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Evaluation of different genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus for winemaking

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ABSTRACT

Several methods based on recombinant DNA techniques have been proposed for yeast strain improvement; however, the most relevant oenological traits depend on a multitude of loci, making these techniques difficult to apply. In this way, hybridization techniques involving two complete genomes became interesting. Natural hybrid strains between different *Saccharomyces* species have been detected in diverse fermented beverages including wine, cider and beer. These hybrids seem to be better adapted to fluctuating situations typically observed in fermentations due to the acquisition of particular physiological properties of both parental strains. In this work we evaluated the usefulness of three different hybridization methods: spore to spore mating, rare-mating and protoplast fusion for the generation of intra- and inter-specific stable hybrids, being the first report about the comparison of different methods to obtain artificial hybrids to be used in fermentations. Spore to spore mating is an easy but time-consuming method; hybrids generated with this technique could lack some of the industrially relevant traits present in the parental strains because of the segregation occurred during meiosis and spore generation prior to hybridization. Hybrids obtained by protoplast fusion get the complete information of both parents but they are currently considered as genetically modified organisms (GMOs). Finally, hybrids obtained by rare-mating are easily obtained by the optimized methodology described in this work, they originally contain a complete set of chromosomes of both parents and they are not considered as GMOs. Hybrids obtained by means of the three methodological approaches showed a high genetic variability; however, a loss of genetic material was detected in most of them. Based on these results, it became evident that a last crucial aspect to be considered in every hybridization program is the genetic stabilization of recently generated hybrids that guarantee its invariability during future industrial utilization. In this work, a wine yeast genetic stabilization process was developed and vegetatively stable hybrids were obtained.

1. Introduction

Wine fermentation has been traditionally performed by *Saccharomyces cerevisiae* strains naturally present on grapes and wine equipment or artificially inoculated as the form of a starter culture. Although hundreds of starter cultures are commercially available in the market, producers and consumers are continuously pressing for new improved yeast strains able to produce distinctive and specific products (Cebollero et al., 2007; Pretorius, 2000).

The availability of natural yeast strains possessing an ideal combination of desirable industrial characteristics, according to the actual requirements in the market, is highly improbable. Additionally, the most important oenological traits such as fermentative vigor, ethanol production and tolerance, volatile acidity production and growth temperature profile among others, depend on a multitude of loci (QTLs) distributed throughout the genome and their unpredictable interactions (Giudici et al., 2005; Marullo et al., 2004). These facts, as well as the presence of a large number of allelic variants, the high heterozygosity degree and the presence of aneuploidies or polyploidies in wine yeasts (Barre et al., 1993; Codón and Benítez, 1995; Naumov et al., 2000), make whole-genomics blind approaches the most adequate methodologies to be used in the generation of new industrial strains (Giudici et al., 2005). In this context, hybridization of two complete genomes is one of the best methods to be taken into account. Hybridization among closely related species of *Saccharomyces* has been proposed as a natural mechanism involved in the adaptation of these yeasts to industrial processes (Barrio et al., 2006; Querol and Bond, 2009). A post-zygotic barrier usually prevents the production of viable spores; however, stable hybrids are generated among different species of this genus. Natural hybrids have been found in different

Abbreviations: P, protoplast fusion; R, rare-mating; S, spore to spore mating.

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fermentation processes (Masneuf et al., 1998; Groth et al., 1999; González et al., 2006), being the most studied examples *Saccharomyces pastorianus* (Vaughan-Martini and Kurtzman, 1985) and the type strain of *Saccharomyces bayanus* (Masneuf et al., 1998; Nguyen et al., 2000).

As a general rule, hybrids are better adapted to intermediate or fluctuating situations due to the acquisition of physiological properties of both parental strains (Belloch et al., 2008; González et al., 2006). In this way, several research laboratories in the world have made use of diverse classic hybridization methods including spores to spores or spores to haploid cells mating, rare-mating or protoplasts fusion to generate hybrid strains with desirable features for different industrial processes or basic studies (Sipiczki, 2008 and Table 1S).

The selection of the hybridization method to be used will be closely related with the final destination of the generated hybrid. The use of genetically modified organisms (GMOs) in food is limited by current legislations in different countries, as well as by public concern (Cebollero et al., 2007; Pretorius and Hoj, 2005; Schilter and Constable, 2002). As it was established in the Directive 2001/18/EC of the European Parliament and the Council of the European Union, a GMO is an organism whose genetic material has been altered in a way that does not occur naturally by mating or natural recombination. According to this definition, hybrids generated by mating of spores and rare-mating—based on the natural rare event of mating type switching in industrial yeasts—must not be considered as GMOs. Contrarily, protoplast fusion is an artificial hybridization method based in the fusion of yeast cells, previously subjected to enzymatic cell wall degradation, in the presence of a fussing agent as polyethylene-glycol (Curran and Bugeja, 1996). Consequently, hybrids generated by protoplast fusion are considered as GMOs according to the European Union legislations and, for that reason, its use has been generally limited to basic studies or industrial processes not involving foods (Kucsera et al., 1998; Law et al., 1993; Nakazawa and Iwano, 2004). On the other hand, hybrids obtained by spore to spore mating could lack some of the industrially relevant traits present in the parental strains because of the segregation occurred during meiosis and spores generation prior to hybridization (Caridi et al., 2002; Gimeno-Alcañiz and Matallana, 2001; Marullo et al., 2004).

In this work we evaluated the usefulness of two different non GMO producing hybridization methods (spore to spore mating and rare-mating) for intra and interspecific wine yeast hybrids generation. The traditional technique of protoplast fusion was also done with comparative purposes. Finally, a genetic stabilization procedure was proposed and inter-specific vegetatively stable hybrids were obtained.

