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Stabilization process in *Saccharomyces* intra- and interspecific hybrids in fermentative conditions

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Summary. We evaluated the genetic stabilization of artificial intra- (*Saccharomyces cerevisiae*) and interspecific (*S. cerevisiae* × *S. kudriavzevii*) hybrids under wine fermentative conditions. Large-scale transitions in genome size and genome reorganizations were observed during this process. Interspecific hybrids seem to need fewer generations to reach genetic stability than intraspecific hybrids. The largest number of molecular patterns recovered among the derived clones was observed for intraspecific hybrids, particularly for those obtained by rare-mating. Molecular marker analyses revealed that unstable clones could change during the industrial process to obtain active dry yeast. When no changes in molecular markers and ploidy were observed after this process, no changes in genetic composition were confirmed by comparative genome hybridization, considering the clone as a stable hybrid. According to our results, under these conditions, fermentation steps 3 and 5 (30–50 generations) would suffice to obtain genetically stable interspecific and intraspecific hybrids, respectively. [**Int Microbiol** 2014; 17(4):213-224]

Keywords: *Saccharomyces cerevisiae* · *Saccharomyces kudriavzevii* · rare-mating in yeast · molecular markers · DNA content evaluation · stabilization of genomes

Introduction

The detection of "natural" *Saccharomyces* hybrid strains in different fermentations [22,29,35], and among the starter cultures used for wine inoculation [9,22,23,33], led to pay attention to the relevance of hybrids in these processes. These hybrids contain an almost complete set of chromosomes from partners in the form of allodiploid or allotetraploid genomes or only portions of the partner's genomes resulting in alloaneuploids, or strains with chimerical chromosomes [5,17,45,48]. The physiological advantage of hybrids has been proposed to be related to their better fitness than parental strains under intermediate or fluctuating conditions [44]. For this reason, the artificial generation of hybrids has become an interesting strategy in recent years to improve industrial yeast strains. Construction of hybrids in the *Saccharomyces* genus has been reported between wine strains of *Saccharomyces uvarum* and various strains of *Saccharomyces cerevisiae* (for a review, see [48]). The artificial hybrids between *S. cerevisiae* and other *Saccharomyces* species, including *S. paradoxus* and *S. kudriavzevii*, have also been reported [6,8,39]. Different procedures, including protoplast fusion, mass-mating, spore-to-

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spore mating and rare-mating, have been used for hybrids generation [48]. However, only those methods occurring naturally by mating or natural recombination can be used for the generation of non-genetically modified organisms (non-GMO), in accordance with Directive 2001/18/EC of the European Parliament and the European Council. Protoplast fusion is thus excluded from the group of non-GMOs-producing techniques [11].

Commercialized wine strains have been selected because of their fermentation qualities and stress adaptability during alcoholic fermentation, and also because these strains ensure the production of consistent wines in successive vintages [42]. This means that the strains developed for industrial processes must have stable genomes. In a previous work carried out in our laboratory, different inter- and intraspecific hybrid strains were obtained by employing several hybridization methodologies [39]. However, we observed that instable hybrids showing high DNA content were generally obtained. In other works, polyploid genomes were known to be unstable in *S. cerevisiae* [20,50] or in hybrids of the *Saccharomyces* genus [2,26,48]. Similarly, many newly formed polyploids in plants have unstable genomes that undergo rapid repatterning during the first generations, which is particularly important for allopolyploids [49,52]. Because of this trend to the reorganization of the genome and the genetic heterogeneity of the new hybrids [26], the development of a method to ensure proper genetic stability of strains used in industrial applications was necessary.

Wine yeast should be adapted to several stress conditions, such as low pH and high sugar content of grape must. The selectivity of fermenting must be further strengthened once anaerobic conditions are established; certain nutrients become depleted and the ethanol level increases [42]. During the process of active dry yeast (ADY) production and the posterior rehidratation, yeast cells are exposed to stressing conditions, such as osmotic, oxidative and thermic stress, and starvation [3,4,14,36,38]. All these stresses exert a strong selective pressure on the microorganisms and could induce changes in unstable genomes. Loss of the type (i.e., parental origin) and content of DNA in the genetic stabilization process during hybrids formation can strongly influence future physiological features and the adaptation of a hybrid to industrial processes. Several examples correlate the influence of genome size differences with phenotypic variations, including cell size [31], generation time [41], and ecological tolerance [19]. Genomic changes such as insertions, deletions and translocations have also been related to yeasts adapting to novel environments [7,16,19].Variations in the number of gene copies occurring in polyploids or aneuploids have also been associated with altered gene expression patterns and metabolic activity [18,51]. Genome reduction and rearrangements occurring during the stabilization process might lead to loss of industrially important traits in hybrids, and can be avoided if a selective pressure, mimicking the desired industrial process, is applied during the stabilization. Understanding the stabilization process can help us to design the experimental conditions to develop a new lab-made hybrid for industrial purposes.This work aimed to validate a fast genetic stabilization method for newly generated *Saccharomyces* hybrids under selective enological conditions, to know how many rounds (or generations) suffice to obtain stable hybrids and to study the changes during the process. The whole stabilization processes in intra- and interspecific hybrids showing different ploidy levels, as a result of using different hybridization methodologies, were also compared. As far as we know, this is the first work that deeply studies the stabilization procedure under enological conditions.

Materials and methods

Yeast strains. Four interspecific *Saccharomyces cerevisiae* × *Saccharomyces kudriavzevii* hybrids, two obtained from rare-mating (R2 and R8) and two from spore-to-spore mating (S2 and S7), and four intraspecific *S. cerevisiae* hybrids, two obtained from rare-mating (R1 and R3) and two from spore-to-spore mating (S5 and S8) were selected from a previous work [39] to undergo a genetic stabilization procedure (see hybrid and parental characterization in Table 1).

