Influence of the Yeast Genotypes on Enological Characteristics of Sherry Wines

J. J. MESA[1], J. J. INFANTE[2], L. REBORDINOS[3]*,
J. A. SANCHEZ[4], and J. M. CANTORAL[5]

The dynamics of yeast strain populations in the flor velum and certain analytical parameters of sherry wine in two "añadas", the static system for aging sherry wine in oak butts, were monitored for 12 months starting in spring 1996, in order to determine the correlations between biological and enological parameters. Metabolic characterization of yeasts from both añadas indicated the presence of *beticus* and *cheresiensis* races of *Saccharomyces cerevisiae*, although their relative proportions were different in each. The alcoholic degree, acetaldehyde content and volatile acidity level also evolved differently. The *cheresiensis* race was more predominant in the añada with the lower ethanol and acetaldehyde contents but higher volatile acidity. Molecular characterization of the strains, by karyotype and mtDNA restriction analyses, revealed a high polymorphism, with certain patterns being predominant. Greater variability was shown in the nuclear DNA than in the mtDNA due to the relatively young age of the wine. Some patterns were specific to either *beticus* or *cheresiensis* race, but several strains shared the same karyotype or mtDNA pattern, indicating similarity between the two types of strain, however, a preferential association between karyotype and mtDNA appeared. Cluster analysis of the combined patterns of karyotype and mtDNA restriction profiles distinguished three main genotypes. Patterns with higher frequencies were specific to each añada and some *beticus* and *cheresiensis* strains showed these same patterns; this was probably due to differences in the analytical conditions of each "añada" resulting in the selection of different yeast genotypes.

KEY WORDS: flor yeasts, sherry wines, acetaldehyde, ethanol, molecular strain identification, genotypic selection

Spanish "fino" sherry wines are pale in color, dry with pungent bouquet and flavor, and mainly characterized by their high alcoholic content. Their aging is biological and involves two phases: a static phase ("añadas") during which the wine of a simple harvest is kept in the same oak butt for a variable number of years, followed by the dynamic phase, known as "criaderas-solera" system (or "soleraje"), consisting of several blending and aging stages before being commercialized. In both phases, wine is kept isolated from the air by a surface film of yeasts, known as the flor velum, which develops an oxidative metabolism responsible for most of the organoleptic properties of sherries [9].

The development of flor velum yeasts depends on various environmental factors and also on the chemical composition of the wine. Sherry wines contain relatively high amounts of acetaldehyde, which plays an important role in aroma and bouquet; its concentration increases during aging, owing to the oxidation of ethyl alcohol and to the activity of velum yeasts [4,12,19]. Moreover, the relationships between acetaldehyde and the total content in higher alcohols suggest that acetaldehyde plays a key role in the biosynthesis of higher alcohols, probably as a precursor for the formation of valine and leucine from which isobutanol and isoamyl alcohol are derived [29]. However, acetaldehyde exerts an inhibitory effect on velum formation when its concentration is above the tolerance threshold [28].

Physiological characterization of the yeasts present in the velum of sherry wines has indicated that more than 95% of the whole population belongs to four races of *S. cerevisiae: beticus*, *montuliensis*, *cheresiensis,* and *rouxii* [18]. Nevertheless, the presence of *montuliensis* and *rouxii* races in sherry wines has only been described in the final blending stages of relatively old sherries, although the presence of these two races is not a constant even in sherries from the same winery [12]. Presence of *montuliensis* in the velum population has been related to high concentrations of acetaldehyde in the wine, and this race and *rouxii* appeared more tolerant to high levels of acetaldehyde than *beticus* and *cheresiensis*. However, results of studies on the relationships between these races and organoleptic characteristics are scarce [5,19].

The differences found in acetaldehyde production are reproducible, and consistent phenotypes of low, medium, and high acetaldehyde producers have been described [29]. The low and high phenotypes also differed considerably in the production of secondary wine fermentation products that affect the flavor of the wines in general, such as acetic acid, acetoin and higher alcohols, esters and others. Crosses between high and low acetoin-producing strains indicated low production as a

[1,2,3,5] Laboratorio de Microbiología y Genética, C.A.S.E.M., Universidad de Cádiz, Polígono Río San Pedro, 11510 Puerto Real, Cádiz, Spain; [4] Departamento de I+D, Bodegas Sandeman-Coprimar, C/ Pizarro nº 10, 11402 Jerez de la Frontera, Spain.

*Corresponding author [Fax 34-956-016180; e-mail < laureana.rebordinos@uca.es>].

Acknowledgements: This research was supported by grant OT106/116/117 from Bodegas Sandeman-Coprimar, S.A. of Jerez de la Frontera (Spain). J. J. Mesa received a postgraduate fellowship from the Spanish Ministry of Education and Culture.