2. Materials and methods

2.1. Yeasts

Two selected *S. cerevisiae* commercial strains provided by Lallemand Inc. (*Sc1* and *Sc2*) and the type strain of *Saccharomyces kudriavzevii* NBRC 1802 (*Sk*) were used in the present study. *Sc1* parental was selected for its high polysaccharide release capacity inducing color stability. *Sc2* was selected for its excellent fermentative vigor and low volatile acidity production. *Sk* was selected for its cryotolerance and its capacity to produce high levels of glycerol. The three analyzed strains were homothallic.

2.2. Generation of natural auxotrophic colonies from parental yeasts

For the selection of natural auxotrophic parental strains, *S. cerevisiae* (Sc1 and Sc2) and *S. kudriavzevii* (Sk) cells were grown on 15 mL of GPY medium (% w/v: yeast extract 0.5, peptone 0.5, glucose 2) during 5 days at 28 °C. Aliquots of each culture were seeded onto α-aminoadipic (α-AA) and fluoroorotic acid (5-FOA) agar plates in order to select lys^- and $ura3^-$ natural mutant colonies respectively (Boeke et al., 1987; Zaret and Sherman, 1985). One milliliter of each culture was also seeded in 15 mL of fresh GPY medium and incubated again in the same conditions. This process was repeated over 4 times.

Colonies that were able to grow on α-AA or 5-FOA plates were picked again on a new α-AA or 5-FOA plate respectively. In order to confirm the presence of the auxotrophy, cells were grown on starvation medium (0.1% w/v of Yeast Nitrogen Base without amino acids supplemented with 0.1% w/v of glucose) during 4 h at 28 °C. Subsequently, cell suspensions were spotted onto GPY-A (GPY medium with 2% w/v agar-agar), minimal medium (MM; 0.17% Yeast Nitrogen Base without amino acids, 2% glucose and 2% agar) and MM supplemented with proline (1 g/L) and uracil (10 mg/L) or lysine (30 mg/L). Plates were incubated during 5 days at 28 °C.

2.3. Hybrid generation

2.3.1. Protoplast fusion

The method described by Curran and Bugeja (1996) was used with slight modifications. Strains carrying the auxotrophic markers were grown separately in GPY medium (25 mL) for 48 h at 28 °C, recovered by centrifugation (3000 × *g* during 5 min at room temperature) and washed twice in sterile cold water. Cells were resuspended in 10 mL of protoplasting solution (sorbitol 1.2 M; Tris 0.1 M; EDTA 0.02 M; pH: 9.8) with 50 μL β-mercapto-ethanol, and incubated for 15 min at room temperature. The cells were washed with sorbitol 1.2 M, resuspended in 10 mL of sorbitol 1.2 M with 500 μg of zymolyase 20 T (1 μg/μl) (Seikagaku Corporation, Tokyo, Japan) and incubated for 60 min at 30 °C. Protoplast formation was monitored by diluting the suspension in water and optical density decrease observation (600 nm) as a consequence of protoplast burst. Protoplasts were washed twice with sorbitol 1.2 M.

For protoplast fusion, 2×10^7 protoplasts from each parental strain were mixed and treated with 2 mL of 60% polyethylene-glycol (molecular weight 6000) and 100 mM $CaCl_2$ for 30 min. Cells were washed twice by centrifugation with sorbitol 1.2 M. Appropriate dilutions of cells (1/10, 1/50 and 1/100) in sorbitol 1.2 M were embedded into 5 mL of GPY-A molten agar medium containing sorbitol 1.2 M, overlaid onto pre-solidified MM and incubated at 26 °C. After 3–7 days, the observed colonies were isolated and purified by restreaking on the same medium.

2.3.2. Rare-mating

Rare-mating assays were carried out according to the procedures proposed by Spencer and Spencer (1996) with some modifications. Strains carrying the auxotrophic markers were grown separately in 25 mL GPY broth for 48 h at 28 °C. Cells were recovered by centrifugation (3000 × *g* for 5 min at room temperature) and resuspended in the residual supernatant. Pairs of yeast cultures to be hybridized were placed together in the same tube and aliquots of these mixed strains were inoculated in 2 mL of fresh GPY medium. After 5–10 days of static incubation in slanted position at 28 °C, cells were recovered by centrifugation (3000 × *g* for 5 min at room temperature), washed in sterile water, resuspended in 1 mL of starvation medium and incubated for 2 h. A heavy suspension of the mixed culture was spread on MM plates and incubated at 28 °C. Prototrophic colonies usually appeared after 3–5 days. These colonies were isolated and purified by restreaking on the same medium.

2.3.3. Spore to spore mating

Yeast sporulation was induced by incubation on acetate medium (% w/v: CH_3COONa 1, Glucose 0.1, yeast extract 0.125 and agar 2) for 5–7 days at 28 °C. Following preliminary digestion of the asci walls with 2 mg/mL glucuronidase (Sigma), pairs of different parental strains were seeded in the same GPY agar plate. Spores from both parental strains were dissected and placed together using a Singer MSM Manual micromanipulator. After incubation at 28 °C for 3–5 days, all

developed colonies were transferred to selection media plates. The colonies capable of growth in the selection media were isolated and purified by restreaking on the same media.

All protoplast, rare-mating and spore to spore generated hybrid strains were immediately placed in cryogenic vials containing glycerol 15% and conserved at −80 °C in order to minimize possible genetic changes.

2.4. Molecular analyses

Yeast total genomic DNA was obtained according to standard procedures (Querol et al., 1992).