Genetic stabilization procedure. A single colony of each hybrid strain was individually inoculated into 15-ml screw-cap tubes containing 10 ml of synthetic must [46] with 50% glucose and 50% fructose, sterilized by filtration. The samples were incubated at 20ºC without shaking. After fermentation (approximately 15–20 days), an aliquot of approximately 107 cells was used to inoculate a new tube containing the same sterile medium (synthetic must) and was incubated under the same conditions, while a second aliquot was seeded on glucose-peptone-yeast agar (GPY-agar) plates and incubated at 20°C. Ten yeast colonies were randomly picked and characterized by inter-δ sequences, random amplified polymorphic DNA–PCR (RAPD–PCR) analyses and mtDNA-restriction fragment length polymorphism (mtDNA-RFLP) patterns. The total DNA content was also measured for each colony showing a different molecular pattern.

All the yeast colonies displaying different molecular profiles, regardless the fermentation step at which they were obtained, were individually inoculated in the same synthetic must and, after these individual fermentations, ten colonies from each one were analyzed by the same methods. When one pattern was recovered more than once, we selected this pattern for the last round in which it appeared. We put the original pattern, selected in the fifth round, in an individual fermentation too. We considered that a clone was genetically stable when the colonies recovered after individual fermentations maintained the same molecular profile (δ elements, RAPD–PCR and mtDNA-RFLP patterns) and the same ploidy level as the previously inoculated culture.

Table 1. Molecular and genetic characterization of hybrids and parental strains used in this study (extracted from [39])

			Molecular patterns[¶]			
Cross	Methodology	Name	mtDNA	δ-PCR	RAPD-R3	DNA content[§]
Parental strains		Sc1	Sc1	δ-Sc1	R3-Sc1	2.7 ± 0.2 [a-c]
		Sc2	Sc2	δ-Sc2	R3-Sc2	2.5 ± 0.3 [a]
		Sk	Sk	δ-Sk	R3-Sk	2.2 ± 0.1 [a]
Sc1xSc2	Rare-mating	R2	Sc2	δ-5	R3-8	5.0 ± 0.1 [j]
		R8	Sc1	δ-4a[&]	R3-7	4.7 ± 0.3 [ij]
	Spore-to-spore mating	S2	Sc2	δ-10	R3-9	2.7 ± 0.1 [a-d]
		S7	Sc1	δ-14	R3-10	2.8 ± 0.2 [a-e]
Sc1xSk	Rare-mating	R1	Sk	δ-4b*	R3-2	3.2 ± 0.2 [a-e]
		R3	Sc1	δ-4b*	R3-4	4.8 ± 0.1 [i-l]
	Spore to spore mating	S5	Sk	δ-9	R3-11	3.4 ± 0.1 [c-f]
		S8	Sc1	δ-8	R3-12	3.2 ± 0.2 [a-d]

[¶]Molecular patterns obtained by mtDNA-RFLP (mtDNA), interdelta sequence DNA polymorphisms (δ-PCR) and RAPD analysis using primer R3 (RAPD-R3).
[§]Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 2$).
[&]Patterns δ4 in Pérez-Través et al. [39], both of them are different.

Random amplified polymorphic DNA (RAPD–PCR) analysis. Primer R3 (5´-ATGCAGCCAC-3´) was used for the RAPD–PCR analysis. This primer showed the highest degree of variability among the hybrid strains—including those analyzed in this work—of the 11 primers described in a previous work [39].The patterns obtained from the RAPD–PCR analysis were codified with lowercase letters.

Amplified elements DNA polymorphism analysis. Primers delta 12 (5´-TCAACAATGGAATCCCAAC-3´) and delta 21 (5´-CATCTA ACACCGTATATGA-3´), as well as the procedures proposed by Legras and Karst [27], were used to amplify yeast genomic DNA. The patterns obtained from the δ elements analysis were codified with Roman numerals.

Mitochondrial DNA-restriction fragment length polymorphism (mtDNA-RFLP) analysis. A mitochondrial DNA restriction analysis was performed by the method of Querol et al. [43] using the endonuclease *Hin*fI (Roche Molecular Biochemicals, Mannheim, Germany). The patterns obtained from the mtDNA-RFLP analysis were codified using capital letters. Irrespective of the molecular marker used, pattern "o" corresponds to the original pattern found in the hybrid prior to the stabilization process.

DNA content evaluation. The DNA content of both hybrid and control strains was assessed by flow cytometry using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, CA, USA) following the methodology described in Lopes et al. [30]. Previously, yeast cells had been grown in GPY during 24 h until stationary phase. DNA content values were scored on the basis of fluorescence intensity compared with haploid (S288c) and diploid (FY1679) reference strains. The value reported for each strain was the result of three independent measures. The results were tested by one-way ANOVA and a Tukey HSD test ($\alpha = 0.05$, $n = 2$).

Active dry yeast (ADY) production. Industrial cultivation and drying were performed according to the Laboratory of Research and Development standard protocols (Lallemand Inc. protocols; Lallemand S.A.S., Montreal, Canada) (not provided). A rehydration step, previous to the use of these yeasts in winemaking, is needed.