Manuscript submitted for publication 4 August 1998; revised 5 October 1999.

dominant trait and also high versus low acetoin production segregated as a single gene [29]. At the same time, the highest acetaldehyde production coincided with the maximum ethanol consumption, and this consumption was highest in *montuliensis* and *rouxii* cultures, so equilibrium between the alcoholic degree and the acetaldehyde concentration needs to be reached in order to improve the process [20]. Considering that the absence, the presence, or variations in the concentrations of acetaldehyde may be an important factor in the ultimate bouquet of a wine, the possible choice between high and low acetaldehyde producers becomes of particular interest from the technological point of view [21].

The aroma compounds of wine subjected to biological aging show many changes as a result of yeast metabolism, as well as from the extraction of various constituents of the wood by wine ethanol. Results suggest that it would be of interest to study the races of film-forming yeasts in relation to the differences observed in the sensory properties and the length of time of the biological aging of wines [17,34]. Some strains have shown differences in the production of compounds involved in aroma; it has been found that in some strains the production was faster suggesting that a more rapid aging of sherry wine is possible [6].

Flor yeasts isolated and grouped as races belonging to *S. cerevisiae* and studies of the dynamics of biological aging have also been based mainly on the metabolic characterization of the yeast strains [12,19]. However morphological and physiological properties cannot always distinguish between strains belonging to the same species and do not include their most important characteristics from the industry point of view. In fact, when molecular techniques have been applied to the study of yeasts, a wide polymorphism has been described both in karyotypes and in mitochondrial restriction patterns [15,27]. These patterns have not previously been shown to be characteristic of races; therefore, a full molecular characterization of the strains, together with the establishment of their relationships with blending/aging stages and sherry characteristics is considered desirable.

In fact, determining the relationship between genotypes and organoleptic properties would enable modification of the aging system in order to improve it or to direct it towards achieving specific characteristics in the wine [6,7,12]. Furthermore, clarification of the genetic control of characteristics that are economically important is needed for modification and selection of the most suitable strain, by either classical genetics or genetic engineering techniques. The study of the relationships between analytical parameters of sherry wines and the yeast races present indicates that there are specific genotypes, rather than metabolic races, that are associated with important enological characteristics.

Materials and Methods

Sampling: To establish particular relationships between organoleptic properties and flor yeast strains of sherry wines, samples were taken from two static systems ("añadas") which were maintained without blending or the addition of ethanol.

Flor velum yeast strains were isolated from the surface of "Fino" sherry wines belonging two different "añadas" referred to as "Añada" A (65 oak butts) and "Añada" B (60 oak butts), both two years old, from the cellars of Sandeman-Coprimar, S.A. of Jerez de la Frontera (Spain). Sampling consisted of the removal of a small amount of the velum, from three different locations on the surface of the wine in each butt. The three samples were then combined and transferred to a solution of peptone (1 g/L). In the laboratory, samples were diluted, and 100 mL of each dilution were seeded on YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose, and 2% agar) and incubated at 28°C for four to five days. Samples were taken every 15 days over a period of one year starting in the spring of 1996.

Twenty colonies per sample were randomly chosen for their microbiological characterization of their morphological and physiological properties. Eighty-three randomly chosen colonies were genotypically characterized by electrophoretic karyotyping, and of these, 60 were analyzed by mtDNA restriction analysis.

Taxonomic classification: Yeasts were grown in complete YPD medium at several dilutions and incubated at 28°C for four to five days. Plates containing from 100 to 300 colonies were examined. Twenty colonies from each sample were randomly selected and identified following the usual criteria for fermentation and assimilation of different carbon and nitrogen sources [16,31]; the media employed for these tests were as described by Barnett *et al.* [3].

Controls consisted of *S. cerevisiae* races *beticus*, *cheresiensis*, *montuliensis,* and *rouxii*; *Saccharomyces cerevisiae* (1329) strain from the CECT (Type Culture Spanish Collection) was used.

Electrophoretic karyotype: The chromosomal DNA was prepared by treating cells with a solution (1 *M* sorbitol, 25 m*M* EDTA) containing 0.3 mg/mL of dithiothreitol and 0.4 mg/mL of Lysing Enzymes (Sigma Chemical Company, St. Louis, USA) in 1 *M* sorbitol. 0.5% low-melting agarose plugs were made in Bio-Rad moulds to a final concentration of 10^8 cells/mL. The electrophoretic karyotype was obtained by contour-clamped homogeneous electric field (CHEF) gel electrophoresis in a CHEF-DRII apparatus (Bio-Rad, Richmond, CA, USA). The electrophoresis was carried out at 6 V/cm (200 V) for 24 hours with initial and final switching times of 60 and 120 seconds, respectively. The buffer used was 0.5 TBE (45 m*M* Tris-borate, 1 m*M* EDTA, pH 8) cooled to 14°C. The chromosome sizes of each molecular karyotype were determined by regression analysis using a standard set of *S. cerevisiae* YNN 295 chromosomes in the Molecular Analyst software (Bio-Rad).