2.4.1. Restriction fragment length polymorphism of amplified DNA products (PCR-RFLP) analysis

The characterization of *Saccharomyces* hybrids obtained from interspecific crosses was performed by PCR amplification of *BRE5* and *PPR1* protein-encoding nuclear genes and subsequent RFLP analysis with the restriction enzyme *Hae* III (Takara Bio Inc., Shiga, Japan) following the methodology proposed by González et al. (2008).

2.4.2. Microsatellite analysis

Two microsatellite loci (named O and P and located in chromosomes XV and XVI in *S. cerevisiae*) were selected for the differentiation of the *Sc1* and *Sc2* parental strains used in intraspecific mating. Microsatellite analysis was carried out according to Bradbury et al. (2006) with modifications. Primer sets YOR267c-3 and YOR267c-5 for microsatellite O and YPL009c-3 and YPL009c-5 for microsatellite P, based on those previously described by Bradbury et al. (2006) and Legras et al. (2005), were enlarged according to the following sequences: YOR267c-3 5′-CTCTTTTCTTTGGATCTACTTGCAGTATACGG-3′, YOR267c-5 5′-AAGTTGATACTAACGTCAACACTGCTGCCAA-3′, YPL009c-3 5′-CGTATTTCTTTTGAATTTCTTCAATTTTCCTCTTTTACCAC-3′ and YPL009-c 5′-CTGCTCAACTTGTGATGGGTTTTGGATTTTTATGGA-3′. Reverse primers were labeled with the fluorogenic compounds 6-carboxyfluorescein (FAM) or hexachlorofluorescein (HEX) (Applied Biosystem, Foster City, USA). PCR products were analyzed in an ABIprism 310 sequencer and the results were evaluated using the Peak Scanner software v1.1 (Applied Biosystems, Foster City, USA).

2.4.3. Random amplified polymorphic DNA (RAPD) analysis

Eleven different primers (OPA2, 3, 7, 8, 9, 10, 11, 15 and 16; R1 and R3) previously reported by Fernández-Espinar et al. (2003), and Corte et al. (2005) were used to carry out RAPD analysis.

2.4.4. Amplified inter-δ sequence DNA polymorphism analysis

Primers delta 12 (5′-TCAACAATGGAATCCCAAC-3′) and delta 21 (5′-CATCTTAACACCGTATATGA-3′) as well as procedures proposed by Legras and Karst (2003) were used to amplify the yeast genomic DNA.

2.4.5. Mitochondrial DNA-restriction fragment length polymorphism (mtDNA-RFLP) analysis

Mitochondrial DNA restriction analysis was performed by the method of Querol et al. (1992) using the endonuclease *Hinf* I (Roche Molecular Biochemicals, Mannheim, Germany).

2.5. Flow cytometry

The DNA content of each parental and hybrid strain was assessed by flow cytometry using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, California, United States) following the methodology described in Lopes et al. (2010). DNA content values were scored on the basis of the fluorescence intensity compared with the *S. cerevisiae* haploid (S288c) and diploid (FY1679) reference strains. DNA content value reported for each strain is the result of two independent measures. Results were tested by one way ANOVA and Tukey HSD test ($\alpha = 0.05$, $n = 2$).

2.6. Spores viability

Sporulation and ascus dissection in the hybrid strains were carried out as mentioned previously for spore to spore mating. Spores viability was calculated as the percentage of spores (from a total of 40 analyzed spores for each hybrid strain) that were able to form a colony on GPY agar after 48–72 h at 26 °C.

2.7. Genetic stabilization

Four hybrid colonies (two obtained by rare-mating and two by spore to spore mating) from each cross (intraspecific and interspecific) were subjected to the same genetic stabilization procedure.

Each selected hybrid strain was individually inoculated into 25 mL screw cap tubes containing 20 mL of synthetic must (Rossignol et al., 2003) and incubated at 20 °C without shaking. After fermentation (approximately 15–20 days), an aliquot was used to inoculate a new tube containing the same sterile medium and incubated in the same conditions. After five successive fermentations, an aliquot of the fifth fermentation was seeded on GPY-agar plates and incubated at 20 °C. Ten yeast colonies were randomly picked and characterized by mtDNA-RFLP, inter-δ sequences and RAPD (using primer R3) analyses, as well as DNA content, as previously mentioned. Simultaneously, the same colonies were inoculated in synthetic must and, after these individual fermentation, ten colonies from each one were analyzed by the same methods. We considered a genetically stable hybrid when the colonies recovered after individual fermentation maintained the same molecular pattern for the three markers and the same DNA content than the previously inoculated (original) culture.

3. Results

In order to generate artificial hybrids, both physiological and molecular differences among the parental strains must be established. Physiological differentiation became necessary to select an appropriate medium for hybrids recovery. Molecular characterization must be focused on finding markers to confirm the success of hybridization as well as the genetic variability among the generated hybrids.

3.1. Selection of hybridization markers in parental strains

α-Aminoadipic (α-AA) and 5-fluoroorotic acid (5-FOA) containing agar plates were used to select natural *lys*$^-$ and *ura3*$^-$ auxotrophic colonies respectively from the three parents. Different numbers of colonies were obtained for each parental strain in both selection media. However, only 54% of them exhibited auxotrophic behavior, i.e. they were able to grow in GPY and minimal medium supplemented with lysine or uracil (depending on the origin of isolation; α-AA and 5-FOA agar plates respectively), but not in minimal medium without supplements. Some of these stable auxotrophic colonies (*Sc1 ura3*$^-$, *Sc2 lys*$^-$ and *Sk lys*$^-$) were used for hybrids generation.

