDNA genes, but we have not tested this hypothesis.

Karyotype is obviously dependent on the pedigree of each particular clone, because each parent haploid cell supplies half of its genome to the zygote. In addition, karyotype patterns are reported to be relatively unstable even in nonselective, normal vegetative growth (14). We are concerned about the possibility that some of this variation arose during our own experimental fermentations or selection tests; that is, clones that we consider independent because they have different karyotypes may be vegetative progeny of a single cell in the original sample. There is probably no good answer to this concern, for we do not know the frequency of these changes in our populations. Presumably, large changes in the number of rDNA gene repeats occur with relatively high frequency, so that populations with the same mtDNA pattern and identical karyotypes except for the hypervariable band are probably very closely related. On the other hand, differences in the mobility of several bands, as observed between mtDNA pattern A strains from the original samples from Macabeu and Xarel.lo (Fig. 2), probably correspond to totally independent clones with a common mtDNA lineage.

The sparkling-wine second fermentation represents a formidable challenge to the yeast metabolism. The search for yeast clones that are able to carry it out led us to develop different selection schemes that gave us three different sets of yeast strains (sets B, C, and D). These yeast strain sets not only have different phenotypical traits but also have different distributions of mtDNA patterns than set A, a random sample from the original yeast stocks (Table 1). Two independent phenotypic traits, the ability to flocculate and galactose utilization, also show a clear correlation with the mtDNA pattern (Table 3). Although the study is not complete yet, we have observed that the ability to produce sparkling wine and to sustain an industrial pied de cup also correlates with specific mtDNA patterns (data not shown).

To our knowledge, our study is the first one that shows a correlation between molecular polymorphisms and specific phenotypic traits in wild *S. cerevisiae* strains from the same vineyard. Mitochondrial metabolism has been related to ethanol tolerance (1), allowing the possibility of a direct relationship between mtDNA genetic polymorphism and this particular phenotype. This relationship is much harder to imagine for flocculation and galactose utilization. For both cases, a great deal of genetic information is available, invariably referring to specific sets of nuclear genes (12, 24). Therefore, we favor the idea that the different mtDNA patterns are indicative only of distinct subpopulations of the natural yeast microflora, each with its own karyotype and mtDNA lineages. These populations are probably genetically isolated from each other; otherwise, phenotypes like galactose utilization and flocculation would have been more widespread among strains with different mtDNA patterns.

We interpret the appearance of strains with the same mtDNA pattern and similar phenotypic characteristics but with somewhat different karyotype profiles as an indication that our sets originated not from a single original cell but from a preexistent population of different, related yeast clones. The presence of such a mosaic of phenotypically distinct natural yeast populations, perhaps reflecting an adaptation to specific microenvironments, has very important implications for the ecology and for the biotechnological use of wild yeast strains. The correlation between different relevant phenotypes and specific mtDNA polymorphisms can facilitate the isolation and characterization of wild yeast populations with the desired metabolic and genetic traits.

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