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Characterization of Yeasts Involved in the Biological Ageing of Sherry Wines

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Yeast strains that form flor velum during the ageing process of sherry have been isolated and characterized. According to their metabolic features, the races beticus *and* cheresiensis *of* Saccharomyces cerevisiae *were the main components of the flor velum, although minority yeasts belonging to either* Saccharomyces *or* Pichia *were also identified. Yeasts were also studied at molecular level, by pulsed field gel electrophoresis, and analysis of variability of mtDNA. Considerable polymorphism was detected by the analysis of the karyotypes, and although most of the patterns were common to both races a specific pattern of* cheresiensis *did appear. Analysis of mtDNA restriction also yielded a high genetic variability; most of the patterns were common to the two races but a unique pattern of* cheresiensis *was also detected. Molecular data were compared and a preferential association between karyotype and mtDNA patterns was shown. A correlation between different blending stages and both chromosomal and mtDNA restriction profiles was found, so that molecular patterns, rather than metabolic races, were associated to specific stages of the biological ageing process. Such a finding has major significance for the improvement of sherry.*

Keywords: *Saccharomyces cerevisiae*; sherry wines; biological ageing; karyotype; electrophoretic profiles

Introduction

'Fino' sherry wines are made in the South of Spain according to a traditional process. The production of the wine starts with an alcoholic fermentation of must, followed by a biological ageing. Biological wine ageing occurs in the so-called 'criaderas-solera' system, or 'soleraje', which consists of the chronological sequencing (or blending stages) and vertical stacking of oak barrels of sherry, in the process of maturation. The youngest stage, and the highest level, is usually the sixth 'criadera', this is followed in turn by the fifth stage, and so on until the oldest stage, known as the 'solera', is reached at ground level. The majority of the sample from this stage is drawn off as the finished wine. The total number of stages in the process can vary, but all barrels contain a characteristic film of yeasts (flor velum) of up to 1 cm in thickness. The films grow on the surface of the wine, which contains about 15 ° of ethanol (1). The yeast film, through its oxidative metabolism, is the major agent producing the organoleptic characteristics of sherry; this yeast metabolism also consumes oxygen and thus prevents the oxidation of the wine (2, 3).

Most of the flor yeasts isolated are grouped into races belonging to the *Saccharomyces cerevisiae* species (4–6), based on growth characteristics. However, morphological and physiological properties can not always distinguish between strains belonging to the same species and do not include the most important characteristics from an industrial perspective. At the same time, readily available methods are needed that allow the reliable identification of different yeast strains to (i) determine unequivocally the composition of yeast strains of sherry wines as the first step for its improvement (7, 8); (ii) relate specific strains to commercially important organoleptic characteristics (9, 10); (iii) improve the process, either by specific yeast inoculations or through directed biological ageing (11, 12). Proposals put forward to achieve these tasks involve the analysis of monocarboxylic acids, the determination of chemical compounds formed by the yeasts (13) or the genetic marking of strains in order to monitor them (14). However, these methods may be neither practical nor applicable to the wild yeasts involved in the biological ageing of sherry wine. Instead, molecular techniques are used to study the genetic material, based on DNA molecular polymorphism, and can accomplish the above requirements. One of these techniques is the determination of the electrophoretic karyotype by pulsed field gel electrophoresis. Using this method, a wide variety of chromosomal patterns are displayed by the wine yeasts (15, 16). In addition, the restriction patterns of mitochondrial DNA appear highly polymorphic and

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Table 1 Characteristics of the biological aging system ('soleraje') studied for the production of sherry wines

Origin[a]	No. casks	No. colonies
6th Cra[b]	180	50
5th Cra	400	190
4th Cra	320	200
3rd Cra	30	50
2nd Cra	30	50
1st Cra	31	50
Solera	32	50

[a] blending stage
[b] Cra: 'criadera'

allow an even better differentiation between strains (17–19).

The aim of the present research was to isolate yeasts of flor velum from a chosen cellar and to characterize them, by both physiological and molecular methods (analysis of karyotype and polymorphism of mtDNA), to compare the results obtained with both techniques, and to determine the composition and dynamics of the yeast populations during the biological ageing process of sherry wines.