Different molecular markers were evaluated to be used to confirm the success of hybridization as well as the genetic variability among the generated hybrids. Two microsatellite regions (O and P) were evaluated to be used in the confirmation of intraspecific (*Sc1*×*Sc2*) hybrids generation. The length of alleles (bp) showed by each parental strain for both O and P microsatellite regions is shown in Table 1. Only one allele was observed for each microsatellite region in *Sc1*, while two different alleles were observed in *Sc2*. The different allele sizes in both *Sc1* and *Sc2* parents allowed us to use them for intraspecific hybrids confirmation.

Following the methodology proposed by González et al. (2008), PCR-RFLP analysis of genes *PPR1* and *BRE5* rendered *S. cerevisiae* and

Table 1
Molecular and genetic characterization of the Sc1 x Sc2 intraspecific hybrids and parental strains.

Methodology	Name	Microsatellite Alleles lenght (bp)		Molecular patterns[a]			DNA Content[b]
		O	P	mtDNA	δ-PCR	RAPD-R3	
Parental	Sc1	295	460	Sc1	δ-Sc1	R3-Sc1	$2.7 \pm 0.2^{a-e}$
	Sc2	328, 340	446, 468	Sc2	δ-Sc2	R3-Sc2	2.5 ± 0.3^{a}
Protoplast fusion	P2	295, 340	460, 468	Sc2	δ-1	R3-1	$3.8 \pm 0.1^{d-j}$
	P3	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-2	4.1 ± 0.3^{hij}
	P4	295, 328, 340	446, 460, 468	Sc2	δ-3	R3-3	4.9 ± 0.5^{j}
	P5	295, 328, 340	446, 460, 468	r1	δ-3	R3-4	$2.7 \pm 0.3^{a-e}$
	P6	295, 340	460, 468	r2	δ-1	R3-1	$3.9 \pm 0.5^{e-j}$
	P8	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-5	$2.8 \pm 0.0^{a-f}$
	P9	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-5	$3.1 \pm 0.6^{a-g}$
	P12	295, 328, 340	446, 460, 468	Sc2	δ-2	R3-6	$3.0 \pm 0.5^{a-g}$
Rare-mating	R1	295, 340	460, 468	r3	δ-4	R3-7	$3.7 \pm 0.3^{b-h}$
	R2	295, 328, 340	446, 460, 468	Sc2	δ-5	R3-8	5.0 ± 0.1^{j}
	R3	295, 340	460, 468	Sc2	δ-4	R3-8	$3.6 \pm 0.3^{a-h}$
	R4	295, 340	460, 468	Sc1	δ-6	R3-7	$4.0 \pm 0.4^{f-j}$
	R5	295, 340	460, 468	r3	δ-7	R3-7	$3.8 \pm 0.4^{c-i}$
	R6	295, 340	460, 468	Sc1	δ-4	R3-7	$3.7 \pm 0.2^{b-h}$
	R7	295, 328	460, 468	Sc2	δ-8	R3-7	$3.5 \pm 0.1^{a-h}$
	R8	295, 328, 340	446, 460, 468	Sc1	δ-4	R3-7	4.7 ± 0.3^{ij}
	R9	295, 328	446, 460	Sc1	δ-9	R3-7	$4.1 \pm 0.5^{g-j}$
	R10	295, 328, 340	446, 460, 468	r3	δ-4	R3-7	4.6 ± 0.1^{ij}
	R11	295, 328	446, 460	Sc2	δ-9	R3-7	$3.8 \pm 0.4^{c-i}$
	R12	295, 328, 340	446, 460, 468	Sc2	δ-4	R3-7	4.5 ± 0.4^{ij}
Spore to spore mating	S1	295, 340	460, 468	Sc2	δ-8	R3-8	$2.8 \pm 0.2^{a-f}$
	S2	295, 340	446, 460	Sc2	δ-10	R3-9	$2.7 \pm 0.1^{a-d}$
	S3	295, 340	446, 460	Sc2	δ-11	R3-9	2.6 ± 0.1^{ab}
	S4	295, 340	460, 468	Sc2	δ-12	R3-8	2.6 ± 0.2^{abc}
	S5	295, 340	460, 468	Sc2	δ-10	R3-8	$4.1 \pm 0.2^{g-j}$
	S6	295, 328	460, 468	r4	δ-13	R3-8	2.6 ± 0.3^{ab}
	S7	295, 340	446, 460	Sc1	δ-14	R3-10	$2.8 \pm 0.2^{a-e}$
	S8	295, 340	460, 468	r2	δ-15	R3-8	$2.9 \pm 0.0^{a-f}$
	S9	295, 328	460, 468	r4	δ-15	R3-8	2.7 ± 0.1^{abc}
	S10	295, 340	446, 460	Sc1	δ-16	R3-11	$2.6 \pm 0.3^{a-f}$
	S14	295, 328	446, 460	Sc1	δ-17	R3-10	4.1 ± 0.5^{ij}

[a] Molecular patterns obtained by mtDNA-RFLP (mtDNA), interdelta sequence DNA polymorphisms (δ-PCR) and RAPD analysis using primer R3 (RAPD-R3).
[b] Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 2$).

S. kudriavzevii specific patterns for *Sc1* and *Sk* parents respectively (data not shown). These differences were then used for the confirmation of interspecific hybrids generation.

Additional molecular markers (mitochondrial DNA restriction analysis; inter δ-elements and RAPD analysis using 11 different primers) were evaluated in parental strains in order to better characterize and to study the genomic stability of the generated artificial hybrids. Differential mtDNA-RFLP and inter δ-elements patterns were obtained for each parental strain (Fig. 1). From a total of 11 analyzed primers, only RAPD analysis with primer R3 allowed us to differentiate the three parental strains (Fig. 1). The DNA content of the three parental strains was also analyzed: *Sk* showed a DNA content of 2.2 ± 0.1, while both *Sc1* and *Sc2* showed higher values (2.7 ± 0.2 and 2.5 ± 0.3 respectively).