Comparative genome hybridization analysis. Array competitive genomic hybridization (aCGH) was performed using a hybrid clone before and after processing as ADY by following the methodology described in Peris et al. [40]. Experiments were carried out in duplicate and the Cy5-dCTP and Cy3-dCTP dye-swap assays were done to reduce the dye-specific bias. Microarray scanning was carried out using a GenePix Personal 4100A scanner (Axon Instruments/Molecular Devices, USA). Microarray images and raw data were produced using the GenePix Pro 6.1 software (Axon Instruments/Molecular Devices) and the background was subtracted by applying the local feature background median option. M-A plots ($M = \log_2$ ratios; $A = \log_2$ of the product of intensities) were represented in order to evaluate if the ratio data were intensity-dependent. The normalization process and filtering were done with Acuity 4.0 (Axon Instruments/Molecular Devices Corp.). Raw data were normalized by the ratio-based option. Features with artifacts or flagged as bad were removed from the analysis. Replicates were averaged after filtering. The data from this study are available from GEO [http://www.ncbi.nlm.nih.gov/geo/]; the accession number is GSE46192.

Natural must fermentation, HPLC analysis of wines and kinetic analysis. The must employed was Albariño. Fermentable sugars were measured using the HPLC (see below), that gave a value of 213.96 g/l. Yeast assimilable nitrogen was determined by the ammonia assay kit (Boehringer Mannheim,Mannehim, Germany), for the inorganic nitrogen (40% of the total nitrogen amount) and nitrogen content was adjusted to a total of

220 mg/l by addition of a nitrogen supplement consisting in NH_4Cl. Prior to the fermentation, dimethyl dicarbamate (DMDC) at 1 ml/l was added for sterilization purposes. Fermentations were carried out in 100-ml bottles containing 80 ml of Albariño must. Must was inoculated independently with the different yeast strains to reach an initial population of 2×10^6 CFU/ml, and maintained at 22°C. Flasks were closed with Müller valves and monitored by weight loss until a constant weight was obtained. Immediately after the end of fermentation, yeast cells were removed by centrifugation and the supernatants analyzed immediately or stored at –20ºC until use. Each fermentation method was carried out by duplicate.

Supernatants were analyzed by HPLC in order to determine the amounts of residual sugars (glucose and fructose), glycerol, and ethanol. A Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a refraction index detector was used. The column was a HyperREZTM XP Carbohydrate H+ 8µm (Thermo Fisher Scientific) which was protected by a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). The conditions used in the analysis were as follows: eluent, 1.5 mM H_2SO_4; flux, 0.6 ml/min; and oven temperature, 50°C. Samples were diluted 5-fold, filtered through a 0.22-µm nylon filter (Symta, Madrid, España) and injected by duplicate.

Before curve fitting, weight loss data were corrected to % of consumed sugar according to the formula:

$$C=\{(m*[S-R])/(mf*S)\}*100$$

were C is the % of sugar consumed at each sample time, m is the weight loss value at this sampling time, S is the sugar concentration in the must at the beginning of experiment (g/l), R is the final sugar concentration in the fermented must (residual sugar, g/l) and mf is the total weight loss value at the end of the fermentation (g).

Curve fitting was carried out using the reparametrized Gompertz equation proposed by Zwietering et al. [53]:

$$y = D* \exp\{-\exp[((\mu max *e)/D)*(\lambda - t)+ 1]\}$$

where y is the % of consumed sugar; D is the maximum sugar consumption value reached (the asymptotic maximum, %); µmax is the maximum sugar consumption rate (h^{-1}), and λ is the lag phase period during which sugar consumption was not observed (h). Data were fitted using the nonlinear regression module of Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), minimizing the sum of squares of the difference between experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to experimental data.

Kinetic parameters and HPLC data were analyzed using Statistica 7.0 software package (StatSoft) by one way ANOVA and Tukey test for means comparison.

Results

Significant differences were observed not only in the stabilization process of the intraspecific (*Saccharomyces cerevisiae* × *S. cerevisiae*) and interspecific (*S. cerevisiae* × *S. kudriavzevii*) hybrids, but also in the stabilization of those strains obtained by different procedures (rare-mating and spore-to-spore mating).

Stabilization of intraspecific hybrids. Different δ elements and RAPD–PCR patterns were detected in the colonies isolated during the successive fermentations inoculated with each particular hybrid strain. Table 2 provides the frequencies in which each particular combined δ elements-RAPD–PCR-mtDNA RFLP pattern appeared.

The genetic variability observed during the stabilization of hybrids generated by rare-mating (R2 and R8) was higher than that obtained by spore-to-spore mating (S2 and S7) for both nuclear and mitochondrial genomes. Six new δ elements patterns were found among the colonies derived from hybrid R2 (patterns I to VI), and eight patterns were obtained among the colonies derived from R8 (patterns I to VIII). Apart from the aforementioned patterns, the δ elements patterns exhibited by the original unstable hybrids R2 and R8 were recovered in the derived colonies isolated from all the successive fermentation steps (Table 2).

Low variations were detected among derived colonies by the RAPD–PCR method using primer R3. Only one different pattern was observed in one colony obtained in fermentation step 4 of hybrid R2 (named pattern a) and two (named patterns a and b) were obtained in the colonies derived from hybrid R8 after fermentation steps 4 and 5 (Table 2).

No variations in RAPD–PCR patterns were detected among the colonies isolated during the five successive fermentation steps inoculated with hybrids S2 and S7 generated by spore-to-spore mating. Only two δ elements patterns, which differed from that present in the original hybrid, were detected during the stabilization of S2 (patterns I and II) (Table 2). Variations in the mtDNA-RFLP patterns were detected only during the stabilization of hybrid R8 obtained after rare-mating. Five different mtDNA-RFLP patterns were identified during the process.