Materials and Methods

Yeast isolates

Wines of different blending stages were sampled from a cellar (Sandeman-Coprimar, S.A.) at Jerez de la Frontera (Spain), by collecting small amounts of the velum from four different points on the surface of the wine in a barrel, and transferring them to a solution of peptone (1 g/L). The total number of barrels comprising the 'soleraje' studied was 10230, samples were taken from 10% of these (**Table 1**).

Once at the laboratory, the samples were diluted and 100 μL samples of each dilution were seeded on YPD medium and incubated at 25 °C over 4–5 d (5). A number of isolates were tested from each blending stage in proportion to the total number of casks (**Table 1**).

All of the colonies chosen were first studied by metabolic and fermentative methods to determine the kind of microorganism and, subsequently a subset of strains was chosen for the molecular analysis.

Taxonomic identification

The morphological and physiological analyses were performed according to Barnett (5, 20), considering the fermentative patterns of sugars such as lactose, galactose, maltose, raffinose, sucrose and glucose, assimilation of nitrates and arbutin hydrolysis.

The assimilation of different carbon sources was tested on a solid minimal medium (YNB) (Difco, U.S.A.) containing 6.7 g/L of yeast nitrogen base and 20 g/L of either galactose, glucose, lactose maltose, raffinose or sucrose. Assimilation of different nitrogen sources was carried out in a minimal medium, containing yeast carbon base (YCB, Difco, U.S.A. 11.7 g/L) supplemented with 5 g/L of potassium nitrate (5, 20).

Fermentative tests were carried out in a liquid medium composed of yeast extract (5 g/L) supplemented with 20 g/L of either lactose, galactose, maltose, sucrose, glucose, or 40 g/L of raffinose. Bromocresol purple (8 g/L) was used as colour marker. Yeasts were inoculated into 5 mL tubes containing 1.5 mL of medium and incubated at 28 °C with rotary shaking of 150 rpm over 20 d. Fermentation is shown by a colour change in the pH indicators. The arbutin hydrolysis was tested in a medium containing yeast extract (1 g/L), arbutin (5 g/L) and a solution of ferric chloride (20 g/L).

The races *beticus*, *cheresiensis*, *montuliensis* and *rouxii* of *S. cerevisiae*, and commercial fermentative strain D3 of *S. cerevisiae* were used as controls. In addition, *Saccharomyces cerevisiae* (1329) and *Pichia anomala* (1114) strains from the CECT (Spanish Collection of Culture Types) were used.

Determination of growth rate and duplication time

The growth rate and duplication time of cells of different races were determined during the linear phase of growth in YPD liquid medium. Cells were cultivated in 500 mL flasks containing 100 mL of medium, after inoculation of 1 mL of an overnight culture. Cultures were incubated at 28 °C with shaking, and the Optical Density (OD) at 660 nm (using a Hitachi U-1100 spectrophotometer) was measured periodically until the stationary phase was reached. The cell number was determined by counting cells in a Neubauer chamber under the light microscope. Results are the average of three experiments with standard deviations of less than 10%.

Determination of cell viability

Samples of cultures of different strains in YPD liquid medium were taken, and the total cell number was measured by counting in a Neubauer chamber. These samples were diluted and spread on YPD plates. Viability was determined by counting the colonies after incubation at 28 °C for 5 d. Results are the average of three experiments, with standard deviations of less than 10%.

Electrophoretic karyotype

Contour-clamped Homogeneous Electric Field (CHEF-DRII, of Bio-Rad) Gel Electrophoresis was used to determine the molecular karyotype. The electrophoresis was carried out at 6 V/cm (200 V) for 24 h, with an initial switching time of 60 s and final switching time of 120 s. The electrophoresis buffer used was TBE 0.5X cooled to 14 °C. A standard set of *Saccharomyces cerevisi* YNN295 chromosomes (Bio-Rad) was used as marker. The plugs containing spheroplasts of the strains were inserted in 10 g/L pulsed field agarose electrophoresis gel. The gel was coloured with ethidium bromide at 0.5 μg/mL for 30 min, and then decoloured for 1 h with migration buffer until the bands

Table 2 Metabolic patterns shown by flor velum yeasts isolated during the biological ageing of sherry wines