Mitochondrial DNA restriction analysis (RFLP): The total DNA of the strains was extracted and purified from spheroplasts by the method of Querol and Barrio [24]. The DNA was digested with the endonucleases *Alu* I (10 units per digest), *Hinf* I (12 units per

digest) and *Rsa* I (10 units per digest) and the restriction fragments were separated by horizontal electrophoresis on 1% agarose gels in 1xTBE at 60 V. The molecular sizes of the fragments were calculated by regression analysis using lambda DNA digested with *Hind* III as molecular markers in the Molecular Analyst software (Bio-Rad).

Analytical parameters: 100 mL of wine were taken from each butt in the two añadas, the samples from the same añada were combined and the mixture was used for the analytical determinations. The ethanol was quantified according to the transflexion technique with the Infraanalyzer™ 450 system from Technicon™ (New York, USA); volatile acidity was determined by flow injection (FIA) on an Autoanalyzer™ II from Technicon™ (New York, USA), according to the method using bromophenol blue [23]; acetaldehyde was estimated according the OIV recommended procedure [22].

Cluster analysis: The similarities between electrophoretic profiles were determined by cluster analysis using the similarity complete linkage method. The unweighted pair group method average (UPGMA method) included in the Statistica package (Stat Soft, Inc., USA) was used.

Results and Discussion

Sherry wines are fortified wines with an alcohol content of 15% on average. Fortification is performed by the addition of grape brandy once the fermentation has been completed. In addition, however, at any time when the ethanol concentration falls to below 15.5%, the wine is alcoholized back up to 15.5%, ensuring an aging system with the ethanol content almost constant and near the upper tolerance limit of yeasts [13].

A valuable consequence of the high alcohol content of these wines is that this produces an almost sterile growing medium. This is enhanced by the absence of oxygen, resulting from the isolation effect produced by the flor velum. However, there are some economic disadvantageous in the system because fortification is an expensive practice. The biological aging of sherry takes several years; therefore, both an accelerated process

Table 1. Microbiological and analytical evolution of Añadas A and B from Spring 1996 to Spring 1997.

Sampling number*	Añada A					Añada B				
	Races*		Ethanol	Acetaldehyde	Volatile acidity	Races*		Ethanol	Acetaldehyde	Volatile acidity
	B	*CH*	(%)	(mg/L)	(g/L)	*B*	*CH*	(%)	(mg/L)	(g/L)
1	100	0	15.50	318	0.20	100	0	15.30	107	0.32
2	100	0	14.80	313	0.20	75	25	15.55	128	0.30
3	100	0	15.50	317	0.16	85	15	15.40	99	0.20
4	100	0	15.30	283	0.20	100	0	15.30	153	0.33
5	100	0	15.20	194	0.20	75	25	15.20	86	0.36
6	100	0	15.05	192	0.27	95	5	15.05	39	0.39
7	100	0	15.10	180	0.22	60	40	15.60	83	0.37
8	100	0	ND	ND	ND	35	65	ND	ND	ND
9	100	0	15.05	160	0.29	95	5	15.30	108	0.38
10	100	0	15.20	155	0.27	30	70	15.35	95	0.39
11	90	10	14.94	150	0.23	75	25	15.05	102	0.32
12	100	0	ND	ND	ND	85	15	ND	ND	ND
13	100	0	14.80	170	0.21	60	40	14.85	105	0.30
14	100	0	15.10	141	0.31	75	25	15.00	97	0.33
15	100	0	15.00	165	0.33	70	30	14.90	104	0.27
16	100	0	14.98	179	0.23	65	35	14.90	85	0.18
17	100	0	14.85	180	0.27	45	55	14.80	90	0.28
18	100	0	14.68	206	0.30	50	50	14.40	110	0.24
19	95	5	14.60	235	0.23	75	25	14.60	107	0.21
20	100	0	14.33	230	0.19	50	50	14.50	109	0.17
21	100	0	14.88	167	0.17	95	5	14.50	127	0.10
22	95	5	14.90	170	0.16	70	30	14.35	137	0.11
23	95	5	14.75	170	0.17	50	50	14.25	129	0.12
24	95	5	14.73	160	0.17	65	35	14.30	116	0.18
25	95	5	14.60	190	0.18	75	25	14.30	93	0.19
26	100	0	14.48	166	0.17	55	45	14.30	95	0.17
27	90	10	14.33	148	0.17	75	25	14.30	83	0.20

ND: Not determined due to technical problems.

* ***B***: *beticus*; ***CH***: *cheresiensis*

** Samples were collected every 15 days: samples 1-5 were collected in spring 1996; samples 6-11 were collected in summer 1996; samples 12-18 were collected in autumn 1996; samples 19-24 were collected in winter 1996; samples 25-27 were collected in spring 1997.

coincident with a reduced alcohol consumption and higher acetaldehyde production would be desirable improvements.