Individual colonies (clones), representative of each hybrid and molecular pattern detected after the complete set of consecutive fermentations, were used to inoculate fresh synthetic must in order to confirm their genetic stability. Of those colonies showing a same molecular pattern, only those from the last fermentation steps were evaluated individually (i.e., the R2ooo "original pattern" was taken from the fifth fermentation, R2Ioo, R2IIIao, R2IVoo and R2Voo from the fourth, and R2IIoo, R2IIIoo and R2VIoo from the fifth). We followed the same methodology used during the stabilization process: after fermentation, ten colonies were isolated and molecularly characterized. As a result of this evaluation, most clones conserved the same molecular patterns as before, except for clones R2Voo, R8ooA, R8ooB, R8ooC, R8IoB and R8IoD and the original R2 and R8 (data not shown).

In order to evaluate if the changes detected between the molecular markers were also coincident with the changes in

Table 2. Molecular characterization of yeast colonies after successive fermentation steps of intraspecific hybrids and frequency

Hybridization method	Hybrid[a]	Molecular patterns and frequency (%)[b]																							
		Original[c]				1st step				2nd step				3rd step				4th step				5th step			
		δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%
Rare-mating	R2	δ–5 (o)	R–8 (o)	Sc2(o)	100	o	o	o	90	o	o	o	100	o	o	o	90	o	o	o	30	o	o	o	20
		–	–	–	–	I	o	o	10	–	–	–	–	–	–	–	–	I	o	o	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	II	o	o	10	II	o	o	20	II	o	o	60
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	III	o	o	10	III	o	o	10
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	III	a	o	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	IV	o	o	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	o	o	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	VI	o	o	10
	R8	δ–4 (o)	R–7 (o)	Sc1(o)	100	o	o	o	20	o	o	o	40	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	o	o	A	40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	o	o	B	30	o	o	B	60	o	o	B	90	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	o	o	C	10	o	o	C	40
		–	–	–	–	I	o	B	10	–	–	–	–	I	o	B	10	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	I	o	D	30	I	o	D	20
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	II	o	E	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	II	a	E	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	III	o	E	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	IV	o	D	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	o	E	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	b	C	10
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	VI	o	C	10	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	VII	o	E	10
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	VIII	o	E	20
Spore to spore mating	S2	δ–10 (o)	R–9 (o)	Sc2(o)	100	o	o	o	100	o	o	o	90	o	o	o	100	o	o	o	90	o	o	o	100
		–	–	–	–	–	–	–	–	I	o	o	10	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	II	o	o	10	–	–	–	–
	S7	δ–14 (o)	R–10 (o)	Sc1(o)	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100

[a]Hybrid names R2, R8, S2 and S7 correspond to intraspecific hybrids in Pérez–Través et al. [39].

[b]δ: patterns obtained by δ elements characterization (identified with roman numbers, patterns exhibited by the original hybrids were designed as "o"); R3: patterns obtained by RAPD–PCR with primer R3 (identified with lowercase letters, patterns exhibited by the original hybrids was designed as "o"). mt: patterns obtained by mtDNA–RFLP analysis (identified with capital letters, patterns exhibited by the original hybrids were designed as "o"). %: percentage of detection of a particular combination of δ elements and RAPD–PCR patterns after a particular fermentation step.

[c]Molecular patterns characterized by Pérez-Través et al. [39]. These patterns were identified as "original patterns (o)" in this work.