3.2. Hybrids generation and characterization

Strains *Sc1* (*ura3*$^-$) and *Sc2* (*lys*$^-$) were used for the generation of intraspecific hybrids and *Sc1* (*ura3*$^-$) and *Sk* IFO1802 (*lys*$^-$) for interspecific hybrids.

3.2.1. Intraspecific hybrids

3.2.1.1. Protoplast fusion (P). After applying a modification of the Curran and Bugeja (1996) method we isolated a total of 30 putative hybrid colonies in minimal medium (MM). Molecular characterization and DNA content analyses were carried out on 12 colonies obtained by protoplast fusion (Table 1). Four colonies (named P1, P7, P10 and P11) presented the same microsatellite allele combination present in one of the parental strains indicating that, despite growth in MM, they were not hybrids (data not shown). The hybrids P3, P4, P5, P8, P9 and P12 showed the complete set of the different alleles present in both *Sc1* and *Sc2* parental strains, while hybrids P2 and P6 only showed two alleles (each one coming from a different parental strain), indicating that they were hybrids that have already lost one allele (Table 1). None of the hybrids showed the same mtDNA-RFLP pattern present in the parental Sc1; 75% exhibited *Sc2* pattern and 25% showed different patterns (r1 and r2) probably obtained by a recombination between *Sc1* and *Sc2* mtDNAs (Table 1 and Fig. 1). All inter δ-elements and RAPD-R3 patterns detected in hybrids generated by protoplast fusion corresponded to new patterns obtained by the combination of the respective parental patterns (Table 1). By combination of all applied molecular techniques, we differentiated 7 molecular patterns out of 8 confirmed hybrids; hybrids P8 and P9 could not be differentiated (Table 1).

In general, DNA content values detected in hybrids (2.7 to 4.9) were significantly lower than the value expected by the addition of the parental values (5.2). Moreover, some hybrids showed DNA content values that were not significantly different than those exhibited by the parental strains, indicating a significant loss of genetic material (Table 1).

3.2.1.2. Rare-mating. The rare-mating hybrids were obtained applying the methodology described by Spencer and Spencer (1996) with some modifications. After mixing of parental strains, samples were taken at 3, 5, 7 and 10 days of incubation. In order to reduce the number of false positive colonies (those colonies growing onto MM by using their own reserves), samples were washed and maintained in starvation medium for 2 h before seeding on MM. The highest number of putative hybrid colonies was recovered from plates seeded after 5 and 7 days of incubation. Twelve randomly selected colonies