Sugar														
Lac		Gal		Mal		Raf		Suc		Glu				
a	*f*	*a*	*f*	*a*	*f*	*a*	*f*	*a*	*f*	*a*	*f*	*Nit*	*Arb*	Control strains
−	−	−	−	+*L*	−	+	+	+	+	+	+	−	−	*S. cerevisae beticus*
−	−	−	−	+	+	+	+	+	+	+	+	−	−	*S. cerevisiae cheresiensis*
−	−	−	−	−	−	−	−	+	−	+	+	−	−	*S. cerevisae montuliensis*
−	−	−	−	+	+	−	−	+	−	+	+	−	−	*S. cerevisae rouxii*
−	−	+	+	+	+	+	+	+	+	+	+	+*L*	−	*S. cerevisiae (D3)*
−	−	−	−	+*L*	−	+	+	+	+	+	+	−	−	*S. cerevisiae (1329)*
−	−	+	+	+	+	+	+	+	+	+	+	+	+	*Pichia anomala (1114)*

Lac: lactose; Gal: galactose; Mal: maltose; Raf: raffinose; Suc: sucrose; Glu: glucose; Nit: nitrate; Arb: arbutin
a: assimilate; f: ferment
+*L*: Low assimilation

clearly appeared. The gel image was then digitalized with a Gel-Doc 1000 (Bio-Rad) Camera. The chromosome sizes of each molecular karyotype were determined using Molecular Analyst Software (Bio-Rad).

Mitochondrial DNA restriction analysis (RFLP of mtDNA)
The total DNA of the strains was purified, precipitated, washed and dried, as described by Querol and Barrio (21), from spheroplasts. Yeast DNA was digested with the restriction enzymes *Alu* I, *Hinf* I and *Rsa* I by incubation at 37 °C for 3 h. The mtDNA restriction patterns were observed on agarose electrophoresis gel (10 g/L).

Cluster analysis
The similarity of chromosomal profiles was determined by cluster analysis through the similarity complete linkage method using the Unweighted Pair Group Method Average (UPGMA) in Statistica 4.5-pc software (StatSoft, Inc. 1993).

Results and Discussion

The objective of this research was the analysis of flor velum yeasts during the ageing of sherry wines, both by metabolic patterns and molecular techniques, to relate specific yeast strains to blending stages. Six hundred and forty isolated flor yeast samples were characterized and classified following fermentation and assimilation tests using different carbon and nitrogen sources, and the hydrolysis of arbutin, based on the results shown in **Table 2**. Strains fell into two main groups: *Saccharomyces cerevisiae* derivatives and non*Saccharomyces* ssp. (4, 5, 6, 22). Most of the yeasts belonging to *S. cerevisiae* species followed the metabolic pattern of races *beticus* or *cheresiensis* (**Table 3**). Eleven strains classified as members of the species *S. cerevisiae* showed the physiological properties of the *cheresiensis* race and, although they fermented maltose considerably faster than the previously mentioned race, their electrophoretic karyotype indicated that they belonged to *S. cerevisiae* species. However, the three non*Saccharomyces* strains showed different biochemical properties in relation to this genus; furthermore, their morphology, as well as their electrophoretic karyotype, supported these differences. Therefore they could be related to the genus *Pichia* (**Tables 2 and 3**).
The metabolic assays used were able to distinguish not only between the four races of flor velum yeast themselves, but also between each of them and commercial fermentative yeasts (D3) belonging to the same species. However, the *S. cerevisiae* (1329) control was not distinguishable from the *beticus* race of the flor velum yeast. The types of yeast found in the flor velum are in agreement with the published data (23), although another two races of *S. cerevisiae* have been described

Table 3 Number of yeast strains isolated of flor velum of different blending stages during the biological ageing of sherry wines

	No. strains			
Origin[a]	*beticus*	*cheresiensis*	*S. cerevisiae spp.*	Non*Saccharomyces*
6th Cra[b]	39	11	0	0
5th Cra	113	65	11	1
4th Cra	149	51	0	0
3rd Cra	39	10	0	1
2nd Cra	37	13	0	0
1st Cra	34	16	0	0
Solera	34	15	0	1

[a] blending stage
[b] Cra: 'criadera'

in addition to *beticus* and *cheresiensis* (*montuliensis* and *rouxii*) (7, 8). Differences in the yeast composition of flor velum could result from several factors, such as different winery practices in relation to the preparation of the musts, frequency of withdrawals of wine and/or the inoculation of strains during the biological ageing, but may also be due to the presence of some yeasts producing killer toxins, and so on. (24, 25).