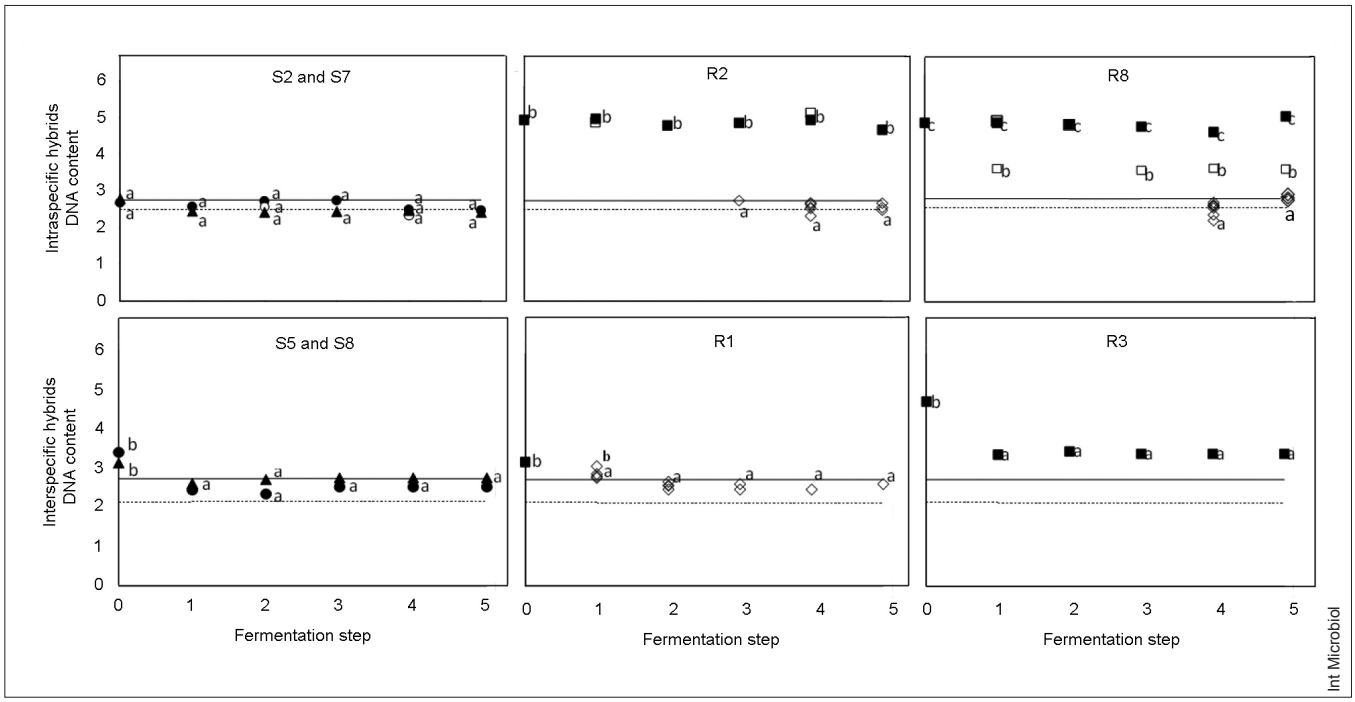

Fig. 1. Changes in DNA content of hybrid cultures during stabilization process of intraspecific (spore-to-spore hybrids S2 and S7, and rare-mating hybrids R2 and R8) and interspecific (spore-to-spore hybrids S5 and S8, and rare-mating hybrids R1 and R3) hybrids. Circles: spore-to-spore hybrids S2 (intraspecific) and S5 (interspecific). Triangles: spore-to-spore hybrids S7 (intraspecific) and S8 (interspecific). Squares: Rare-mating hybrids. Diamonds: stable rare-mating hybrids. Solid line indicate the ploidy value showed by the parental Sc1. Dotted line indicate the ploidy value showed by the parental Sc2 (intraspecific hybrids stabilization) and parent Sk (interspecific hybrids stabilization). Filled symbols indicate cultures with the same molecular pattern found in the original hybrid inoculated in the first fermentation step. Empty symbols indicate cultures with molecular patterns different from the original. Symbols with different letters among cultures derived from a same original hybrid, indicate statistically significant differences (ANOVA and HSD Tukey test, $\alpha = 0.05$).

total DNA content, the clones having each different molecular pattern were subjected to measuring DNA content by flow cytometry (see supplementary Table 1, ST1; it can be requested to authors). Figure 1 shows the evolution in the total DNA content values obtained for all analyzed clones derived from each original hybrid strain during the stabilization process. After this analysis, we observed that all the clones obtained after the consecutive fermentation steps of the spore-to-spore-generated hybrids conserved the same ploidy values found in original hybrids S2 and S7, including those showing different δ elements patterns (Fig. 1, ST1).

Among the clones derived from rare-mating-generated hybrids R2 and R8, the DNA content values varied from 5n (n being the DNA content of a haploid laboratory strain) in the original inoculated hybrids to approximately 2.5n in the clones (Fig. 1, ST1). Most of the clones derived from original hybrid R2 (obtained from fermentations steps 3, 4 and 5) had significantly different DNA content values from the value obtained in the original hybrid (close to 2.5n). An exception was observed for clone R2Ioo and clone R2Voo from fermentation steps 1 and 4, respectively, whose values came close to 5n (Fig. 1, ST1). Finally, all the clones isolated from the different fermentation steps, but showing the original molecular pattern, also conserved the same ploidy value of around 5n (Fig. 1, ST1). Three different situations were observed for the ploidy values shown by the clones derived from original hybrid R8. All the clones having an original molecular pattern in the nuclear genome (R8ooo, R8ooA, R8ooB and R8ooC) conserved high ploidy values ranging from 4.5n to 5n (Fig. 1, ST1). The DNA content of clones R8IoB and R8IoD, bearing δ elements pattern I, which emerged in fermentation step 1, was near 3.5n. The remaining clones, isolated from fermentations 4 and 5, exhibited ploidy values which came close to 2.5n (Fig. 1, ST1).

The DNA content analysis carried out in the colonies obtained after individual clone fermentation revealed high ploidy variability among the colonies derived from the clones with high DNA contents (R2ooo, R2Voo, R8ooo, R8ooA, R8ooB, R8ooC, R8IoB and R8IoD). In their δ pattern, R8IoB and R8IoD also changed. The clones whose DNA content came close to 2.5n maintained