To obtain this improvement, reliable data on the characterization of yeast strains are necessary, since considerable variability has been described for these yeasts; this is not revealed by the classical methods based on morphological and physiological properties [25].

The flor yeast strains analyzed in this study show the metabolic pattern of the *beticus* and *cheresiensis* races of the *S. cerevisiae* species. The distribution of races between the añadas and their dynamics over time can be seen in Table 1.

The types of yeasts we found in flor velum are in agreement with the published data [8], although another two races of *S. cerevisiae* have been described in addition to *beticus* and *cheresiensis* (*montuliensis* and *rouxii*) [12,18]. There could be several reasons for the absence of these races in our samples. *(1)* They have always been described in very low proportions, and even so, they have not been found in all the samples from the same winery [19]. *(2)* They can be considered as markers of later blending stages; therefore, they are likely to be absent from the relatively young wine of the añadas studied here. Although absence of these races could also be explained by other reasons, such as differences in winery practices between cellars [2], or the presence of other minority yeasts producing killer toxins [30], other factors such as practices related to the preparation of musts, the inoculation of strains during the biological aging, *etc.*, cannot be discounted [32].

Molecular characterization of the strains involved the determination of the electrophoretic karyotype and analysis of restriction fragment length polymorphism of the mtDNA. Polymorphism in flor yeasts is produced by a balance of forces tending to induce chromosomal changes (high concentrations of ethanol or acetaldehyde) [28] against a high selection pressure acting upon them (lack of fermentable sugars, absence of sexual reproduction, *etc.*) [4] and low proportions of Y' and Ty elements [14] in terms of selecting the karyotype best adapted to such conditions.

Results showed a higher polymorphism in the *beticus* compared to the *cheresiensis* race; 14 different patterns were displayed by *beticus* but only seven by *cheresiensis* (Fig. 1 and Table 2). Only three patterns were common to both races, and the frequency of patterns between the races was also different because four patterns (I, III, V, XVI) appeared predominant in *beticus*, but only 1 (III) in *cheresiensis*. Comparing the distribution of these patterns between the añadas, there was not only a difference in the number of patterns appearing in each, but also the most frequent pattern was different in each añada: *i.e.*, pattern III in Añada B, but pattern I in Añada A (Table 2).

The mitochondrial DNA restriction analysis has been described as a practical method for monitoring *Saccharomyces* strains in wine fermentation [26]. RFLPs of mitochondrial DNA of 60 strains with the enzymes *Alu* I, *Hinf* I, and *Rsa* I yielded three, four, and four different patterns, respectively, indicating a high degree of polymorphism (Fig. 2).

Table 2. Electrophoretic karyotypes of flor velum yeasts isolated from two añadas of sherry wine rendered by the analysis of the chromosomal patterns through Contour-Clamped Homogeneous Electric Field (CHEF) pulsed gel electrophoresis.

Karyotype	No. of strains	% strains	— Races — *beticus*	— Races — *cheresiensis*	— Añada — A	— Añada — B
I	21	25.30	19	2	17	4
II	1	1.20	1	0	1	0
III	28	33.90	11	17	1	27
IV	1	1.20	1	0	1	0
V	6	7.20	6	0	5	1
VI	1	1.20	1	0	1	0
VII	1	1.20	1	0	1	0
VIII	1	1.20	1	0	0	1
IX	1	1.20	1	0	1	0
X	4	4.80	2	2	2	2
XV	2	2.40	2	0	1	1
XVI	6	7.20	6	0	5	1
XVII	3	3.60	3	0	3	0
XVIII	1	1.20	0	1	0	1
XIX	2	2.40	0	2	1	1
XX	2	2.40	0	2	0	2
XXI	1	1.20	0	1	0	1
XXIII	1	1.20	1	0	1	0
Total	83	100	56	27	41	42

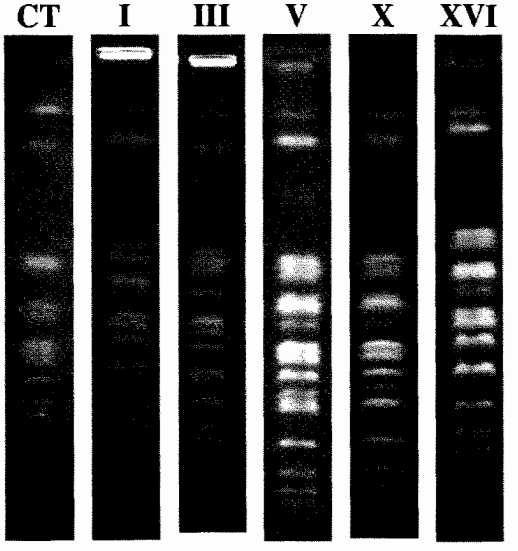

Fig.1. Predominant electrophoretic karyotype patterns of the flor velum yeast strains isolated from two "añadas" of sherry wine. Chromosomes from the *S. cerevisiae* YNN295 were used as a reference (CT) of molecular weights with chromosome sizes from 2,2 kb to 245 pb. Five different patterns appeared with frequencies higher than 5% in the overall set of flor yeasts studied and refereed in this work as pattern I (I), pattern III (III), pattern V (V), pattern X (X) and pattern XVI (XVI).