The yeast strains present in each blending stage were isolated and characterized to determine the natural evolution of populations, and to relate specific strains to blending stages. The total number of each strain related to blending stages is shown in **Table 3**, with the *beticus* race always the most frequent. The evolution of the proportions through the stages can be seen in **Fig. 1**. The dynamics of the population follows a cyclical distribution through the biological ageing, with the proportions of each race increasing and decreasing consecutively. This is probably due to the withdrawals and refillings from and into the barrels. This will cyclically increase the nutrients in the wine which are assimilated in different ways by the two races. The determination of growth rate indicated a higher rate of growth for *beticus* in relation to *cheresiensis*, with the result that its duplication time is lower; this may also be the reason why it is the first race to colonize the velum. The growth rate of *beticus* was 0.526 ± 0.016 cell/h and its time of generation 1.32 ± 0.04 h, whereas for *cheresiensis*, the growth rate was of 0.298 ± 0.032 cell/h, and the generation time 2.36 ± 0.14 h.

Molecular techniques are used to study the genetic material of yeasts, and allow yeast identification based on the polymorphism of DNA. The electrophoretic karyotyping of the strains and analysis of restriction fragment length polymorphism of the mtDNA were carried out (**Figs 2 and 3**). Polymorphism in flor yeasts is achieved by a balance of forces that tend to induce chromosomal changes (high concentrations of ethanol or acetaldehyde) (26) against a high selection pressure that acts upon them (lack of fermentable sugars, absence of sexual reproduction, etc.) (2), and low proportions of Y′ and Ty elements (27) in terms of selecting the best adapted karyotype to such conditions.

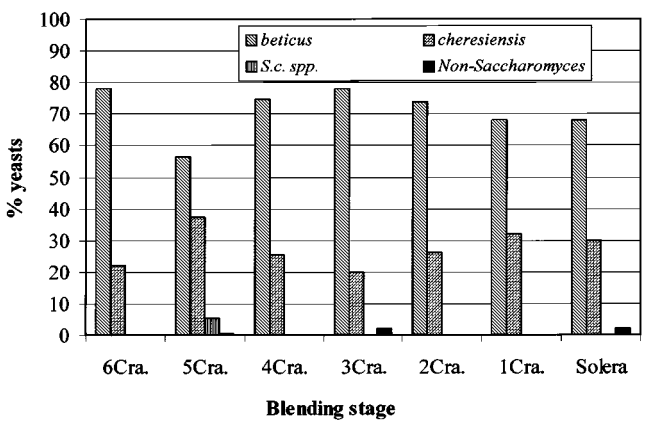

Fig. 1 Dynamics of the yeast strains isolated from flor velum during the biological aging for the production of sherry wines. Cra: 'Criadera'

Thirty one yeast strains (15 previously classified as *beticus*, and 16 as *cheresiensis*) were subjected to karyotype analysis by the pulsed field gel electrophoresis technique (28). Four different patterns were displayed for both *beticus* and *cheresiensis*, but these were also different from the four patterns shown by the velum yeast controls used (**Fig. 2**).

The distribution of karyotype profiles between yeast strains can be seen in **Table 4**. There was one pattern (XXIV) that appeared only in the race *cheresiensis*, but the rest of the patterns were common. The most common pattern (III) was present in both races (**Table 4**). Interestingly, rather than patterns specific to races, there were more patterns related to the blending stages. Three different kinds of genotypes could be distinguished: one present only in 'soleras' or the final stages, of both races (pattern VIII); one representative of the youngest or '5th criadera' stages (pattern XXII); and a transition pattern (III) which was found in both the youngest and the oldest stages.

The significance of the high genetic variability shown by the karyotype analysis was determined by comparing and grouping similar patterns. When cluster analysis was applied to profiles obtained by pulsed field gel electrophoresis, three groups could be distinguished with a similarity of 88%: one group was composed of the non*Saccharomyces* strains; another group was composed of the main two patterns found; and a third, large group consisted of the minority patterns of the study, the fermentative strains and the control races (**Fig. 4**). However, if we consider a similarity of 93%, the largest group can be subdivided into another three: one consisted of the *beticus* and *cheresiensis* controls; *montuliensis* and *rouxii* were also clustered together; the third contained the remainder of the yeasts. These results indicate the validity of the methodology presented, which can discriminate not only between the races described in flor velum but also between different strains belonging to same (*beticus* and *cheresiensis* in