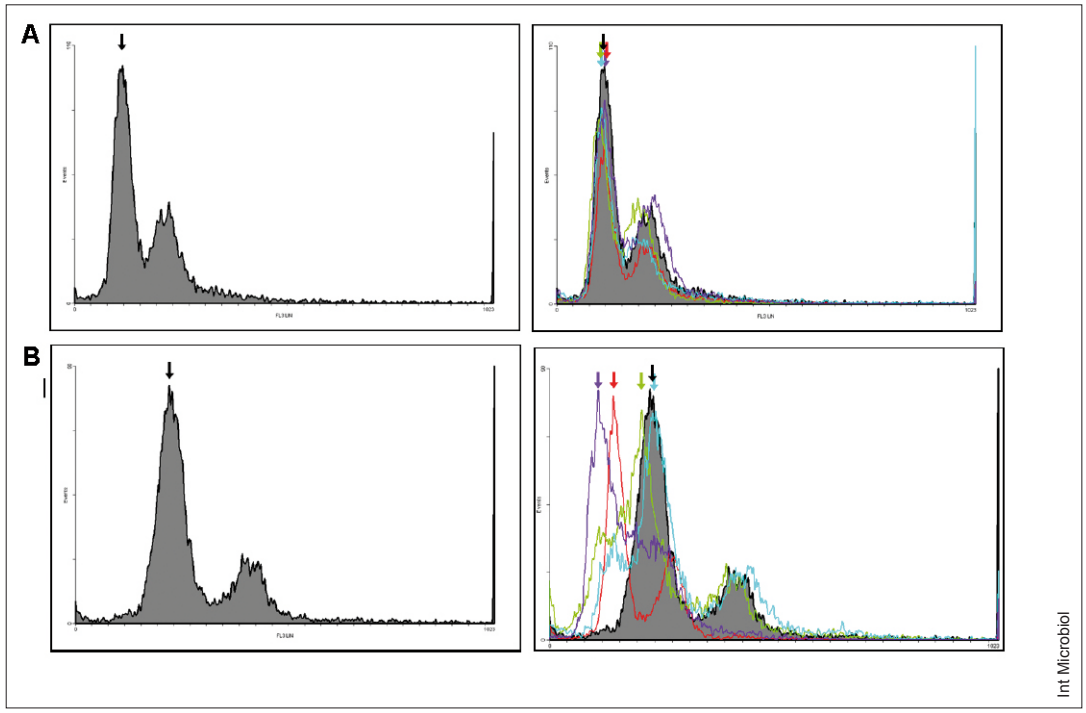

Fig. 2. Analysis of total DNA content (as fluorescence intensity) carried out by flow cytometry in the stable hybrid R2IIIo (**A**) and in the instable hybrid R2Vo (**B**) before (left) and after (right) individual inoculation of synthetic must. Shadowed areas indicate the total DNA content of the cultures before inoculation. Lines in color indicate fluorescence intensity of colonies recovered after individual fermentations. Arrows indicate picks considered for DNA content determinations.

the same values after individual fermentation. An example about the variation or the maintenance in the ploidy levels after individual clone fermentation is shown in Fig. 2.

Stabilization of interspecific hybrids. For interspecific hybrid R3 (generated by rare-mating), and S5 and S8 (generated by spore-to-spore mating), all the clones obtained during the five fermentation steps showed the same molecular pattern at both the nuclear and mitochondrial levels, as detected in the original hybrid strains (data not shown). The stabilization process of hybrid R1 evidenced no variation in genomic DNA patterns, event though new mtDNA patterns appeared, particularly in early process stages (fermentation step1 and 2; Table 3). The emergence of new mtDNA patterns could indicate that rearrangements have occurred. One of the new patterns was present until the end of the stabilization assay. In all cases, significantly different ploidy values were observed between the originally inoculated hybrid strains and all the clones recovered after each fermentation step, irrespective of the hybridization method employed for hybrid generation (rare-mating or spore-to-spore mating). After the first step, clones maintained the same ploidy value until the end of the process (Fig. 1, Table 4). After inoculating fresh media with individual clones, no changes were observed in molecular patterns and ploidy levels (data not shown).

Stability evaluation after active dry yeast (ADY) production. A decision was made to evaluate if clones, obtained by the methodology proposed in this work, maintained their genetic stability after undergoing the ADY production process (Lallemand Inc. protocols). For this purpose, stable intraspecific hybrid clones were selected to undergo the ADY preparation process. These clones were selected because intraspecific hybrids were more variable during the stabilization process than interspecific ones. Furthermore, our approach based on employing an *S. cerevisiae*-based microarray is not useful for detecting genes from *S. kudriavzevii,* which greatly diverge with *S. cerevisiae*.

Stabilized clones R2IVoo and R8IIaE were used for ADY production under the habitual conditions (Lallemand Inc. protocols). After the process, the produced ADY samples were rehydrated and seeded in the complete medium. Ten colonies of each sample, obtained after incubation, were evaluated by the same genetic markers and ploidy previously employed

Table 3. Molecular characterization of yeast colonies after successive fermentation steps of interspecific hybrids and frequency

Hybridization method	Hybrid[a]	Molecular patterns and frequency (%)[b]																							
		Original[c]				1st step				2nd step				3rd step				4th step				5th step			
		δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%	δ	R3	mt	%
Rare–mating	R1	δ–4 (o)	R–2 (o)	Sk (o)	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	o	o	A	20	o	o	A	40	o	o	A	20	o	o	A	100	o	o	A	100
		–	–	–	–	o	o	B	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	o	o	C	40	o	o	C	20	o	o	C	80	–	–	–	–	–	–	–	–
		–	–	–	–	o	o	D	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	o	o	E	20	–	–	–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–	o	o	F	20	–	–	–	–	–	–	–	–	–	–	–	–
	R3	δ–4 (o)	R–4 (o)	Sc1(o)	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100
Spore-to-spore mating	S5	δ–9 (o)	R–11 (o)	Sk (o)	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100
	S8	δ–8 (o)	R–12 (o)	Sc1(o)	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100	o	o	o	100

[a]Hybrid names R1, R3, S5 and S8 correspond to interspecific hybrids in Pérez–Través et al. [39].

[b]δ: patterns obtained by δ elements characterization (identified with roman numbers, patterns exhibited by the original hybrids were designed as "o"); R3: patterns obtained by RAPD–PCR with primer R3 (identified with lowercase letters, patterns exhibited by the original hybrids was designed as "o"). mt: patterns obtained by mtDNA–RFLP analysis (identified with capital letters, patterns exhibited by the original hybrids were designed as "o"). %: percentage of detection of a particular combination of δ elements and RAPD–PCR patterns after a particular fermentation step.

[c]Molecular patterns characterized by Pérez-Través et al. [39]. These patterns were identified as "original patterns (o)" in this work.