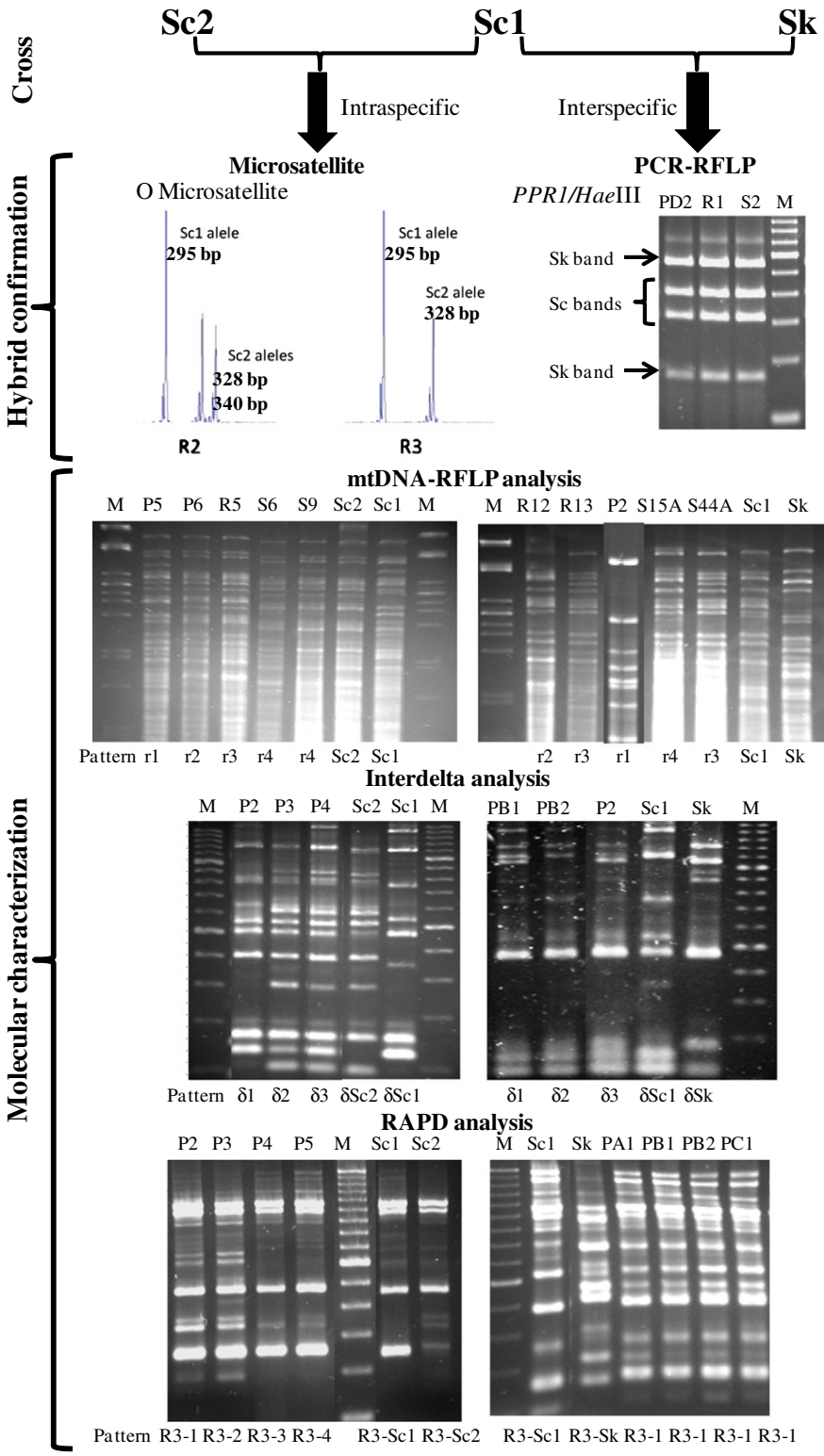

Fig. 1. Schematic representation of molecular markers used for both hybrids confirmation and molecular characterization. The codes at the top of the figures indicate the identity of some strains showed as illustrative examples and the codes at the bottom indicate their respective molecular patterns. M: molecular marker; 100-bp DNA ladder marker for PCR-RFLP and RAPD analyses, mix of 100 bp and 50 bp DNA ladder market for interdelta analyses and λ-*Pst* I for mtDNA-RFLP analysis.

were reisolated in MM for further characterization (Table 2). The microsatellite analysis revealed that all colonies corresponded to *Sc1*×*Sc2* hybrids, i.e. they showed alleles coming from both parental strains for both O and P analyzed gene regions (Table 1). However, as happened after protoplast fusion, not all the strains showed the complete set of the alleles coming from both parental strains (Table 1). Regarding the mtDNA-RFLP patterns, 33.33% of the selected hybrids showed the profile presented in the parental *Sc1*, 41.67% Sc2, and the remaining 25% showed a new profile (r3) that could correspond to a recombinant pattern (Table 1). The analysis of inter δ-elements revealed 6 new profiles; the profile δ-4 was exhibited by 6 hybrid strains (R1, 3, 6, 8, 10 and 12), δ-9 by two hybrids (R9 and R11) and δ-5, δ-6, δ-7 and δ-8 by only one

Table 2
Molecular and genetic characterization of the Sc1 x Sk interspecific hybrids and parental strains.

Methodology	Name	Molecular patterns[a]			DNA Content[b]
		mtDNA	δ-PCR	RAPD-R3	
Parental	Sc1	Sc1	δ-Sc1	R3-Sc1	2.7 ± 0.2^{abc}
	Sk	Sk	δ-Sk	R3-Sk	2.2 ± 0.1^{a}
Protoplast fusion	PA1	Sc1	δ-1	R3-1	$5.0 \pm 0.2^{j-l}$
	PB1	Sc1	δ-1	R3-1	$4.8 \pm 0.2^{i-l}$
	PB2	Sc1	δ-2	R3-1	$4.1 \pm 0.4^{d-l}$
	PB3	Sc1	δ-1	R3-1	$4.7 \pm 0.5^{h-l}$
	PC1	Sc1	δ-1	R3-1	$4.6 \pm 0.3^{h-l}$
	PC2	Sc1	δ-1	R3-1	$5.1 \pm 0.4^{j-l}$
	PC3	Sk	δ-1	R3-1	$4.2 \pm 0.3^{e-l}$
	PD1	Sc1	δ-1	R3-1	$4.5 \pm 0.3^{f-l}$
	PD2	Sc1	δ-1	R3-1	$4.8 \pm 0.1^{i-l}$
	PD3	Sc1	δ-1	R3-1	$4.8 \pm 0.1^{i-l}$
	PD4	Sc1	δ-1	R3-1	$4.7 \pm 0.1^{i-l}$
	P2	r1	δ-3	R3-1	5.1 ± 0.5^{l}
Rare-mating	R1	Sk	δ-4	R3-2	$3.2 \pm 0.2^{a-e}$
	R2	Sk	δ-4	R3-3	$4.9 \pm 0.1^{i-l}$
	R3	Sc1	δ-4	R3-4	$4.8 \pm 0.1^{i-l}$
	R4	Sk	δ-4	R3-2	$4.0 \pm 0.2^{d-j}$
	R5	Sc1	δ-5	R3-2	$3.1 \pm 0.6^{a-d}$
	R6	Sc1	δ-4	R3-4	$3.6 \pm 0.4^{c-h}$
	R7	Sk	δ-4	R3-5	5.1 ± 0.2^{l}
	R9	Sc1	δ-6	R3-6	$4.5 \pm 0.2^{g-l}$
	R10	Sc1	δ-6	R3-6	2.3 ± 0.2^{ab}
	R11	Sk	δ-6	R3-7	5.1 ± 0.1^{kl}
	R12	r2	δ-6	R3-6	$3.9 \pm 0.0^{d-i}$
	R13	r3	δ-6	R3-7	$4.6 \pm 0.3^{g-l}$
	R14	Sc1	δ-6	R3-6	$3.0 \pm 0.3^{a-d}$
	R15	Sk	δ-6	R3-8	$4.1 \pm 0.1^{d-k}$
Spore to spore mating	S1	Sc1	δ-7	R3-9	$3.1 \pm 0.4^{a-d}$
	S2	Sc1	δ-8	R3-10	$3.5 \pm 0.2^{c-g}$
	S3	Sc1	δ-8	R3-10	$3.0 \pm 0.2^{a-d}$
	S4	Sc1	δ-8	R3-10	$3.3 \pm 0.1^{b-e}$
	S5	Sk	δ-9	R3-11	$3.4 \pm 0.1^{c-f}$
	S6	Sc1	δ-8	R3-10	$3.2 \pm 0.0^{a-e}$
	S8	Sc1	δ-8	R3-12	$3.2 \pm 0.2^{a-d}$
	S15A	r4	δ-9	R3-13	$3.2 \pm 0.3^{a-e}$
	S15B	Sc1	δ-9	R3-14	$3.1 \pm 0.3^{a-d}$
	S44A	r3	δ-9	R3-15	$3.3 \pm 0.3^{b-e}$
	S44B	Sk	δ-9	R3-13	$3.6 \pm 0.4^{c-h}$
	S45	Sc1	δ-8	R3-16	$3.1 \pm 0.1^{a-d}$