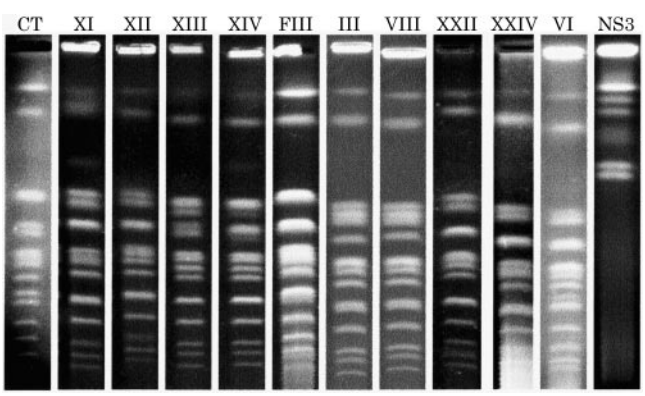

Fig. 2 Chromosomal patterns of *S. cerevisiae* strains of flor velum yeasts isolated during the biological aging of sherry wines. CT: pattern of the size markers. XI, XII, XIII and XIV: patterns of the flor velum controls (*beticus*, *cheresiensis*, *montuliensis* and *rouxii*, respectively). FIII: profile of commercial fermentative strains. III, VIII, XXII and XXIV: patterns of race *beticus* and *cheresiensis* of this study. VI and NS3: patterns of the minority strains, classified as *S. cerevisiae* strains and non*Saccharomyces*, respectively

Table 4 Frequency and distribution of electrophoretic chromosomal and mtDNA restriction patterns of *S. cerevisiae* strains isolated the different blending stages of flor velum during the biological ageing of sherry wines

Race	Karyotype	RFLP			Origin[a]	Strains
		Alu I	*Hinf* I	*Rsa* I		
beticus	III	A2	H2	R2	5th Cra[b]	3
beticus	III	A2	H2	R2	4th Cra	2
beticus	III	—	—	—	6th Cra	1
beticus	III	—	—	—	4th Cra	3
beticus	III	—	—	—	5th Cra	1
beticus	III	A2	H2	R2	Solera	1
beticus	VIII	A2	H4	R4	Solera	2
beticus	XXII	A2	H3	R5	5th Cra	2
cheresiensis	III	—	—	—	4th Cra	3
cheresiensis	III	A2	H2	R2	6th Cra	1
cheresiensis	III	A2	H3	R3	4th Cra	1
cheresiensis	III	A2	H2	R2	5th Cra	1
cheresiensis	III	—	—	—	5th Cra	2
cheresiensis	III	A2	H2	R2	Solera	2
cheresiensis	III	—	—	—	1st Cra	1
cheresiensis	III	A2	H2	R2	2nd Cra	1
cheresiensis	VIII	A2	H4	R4	Solera	1
cheresiensis	XXII	A2	H3	R3	5th Cra	1
cheresiensis	XXII	—	—	—	3rd Cra	1
cheresiensis	XXIV	A2	H3	R5	5th Cra	1

[a] blending stage
[b] Cra: 'criadera'

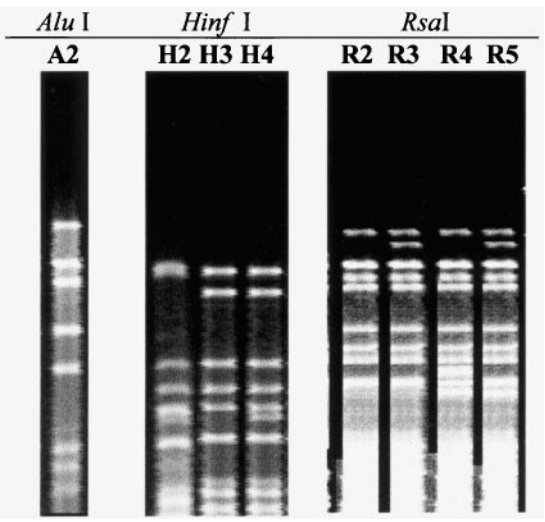

Fig. 3 Mitochondrial DNA restriction analysis of yeasts of flor velum isolated from different blending stages of sherry wines