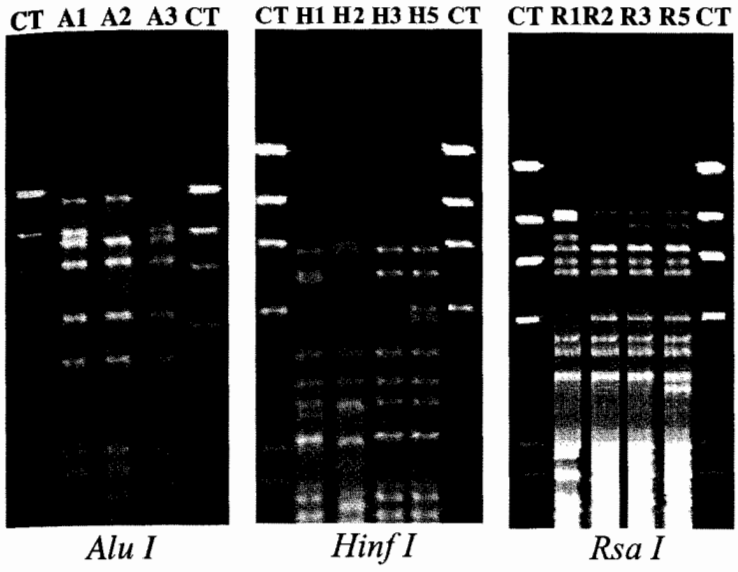

Fig. 2. Restriction analysis of the mtDNA of flor velum yeasts digested with *Alu I* (A1, A2, A3), *Hinf I* (H1, H2, H3, H5) and *Rsa I* (R1, R2, R3, R5). Lambda DNA digested with *Hind III* was used as standards (CT).

Mitochondrial combined patterns with the three enzymes can be seen in Table 3, where only seven out of 36 possible patterns appeared. These patterns were more specific than the karyotype ones, both in races and in añadas, with two predominant profiles in both strains. Distribution of these profiles between añadas was also specific; only three patterns appeared in Añada A, with RP1 being the most frequent, whereas five appeared in Añada B, with RP2 being the most frequent combination, which was not present in Añada A. The existence of predominant patterns could be due to the selection exerted by the sherry conditions. Comparing the polymorphism found, this appeared lower in chromosomal than in mitochondrial DNA; however, a greater uniformity in the chromosomal compared to the mitochondrial patterns of flor velum yeasts from the dynamic blending/aging phase has been described [18]. The authors explained the difference as due to the selection of an optimum karyotype while the mtDNA was variable because of the mutagenicity of the ethanol concentration present. The differences in our results might be the result of the yeast strains of the añadas being under selective pressure for less time, with the effects between DNA types not yet distinguishable. However, when the wine is undergoing dynamic biological aging, the karyotypes of flor yeasts present are reduced by selection, with only a few karyotypes remaining; instead, the sensitivity of mtDNA to alcohol is being displayed as higher variability.

Combined patterns between chromosomal and mtDNA profiles yielded two predominant frequencies. Although results suggested that the different strains identified are very closely related, since they may share either of these patterns in their karyotype or in their mtDNA restriction analysis, combined patterns were not random, and some preferential associations could be distinguished, since only 24 out of 648 possible combinations

Table 3. Mitochondrial DNA patterns (RFLP) of flor velum yeasts isolated from two "añadas" of sherry wine through restriction with the enzymes *Alu I*, *Hinf I* and *Rsa I*.

Pattern*	No. of strains	% strains	— Races — *beticus*	*cheresi-ensis*	— Añada — A	B
RP1	28	46.67	27	1	25	3
RP2	23	38.30	6	17	0	23
RP3	1	1.67	1	0	1	0
RP4	1	1.67	1	0	0	1
RP7	4	6.68	0	4	0	4
RP8	1	1.67	0	1	0	1
RP10	2	3.34	2	0	2	0
Total	60	100	37	23	28	32

* Patterns are the combination of the individual patterns yielded by each enzyme (Fig. 2): RP1: A1H1R1; RP2: A2H2R2; RP3: A3H1R1; RP4: A1H2R2; RP7: A2H3R3; RP8: A2H3R2; RP10: A2H5R5.

Table 4. Genotypic patterns of flor velum yeasts isolated from two añadas of sherry wine according to their molecular karyotypes and RFLP of mtDNA.