[a] Molecular patterns obtained by mtDNA-RFLP (mtDNA), interdelta sequence DNA polymorphisms (δ-PCR) and RAPD analysis using primer R3 (RAPD-R3).
[b] Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 2$).

strain each. In the analysis of RAPD-R3 only two profiles (R3-7 and R3-8) were identified. Combining all the molecular markers was possible to differentiate the 12 different hybrids analyzed. The DNA content presented by the hybrids ranged from 3.5 to 5.0; these values were generally higher than those exhibited by hybrids generated by protoplast fusion (Table 1).

3.2.1.3. Spore to spore mating. No colonies were obtained when spores dissection and mating were conducted on MM according to the standard methods. In order to improve the germination and consequently the hybridization process, asci dissection and crosses were repeated on complete medium (GPY). All colonies developed onto GPY agar plates were subsequently replica-plated in MM in order to select real hybrids bearing complementary auxotrophies. Sixty-four spore to spore crosses were made and the same number of colonies was obtained on GPY-agar plates. However, only 14 colonies were able to grow when transferred to MM. According to O and P microsatellite analysis, 11 colonies (17.2%) demonstrated to be hybrids; i.e. they presented one allele from each parent (Table 1). According to mtDNA, 45.4% showed a *Sc2* parental profile, 27.3% *Sc1* and the remaining 27.3% presented different mtDNA-RFLP patterns named r2 and r4 (Table 1). Interestingly, the pattern r2 was indistinguishable to pattern r2 found in one of the hybrids obtained by protoplast fusion (P6) (Table 1). As a result of inter δ-element analysis, nine profiles were identified including the pattern δ-8, that was also detected in hybrid R7 obtained by rare-mating (Table 1). Finally, 4 different profiles were found after RAPD-R3 analysis. Most colonies exhibited the profile R3-8, also observed in two isolates obtained after rare-mating (R2 and R3) (Table 1). Combining all molecular markers, we differentiated the 11 hybrid colonies recovered. The value of the DNA content detected for most generated hybrids was around 2.7; however, the hybrid strains S5 and S14 showed values as high as 4.1 (Table 1).

3.2.2. Interspecific hybrids

3.2.2.1. Protoplasts fusion. Twelve colonies growing in MM after protoplast fusion were randomly selected and the hybrid nature of all of them was confirmed by PCR-RFLP. As a result of mtDNA-RFLP analysis, 10 colonies (83.3%) showed Sc1 parental pattern, the hybrid strain PC3 showed *Sk* parental pattern and P2 a new pattern (r1) (Table 2 and Fig. 1). Three different profiles were found after inter δ-element polymorphism analysis (δ-1, δ-2 and δ-3) and, as in intraspecific mating, they were a combination of both parental strain patterns. All the hybrids showed the same RAPD-R3 pattern. Combining the three markers we differentiated 4 out of 12 generated hybrids. The DNA content expected for these hybrids is 4.9, resulting from the addition of *Sc1* (2.7) and *Sk* (2.2) contents. However, the real values obtained for these hybrids ranged between 4 and 5 (Table 2).

3.2.2.2. Rare-mating. After rare-mating assays, fifteen colonies were isolated randomly from MM plates and only one of them (R8) was not confirmed as hybrid by PCR-RFLP of *BRE5* and *PPR1* genes. 42.9% of the hybrids had a *Sc1* mtDNA-RFLP pattern and 42.9% exhibited *Sk* pattern (Table 2). The remaining 14.2% of the colonies showed two different recombinant mtDNA-RFLP profiles (r2 and r3). Additionally, we identified 3 inter δ-element profiles (δ-4 to δ-6) and 7 RAPD-R3 patterns (Table 2). Combining the three markers we differentiated 10 out of 14 hybrids studied. The DNA content of these hybrids was quite diverse and ranged from 2.3 (R10) to 5.1 (R7 and R11) (Table 2).

3.2.2.3. Spore to spore mating. From a total of 40 spore to spore crosses performed on GPY agar plates, only 12 (30%) were confirmed to be hybrids after seeding on MM and PCR-RFLP evaluation. Among them, 66.7% presented *Sc1* mtDNA-RFLP pattern, 33.3% *Sk* and the remaining 33.3% showed new patterns (r3 and r4) (Table 2).

We identified three new inter δ-element profiles, including δ-7 profile detected in only one strain (S1). RAPD analysis using primer R3 showed 8 new profiles. Combining the three markers we distinguished 9 out of 12 hybrid colonies evaluated. On the other hand, the mean DNA content value obtained in spore to spore hybrids was 3.2 (Table 2).

3.3. Genetic stabilization of hybrids during vegetative propagation

In order to obtain genetically stable hybrids to be used in future commercial fermentations, we developed a genetic stabilization procedure. This method was based on five consecutive fermentations on synthetic must at 20 °C (selective conditions). The stabilization process was evaluated by analyzing the variability of different molecular markers (mtDNA-RFLP, interdelta and RAPD) as well as the changes in total DNA content. A total of eight hybrid strains obtained by rare-mating and spore to spore mating of both intra and interspecific crosses were stabilized (Table 3). Rare-mating hybrid strains were selected among those with the highest values of DNA content. Significant differences were observed in the stabilization process of intraspecific and interspecific hybrids, particularly for those strains generated by rare-mating.