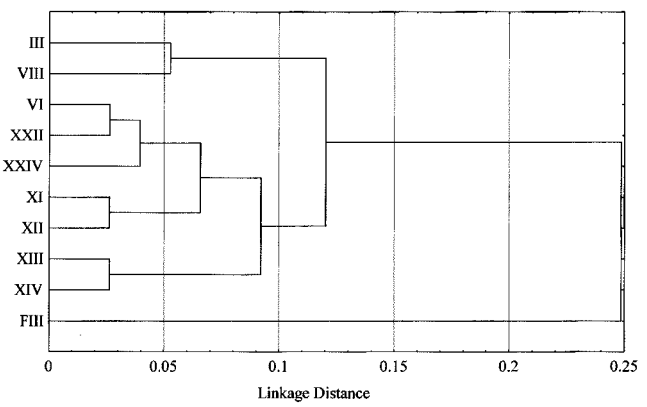

Fig. 4 Cluster analysis applied to the chromosomal profiles of *S. cerevisiae* strains isolated from flor velum during the biological aging of sherry wines. X axis: linkage distance. Y axis: chromosomal patterns. CT: pattern of the site markers. XI, XII, XIII and XIV: patterns of the flor velum controls (*beticus*, *cheresiensis*, *montuliensis* and *rouxii*, respectively). FIII: profile of commercial fermentative strains. III, VIII, XXII and XXIV: patterns of race *beticus* and *cheresiensis* of this study. VI: pattern of the minority strains, classified as *S. cerevisiae* strains

this study). These strains could also be differentiated from *beticus* and *cheresiensis* controls.

Mitochondrial DNA restriction analysis has been described as a practical method for monitoring *Saccharomyces* strains in wine fermentations (19). Mitochondria are especially relevant in flor velum yeasts because they accommodate all of the enzymatic systems necessary for the oxidative metabolism that differentiates these yeasts from the fermentative ones. There are some respiratory mutants that generate a phenotype called 'petite', due to the small size of the colonies developed, which can not develop normally because they carry some deletions in their mtDNA (29). The polymorphism of mitochondrial DNA has been attributed to forces selecting a combination resistant to ethanol but conversely, to the mutagenic effects of the ethanol upon the mitochondrial genome (30). RFLPs of mitochondrial DNA of 19 strains with the enzymes *Alu* I, *Hinf* I and *Rsa* I yielded one, three and four different patterns respectively indicating a high degree of polymorphism (**Fig. 3**).

Restriction enzyme *Alu* I, which only produced one

pattern, can not discriminate between strains; *Hinf* I originated three different patterns, although they were common to the races *beticus* and *cheresiensis*. However, *Rsa* I not only produced a higher number of different patterns, but also the pattern R3 that was specific to *cheresiensis*.

Although mitochondrial and nuclear gene products are closely related, some characteristics like size, kind of inheritance, sensitivity to ethanol, and so on, mean that the two DNAs differ in terms of genetic variability (7). In our data, only six out of 12 possible combined patterns that would result from combining mitochondrial and karyotype profiles were shown. The most frequent association was III, A2, H2, R2, which appeared in both races analyzed, *beticus* and *cheresiensis*. However, another two combinations were present in *beticus*, one of which was also present in *cheresiensis*, and another three combinations in *cheresiensis* (**Table 4**). Results suggested that the different strains identified are very closely related, since they may share either pattern in their karyotype or mtDNA restriction analysis. Pattern XXIV is specific to *cheresiensis* and yielded a mitochondrial profile common to both strains. However, combined patterns were not random and some preferential associations could be distinguished. More specifically, pattern H2 was always present with R2, and H3 was associated with R5 in both races but only with R3 in *cheresiensis*. Finally, only H4 was associated with R4 in both races. In relation to the distribution of patterns during the biological ageing the trend described previously for karyotypes was confirmed: it was found that the combination H4 R4 only appeared in the 'solera', whilst H3 R3 and H3 R5 were characteristics of the youngest blending stages (4th and 5th 'criaderas'), and H2 R2 appeared both in the youngest and in the 'soleras' of both strains.

The use of high-resolution techniques is of great interest not only for the characterization of yeasts, but also because they enable the monitoring of strains during the process of biological ageing, for detecting the presence of contaminants or for following the dynamics of the yeast population. At the same time, a detailed molecular knowledge of the strains would facilitate the identification of relationships between the organoleptic characteristics of particular sherries and their yeast properties. Such relationships may not be obvious by other means, but are important for maintaining and improving quality control and production methods.

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