Pattern*	No. of strains	% strains	— Races — *beticus*	*cheresi-ensis*	— Añada — A	B
PC1	12	20.00	12	0	12	0
PC3	1	1.67	0	1	1	0
PC4	1	1.67	1	0	1	0
PC5	1	1.67	1	0	1	0
PC6	1	1.67	1	0	0	1
PC7	20	33.24	5	15	0	20
PC8	1	1.67	1	0	1	0
PC9	3	5.01	3	0	3	0
PC10	2	3.34	2	0	1	1
PC11	1	1.67	1	0	1	0
PC12	1	1.67	1	0	1	0
PC13	1	1.67	1	0	0	1
PC15	2	3.34	1	1	1	1
PC16	1	1.67	0	1	0	1
PC17	1	1.67	1	0	1	0
PC18	1	1.67	0	1	0	1
PC19	1	1.67	1	0	1	0
PC20	2	3.34	2	0	1	1
PC21	1	1.67	0	1	0	1
PC22	1	1.67	0	1	0	1
PC23	2	3.34	0	2	0	2
PC24	1	1.67	0	1	0	1
PC28	1	1.67	1	0	1	0
PC30	1	1.67	0	1	1	0

* Combined patterns of karyotype and mtDNA: Combined patterns with RP1: PC1, PC5, PC9, PC10, PC11, PC12, PC15, PC17, PC19, PC20, PC28 containing chromosomal patterns I, II, IV, V, VI, VII, X, III, XV, XVI and XXIII, respectively; Combined patterns with RP2: PC7, PC13, PC18, PC24 and chromosomal patterns III, VIII, I, and XXI, respectively; PC4: I-RP3; PC6: III-RP4; Combined patterns with RP7: PC8, PC16, PC22, PC23 containing chromosomal patterns III, X, XIX and XX, respectively; PC21: XVIII-RP8; Combined patterns with RP10: PC3 and PC30: I and XIX, respectively.

appeared (Table 4). Moreover, preferential distribution of combinations between the races was found, PC1 being specific to *beticus* and PC7 appearing with higher frequency in *cheresiensis* than in *beticus*. In relation to the distribution of patterns between the añadas, the tendency described previously was confirmed, with PC1 only found in Añada A and PC7 only in Añada B.

When cluster analysis was applied to the combined patterns, three groups could be distinguished at 86% similarity; one group was composed of patterns with very low frequencies in both races. In contrast, in each of the other two groups, specific combination, of either one or the other añada appeared; *i.e.,* the combined pattern PC7 was present in five *beticus* and 15 *cheresiensis* strains, but all of them in Añada B appeared in one cluster, whereas PC1 was only present in *beticus* of Añada A and was clustered in a third group (Fig. 3). Therefore, these results confirmed the presence of specific patterns for different race strains growing under the same conditions and are in agreement with data showing a correlation between specific genotype patterns and blending/aging stages [21].

Relationships between yeast populations and analytical parameters showed that Añada A, containing mainly the *beticus* race, showed the best values of the three parameters studied: ethanol, acetaldehyde, and volatile acidity. Añada B, which also started with 100% *beticus*, showed a lower ethanol content, and due mainly to the acetaldehyde concentration, *cheresiensis* was able to develop. Data on growth rate indicated a faster growth for *beticus* in comparison with *cheresiensis*. Hence its duplication time is lower, and this may be the reason why *beticus* is first to colonize the velum. Only when conditions are less favorable for the development of *beticus* are the *cheresiensis* strains able to develop (data not shown). The results in Table 4 indicate that differences in analytical parameters depend on seasonal effects, mainly temperature differences. The study started in Spring 1996; by the end of that season when the temperature was rising and all through the summer, a decrease in the acetaldehyde content was observed in both añadas. At the end of the summer, when the temperature decreased again, a higher production rate of acetaldehyde was found; this decreased again at the beginning of the next Spring. Volatile acidity was inversely related to acetaldehyde content; increases of acetaldehyde corresponded to decreases of volatile acidity and vice versa. Ethanol decreased continuously, since the añadas were not fortified.