Table 4. DNA content of hybrids showing different combined molecular patterns during the whole process of interspecific hybrids stabilization

Original hybrid[$]	DNA content[&]	1st step		2nd step		3rd step		4th step		5th step	
		Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]	Pattern[#]	DNA content[&]
R1	3.2 ± 0.2[b]	ooA	2.8 ± 0.2[a]	ooA	2.7 ± 0.1[a]	ooA	2.65 ± 0.13[a]	ooA	2.50± 0.09[a]	ooA	2.64 ± 0.03[a]
		ooB	3.1 ± 0.1[b]	–	–	–	–	–	–	–	–
		ooC	2.8 ± 0.3[a]	ooC	2.6 ± 0.1[a]	ooC	2.52 ± 0.22[a]	–	–	–	–
		ooD	2.9 ± 0.2[a]	–	–	–	–	–	–	–	–
		–	–	ooE	2.5 ± 0.2[a]	–	–	–	–	–	–
		–	–	ooF	2.6 ± 0.2[a]	–	–	–	–	–	–
R3	4.8 ± 0,1[b]	ooo	3.4 ± 0.1[a]	ooo	3.5 ± 0.1[a]	ooo	3.4 ± 0.3[a]	ooo	3.5 ± 0.1[a]	ooo	3.6 ± 0.1[a]
S5	3.4 ± 0,1[b]	ooo	2.5 ± 0.2[a]	ooo	2.4 ± 0.2[a]	ooo	2.5 ± 0.3[a]	ooo	2.5 ± 0.1[a]	ooo	2.4 ± 0.2[a]
S8	3.2 ± 0,2[b]	ooo	2.6 ± 0.2[a]	ooo	2.7 ± 0,1[a]	ooo	2.8 ± 0.2[a]	ooo	2.8 ± 0,3[a]	ooo	2.7 ± 0.2[a]

[$]Hybrid names R1, R3, S5 and S8 correspond to interspecific hybrids in Pérez-Través et al. [39].

[&]Values expressed as mean ± standard deviation. Values not sharing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α = 0.05, n=2).

[#]Molecular patterns obtained by combination of interdelta, R3 and mtDNA-RFLP profiles.

All colonies were considered as stable when both molecular patterns and DNA content did not change after individual colony fermentation.

Table 5. Main kinetic parameters of the fermentations carried out with both parental and hybrid strains on Albariño must at 22°C and chemical analysis of the final fermented products

	Kinetic parameters[a]			Chemical parameters[a]			
Strain	K (h^{-1})[b]	l (h)	t_{95}(h)[c]	Glucose (g/l)[d]	Fructose (g/l)	Glycerol (g/l)	Ethanol (% v/v)
R2IVo	1.57 ± 0.02	19.38 ± 0.92	125.20 ± 1.20	bdl	1.09 ± 0.11	5.35 ± 0.06	11.81 ± 0.01
R2IVo LSA	1.54 ± 0.01	20.39 ± 0.50	125.97 ± 0.95	bdl	0.89 ± 0.02	5.38 ± 0.03	11.79 ± 0.03

[a]Values expressed as mean ± standard deviation. Values not sharing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α = 0.05, n = 2).
[b]K: kinetic constant.
[c]t_{95}: time necessary to consume 95% of fermented sugars.
[d]bdl: value below detection limit (0.05 g/l).

during stabilization. No changes were observed in the evaluated parameters of the obtained colonies in relation to the clone R2IVoo before the dryness process, otherwise their happened for the clone R8IIaE, which changed in its δ profile (data not shown).

Additionally, in order to ensure that no changes in genomic constitution—including variation in genes copy number—occurred during ADY production for R2IVoo clone, the rehydrated culture was compared at a single gene resolution with the same strain without being subjected to the dryness process. For this comparison, genomic DNA isolated from the clone before dryness and labelled with one fluorescent dye was mixed with the DNA from the colonies obtained after ADY production and rehydration, which was labelled with a different dye. This mixture was then co-hybridized in a *S. cerevisiae* DNA microarray (see Materials and methods).

Differences in the $\log_2$ of the Cy5/Cy3 signal ratio obtained for each open reading frame (ORF) probably indicated variations in the relative copy number of *S. cerevisiae* genes present in the hybrid strain before and after the dryness process. Log_2 ratios close to zero for a particular ORF indicated the presence of the same number of DNA copies in both genomes, while higher or lower $\log_2$ ratios than zero might indicate more or less copies, or even depleted genes (ORF deletions). Our results do not evidenced changes in the gene copy numbers between the two analyzed genomes, suggesting that no changes in the DNA composition of clone R2IVoo had occurred in the industrial dry yeast generation process (data not shown). Finally we decided to carry out a fermentation in natural must with the hybrid clone before and after ADY production. No differences were found in residual sugars content, glycerol and ethanol production, neither in parameters analysis (latency, maximum fermentation rate and time necessary to consume 95% of fermentable sugars) (Table 5).

Discussion

Interspecific hybrids have been isolated from different fermented beverages, including wine, cider and beer [45,48]. Even one of the most popular beverages, lager beer, is prepared by hybrid yeast *S. pastorianus* containing both the *S. cerevisiae* and *S. eubayanus* subgenomes [28]. In most cases, hybrids acquire interesting combinations of physiological features from parental strains, and prove to be promising tools for specific technological uses. For this reason, many artificial hybrid yeasts have been constructed in recent decades to improve different industrial processes such as winemaking [6,12,39], brewing [47] and bakery [25,47], and also for basic studies [13,34]. However, only a few works mention and evaluate the necessary genetic stabilization process occurring immediately after hybridization [2,6,26,39], an important aspect when the strains are going to be used in industrial processes, where the product homogeneity is desired because starters ensures the production of consistent products in successive vintages [42].

Genome reduction and rearrangements occurring during the stabilization of newly formed hybrids have been reported, and these processes seemed to be different in unstressed or in a salt-stressed media [19,20]. These phenomena might lead to loss of industrially important traits in hybrids, and could be avoided if a selective pressure, mimicking the desired industrial process, were applied during the stabilization. For this reason, a major aspect in the hybrids study is the careful selection of stabilization conditions.