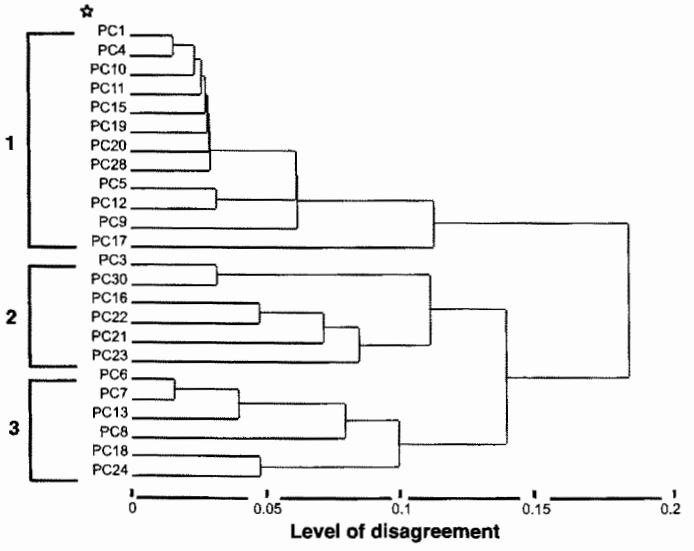

Fig.3. Cluster analysis of combined patterns (karyotype and mtDNA) of flor velum yeasts of two "añadas (static system) of sherry wine.
☆ Combined patterns shown on Table 3. Their distribution and frequencies between the races and "añadas" were as follows:
Cluster 1: PC4, PC5, PC11, PC12, PC17, PC19 and PC28 appeared in one strain of *beticus* of "Añada" A; PC9 in three *beticus* of "Añada" A; PC1 in twelve *beticus* of "Añada" A; PC10 and PC20 in one *beticus* of "Añada" A and one *beticus* of "Añada" B; PC15 in one *beticus* of "Añada" A and one *cheresiensis* of "Añada" B.
Cluster 2: PC3 and PC30 in one *cheresiensis* of "Añada" A; PC16, PC21 and PC22 in one *cheresiensis* of "Añada" B; PC23 in two *cheresiensis* of "Añada" B.
Cluster 3: PC13 and PC18 in one *beticus* of "Añada" A and in one *beticus* of "Añada" B; PC6, PC18 and PC24 in one *cheresiensis* of "Añada" B; PC7 in five *beticus* and in fifteen *cheresiensis* both in "Añada" B.

The ability of yeasts to resist the inhibitory effect of ethanol decreases as the temperature increases; this explains the significant deterioration of the yeast film observed as the temperature of the wine rises during the summer. Moreover, elevated temperatures and inhibitory ethanol concentrations have a lethal effect on yeast cells, and it has been proposed that these factors act upon the mitochondria, suggesting that the production of respiratory deficient mutants in the flor yeast population may cause the seasonal deterioration of the yeast film. On the other hand, thermal tolerance and ethanol tolerance are genetically determined properties that may vary from one strain to another and, hence, would be susceptible biological improvement [13,33], although the deterioration may be attributed to factors other than heat, such as limited amounts of essential nutrients or the opportunistic proliferation of contaminant yeasts [10,11]. However, the large number of genes involved in the control of some of these characteristics must be considered because this may impede the genetic improvement, and other changes, such as the thermal acclimation of the winery, could produce improved results more quickly [12].

Our results indicate that not only is the biological composition of the wine in terms of races important in order to improve certain analytical parameters, but also that the genotypes of yeast races developing in two separate añadas of the same age are different due to the selective effect exerted by the evolution of the compounds in the wine.

Conclusions

Molecular techniques are very useful not only for the characterization of yeasts, but also because they allow monitoring of strains during the process of biological aging to detect the presence of contaminants and the dynamics of the yeast population to be followed, detect-

ing differences not revealed by microbiological methods. The study of two añadas with different analytical parameters showed different proportions of races *beticus* and *cheresiensis,* and they evolved differently depending on the season and also varied over time. The presence of *cheresiensis* is an indicator of undesirable enological conditions for fino sherry. Molecular analysis of both races by karyotype and mtDNA restriction analyses showed that certain patterns were specific to only one or the other of the races; other strains of both races shared the pattern of either karyotype and/or mtDNA. However, when the origin of the strains was considered, patterns were found to be specific to the particular añada, with some *beticus* and *cheresiensis* strains showing the same molecular pattern, indicating the selection of genotypes according to the individual conditions of growth in the añada.

Literature Cited

1. Adams, J., S. Puskas-Rozsa, *et al.* Adaptation and major chromosomal changes in populations of *Saccharomyces cerevisiae.* Curr. Genet. 22:13-19 (1992).

2. Angulo, L., C. Lema, and J. E. López. Influence of viticultural and enological practices on the development of yeast populations during winemaking. Am. J. Enol. Vitic. 44:405-408 (1993).

3. Barnett, J. A., R. W. Payne, and D. Yarrow. Yeast: Characteristics and Identification (2nd ed.). Cambridge University Press, Cambridge, England. (1990).

4. Botella, M. A., L. Pérez, *et al.* Amino acid content of Fino and Olorosos sherry wines. Am. J. Enol. Vitic. 41:12-15 (1990).

5. Cabrera, J., J. Moreno, *et al.* Formation of ethanol, higher alcohols, esters, and terpenes by five yeast strains in musts from Pedro Ximénez grapes in various degrees of ripeness. Am. J. Enol. Vitic. 39:283-287 (1988).

6. Cortés, M. B., J. Moreno, *et al.* Changes in aroma compounds of sherry wines during their biological aging carried out by *Saccharomyces cerevisiae* races *bayanus* and *capensis.* J. Agric. Food Chem. 46:2389-2394 (1998).

7. Fatichenti, F., G. A. Farris, and P. Deiana. Improved production of a Spanish-type sherry by using selected indigenous film-forming yeasts as starters. Am. J. Enol. Vitic. 34:216-220 (1983).

8. García-Maiquez, E. Los microorganismos del jerez. Microbiología SEM 11:51-58 (1995).

9. González Gordon, M. M. Jerez-Xérès-Sherry. Editorial Jerez Industrial, Jerez, Spain. (1948).

10. Guilliland, R. B. *Brettanomyces.* I. Occurrence, characteristics and effects on beer flavour. J. Inst. Brew. 67:257-261 (1961).

11. Ibeas, J. I., I. Lozano, *et al.* Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. Appl. Environ. Microbiol. 62:998-1003 (1996).

12. Ibeas, J. I., I. Lozano, *et al.* Dynamics of flor yeast populations during the biological aging of sherry wines. Am. J. Enol. Vitic. 48:75-79 (1997).

13. Jiménez, J., and T. Benítez. Genetic analysis of highly ethanol-tolerant wine yeasts. Curr. Genet. 12:421-428 (1987).

14. Johnston, J. R. Molecular genetics of yeasts. Oxford University Press, Oxford, England (1994).

15. Lavallée, F., Y. Salvas, *et al.* PCR and DNA fingerprinting used as quality control in the production of wine yeast strains. Am. J. Enol. Vitic. 45:86-91 (1994).

16. Lodder, J. The Yeast: A Taxonomic Study (1st ed.). North-Holland Publishing Company, Amsterdam-Oxford (1970).

17. Martínez de la Ossa, E., L. Pérez, and I. Caro. Variations of the major volatiles through aging of sherry. Am. J. Enol. Vitic. 38:293-297 (1987).

18. Martínez, P., A. C. Codón, *et al.* Physiological and molecular characterization of flor yeasts: polymorphism of flor yeast populations. Yeast 11:1399-1411 (1995).

19. Martínez, P., L. Pérez, and T. Benítez. Velum formation by flor yeasts isolated from sherry wine. Am. J. Enol. Vitic. 48:55-62 (1997).

20. Martínez, P., M. J. Valcárcel, *et al.* Metabolism of *Saccharomyces cerevisiae* flor yeasts during fermentation and biological aging of fino sherry: by-products and aroma compounds. Am. J. Enol. Vitic. 49:240-250 (1998).

21. Mesa, J. J., J. J. Infante, *et al.* Characterization of yeasts involved in the biological aging of sherry wines. Food Sci. Technol. 32:114-120 (1999).

22. Office International de la Vigne et du Vin. Recopilación de los Métodos Internacionales de Análisis de Vinos. OIV, París, France. (1979).

23. Owades, J. L., and J.M. Dono. Determination of volatile acids in wine by microdiffusion. Am. J. Enol. Vitic. 19:46-51 (1968).

24. Querol, A., and E. Barrio. A rapid and simple method for the preparation of yeast mitochondrial DNA. Nucl. Ac. Res. 18:1657 (1990).

25. Querol, A., E. Barrio, and D. Ramón. A comparative study of different methods of yeast strain characterization. System. Appl. Microbiol. 15:439-446 (1992).

26. Querol, A., E. Barrio, *et al.* Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl. Environ. Microbiol. 58:2948-2953 (1992).

27. Quesada, M. P., and J. L. Cenis. Use of random amplified polymorphic DNA (RAPD-PCR) in the characterization of wine yeasts. Am. J. Enol. Vitic. 46:204-208 (1995).

28. Ristow, H., A. Seyfarth, and E. R. Lochmann. Chromosomal damages by ethanol and acetaldehyde in *Saccharomyces cerevisiae* as studied by pulsed field gel electrophoresis. Mutation Res. 326:165-170 (1995).

29. Romano, P., G. Suzzi, *et al.* Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiol. Lett. 118:213-218 (1994).

30. Shimizu, K. Wine Microbiology and Biotechnology. Harwood Academic Publishers, Sydney, Australia (1993).

31. Súarez Lepe, J. A., and Íñigo Leal. La crianza biológica del vino. *In:* Microbiología Enológica, Fundamentos en Vinificación. pp 503-539. Mundi-Prensa, Madrid, Spain. (1990).

32. Suárez, J. A., A. T. Palacios, *et al.* Applying statistical analysis to determine the optimum parameters for developing yeasts in sherry. Bull. OIV 771-772 (1995).

33. van Uden, N. Ethanol toxicity and ethanol tolerance in yeasts. Ann. Rep. Ferment. Proc. 8:11-58 (1985).

34. Webb, A. D., and A. Noble. Aroma of sherry wine. Biotechnol. Bioeng. 13:939-952 (1976).