In a previous work carried out in our laboratory [39], intraspecific *S. cerevisiae* × *S. cerevisiae* and interspecific *S. cerevisiae* × *S. kudriavzevii* hybrids were successfully obtained by means of different hybridization methods, which

included protoplast fusion, rare-mating and spore-to-spore mating. Here we present the changes observed in some interspecific and intraspecific hybrid strains generated in that previous work throughout the genetic stabilization process carried out in selective media (in this case, by successive fermentation steps in synthetic must). We compared the stabilization process in the inter- and intraspecific hybrids showing high ploidy values (resulting from the rare-mating of two parental strains close to diploidy) and the stabilization of hybrids close to diploidy (most hybrids resulted from spore-to-spore mating).

Flow cytometry identified large-scale (ploidy level) changes in genome size throughout the stabilization process in most hybrids. This reduction was significant, particularly for the hybrids generated by rare-mating, which originally had two diploid parental sets of chromosomes. Genome reduction in intraspecific rare-mating hybrids R2 and R8 seemed to occur drastically in fermentation steps 3 and 4 (Fig. 1), although an intermediate reduction occurred in hybrid R8 in fermentation step 1. According to the results obtained after fermentation with the individual R2 and R8 derived colonies, stable clones corresponded only to those having the same ploidy values found in parental strains Sc1 (2.7n) and Sc2 (2.3n).

The genome reduction in interspecific hybrids was faster than that observed for intraspecific ones. This reduction occurred in fermentation step1 (Fig. 1). All the colonies recovered in fermentation steps 2 to 5 had the same ploidy values. The ploidy values at which hybrids became stable were similar to the parental strains ploidy (in S5, S8 and R1) or to a higher one (R3). A similar genome reduction process has been also evidenced by Antunovics et al. [2] after the stabilization of *S. cerevisiae* × *S. uvarum* hybrids by successive sporulation events, and also by Marinoni et al. [32] after interspecific hybridization by mass-mating. In experimental evolution studies, Gerstein et al. [19] observed that the DNA content of triploid and tetraploid cultures of *S. cerevisiae* diminished. This reduction occurs in the first generations and all the clones show a tendency to stabilize, with ploidy values close to 2n (historical ploidy values, the ploidy shown by the original strain). The authors also observed that cultures maintain a higher ploidy under stress conditions.

Chromosomal instability in artificial polyploid *S. cerevisiae* strains has been previously observed by several authors [1,19,50], together with high mutation and recombination levels.

In this work, apart from a reduced ploidy, variation in nuclear (evidenced in new δ elements and RAPD–PCR profiles) and mitochondrial (evidenced in new mtDNA-RFLP patterns) genomes was observed during the stabilization process. All these changes resulted in a large number of clones derived from an individual hybrid. Thus, the stabilization process generated a genetic variability among the recovered colonies. These new molecular patterns were observed mainly during the stabilization of the intraspecific hybrids obtained by rare-mating, which evidenced the existence of extensive genetic rearrangements among genetically similar genomes. This phenomenon was not observed for interspecific hybrids, irrespective of the hybridization method used for their generation; only hybrid R1 showed mitochondrial genome variability after fermentation step 1, but only one pattern consecutively appeared until the end of the process (R1ooA). Contrarily to our results, Bellon et al. [6] have not detected changes in DNA molecular patterns in recently generated interspecific hybrid strains after 50 generations in the model medium and grape juice. However, those authors reported neither changes in ploidy values nor having monitoring these changes throughout the stabilization process.

To sum up, different situations emerged throughout the process after analyzing hybrids: (i) stabilization by gradual loss of genetic material with no detectable changes in nuclear or mitochondrial DNA patterns (interspecific hybrids R3, S5 and S8); (ii) stabilization after nuclear genetic rearrangements and ploidy reduction until historical values in parental strains (rare-mating intraspecific hybrids) with (R8) or without (R2) mitochondrial genome changes; (iii) stabilization after rapid loss of genetic material with no changes in genomic markers, but in the mtDNA-RFLP patterns (interspecific hybrid R1).

From our results, we could conclude that both nuclear and mitochondrial genomes could undergo changes during the stabilization process of newly generated intra- and interspecific hybrids in the genus *Saccharomyces*. Intraspecific hybrids seemed to require more generations to produce genetically stable cells, while interspecific hybrids underwent a faster stabilization process and were active mainly in early stages.

ADY production is an essential step to prepare a wine yeast starter culture, during which yeast is affected by a number of different stresses [3,4,14,21,36]. As changes in the ploidy level, genes copy number, and chromosomal rearrangements have been observed in *Saccharomyces* strains subjected to different stress [15,19,37] or culture conditions [10,16,24], we evaluated the genomic stability of two representative hybrids strains by molecular markers and ploidy analyses before and after ADY production. Two clones were selected, as representative of the set of hybrids obtained from intraspecific rare-mating, because the stabilization of such hybrids shows the highest variability in ploidy and molecular patterns. We observed no changes in DNA content of both

strains, but molecular patterns changed in one of them (R8I-IaE hybrid strain). We observed no large amplification or deletion in the genome of R2IVoo clone after the process, and no differences were found when we compared, in fermentation, the hybrid before and after ADY production. These results evidenced the success of both the stabilization protocol and the selection of stable hybrids proposed in this work. Our results suggest that molecular markers such as δ elements and mtDNA-RFLP patterns, as well as ploidy evaluation, would allow the quick assessment of the genotypic stability of recently generated inter- and intraspecific *Saccharomyces* hybrid strains, and that the evaluation of these parameters should be done before and after ADY production. According to our results, and by considering that a stable hybrid must maintain the same molecular pattern and the same ploidy level during successive cell divisions, we found that fermentation steps 3 and 5 (30 and 50 generations) sufficed to obtain genetically stable interspecific and intraspecific hybrids, respectively, regardless of the hybridization methodology used for their generation.

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