Table 3
Genetic stabilization process in selected intraspecific and interspecific hybrids.

Cross, methodology and hybrid name			Original[1]				5° step of fermentation[2]						Stable[3]
			Molecular patterns[4]			DNA content[5]	Derived hybrid	Molecular patterns[4]			DNA content[5]	%	
			δ-PCR	RAPD-R3	mtDNA-RFLP			δ-PCR	RAPD-R3	mtDNA-RFLP			
Intraspecific	Rare-mating	R2	δ-5	R3-8	Sc2	5.0 ± 0.1^{d}	R2-original	δ-5	R3-8	Sc2	4.7 ± 0.0^{d}	20	No
							R2-A	δ-18	R3-8	Sc2	2.7 ± 0.2^{a}	60	Yes
							R2-B	δ-19	R3-8	Sc2	2.5 ± 0.3^{a}	10	Yes
							R2-C	δ-20	R3-8	Sc2	2.6 ± 0.2^{a}	10	Yes
		R8	δ-4	R3-7	Sc1	4.7 ± 0.3^{c}	R8-original	δ-4	R3-7	Sc1	4.9 ± 0.2^{c}	40	No
							R8-A	δ-21	R3-7	Sc1-derived1	3.5 ± 0.4^{b}	20	No
							R8-B	δ-22	R3-12	Sc1-derived2	2.7 ± 0.1^{a}	10	Yes
							R8-C	δ-23	R3-7	Sc1-derived3	2.9 ± 0.1^{ab}	10	Yes
							R8-D	δ-24	R3-7	Sc1-derived3	2.6 ± 0.1^{a}	20	Yes
	Spore to spore mating	S2	δ-10	R3-9	Sc2	2.7 ± 0.1^{a}	S2-original	δ-10	R3-9	Sc2	2.5 ± 0.4^{a}	100	Yes
		S7	δ-14	R3-10	Sc1	2.8 ± 0.2^{a}	S7-original	δ-14	R3-10	Sc1	2.4 ± 0.2^{a}	100	Yes
Interspecific	Rare-mating	R1	δ-4	R3-2	Sk	3.2 ± 0.2^{b}	R1-original	δ-4	R3-2	Sk-derived	2.6 ± 0.0^{a}	100	Yes
		R3	δ-4	R3-4	Sc1	4.8 ± 0.1^{b}	R3-original	δ-4	R3-4	Sc1	3.6 ± 0.1^{a}	100	Yes
	Spore to spore mating	S5	δ-9	R3-11	Sk	3.4 ± 0.1^{b}	S5-original	δ-9	R3-11	Sk	2.4 ± 0.2^{a}	100	Yes
		S8	δ-8	R3-12	Sc1	3.2 ± 0.2^{a}	S8-original	δ-8	R3-12	Sc1	2.8 ± 0.2^{a}	100	Yes

[1] Hybrid used to inoculate wine-like medium for the five-step stabilization procedure.
[2] Hybrid colonies isolated after five fermentation steps. Percentages (%) were calculated from a total of 10 analyzed colonies.
[3] Strains were considered as genetically stable when no changes in both molecular patterns and DNA content were detected after fermentation with a single derived hybrid colony (from a total of 10 colonies analyzed).
[4] Interdelta patterns (δ-PCR) are indicated as δ- followed by a Latin number. Patterns obtained by RAPD-PCR with primer R3 (RAPD-R3) are indicated as R- followed by a Latin number.
[5] Values expressed as mean ± standard deviation. Values not sharing the same superscript letter within the columns of both original and derived hybrids are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 2$).

For interspecific hybrids, all colonies obtained after five fermentation steps showed the same molecular pattern detected in the original hybrid strain for interdelta and RAPD analyses, independently of the hybridization procedure used for generation (Table 3). Although no nuclear genetic variability was detected among original and derived colonies, significant differences in DNA content values, as well as changes in their mtDNA-RFLP profile, were observed in most cases. Colonies derived from R1, R3 and S5 showed values of DNA content significantly lower than those exhibited by the originals (Table 3), indicating that the DNA content is a good marker to analyze the genetic stabilization of the artificial hybrids, additionally to molecular markers. Colonies derived from R1 hybrid changed their mtDNA-RFLP profile (Fig. 2A). All interspecific hybrids were able to sporulate in acetate medium; however, most of them showed spores unable to develop colonies in GPY. The only exception was vegetatively stable hybrid R3, that showed viability values of 50%.

Most hybrids obtained from intraspecific spore to spore mating exhibited the same behavior during vegetative stabilization than interspecific spore to spore hybrids: no genetic variability and no changes in DNA content. Contrarily, colonies isolated after five consecutive fermentations of intraspecific hybrids obtained by rare-mating showed new molecular patterns (Table 3). Three new molecular patterns were detected among R2 derived colonies and four patterns among R8 derived colonies. Only R8 derived colonies changed their mtDNA-RFLP profile. DNA content analysis evidenced that those derived colonies exhibiting the same molecular pattern detected in the original hybrid (R2-original and R8-original, as well as all spore to spore hybrids) showed similar DNA content to that present in the original hybrid. Contrarily, derived colonies exhibiting new molecular patterns (R2-A, R2-B, R2-C, R8-A, R8-B, R8-C and R8-D) showed significantly lower DNA content values (Table 3).

Finally, individual colonies representative of each derived hybrid detected after the complete set of five consecutive fermentations were used to inoculate fresh synthetic must in order to confirm their vegetatively genetic stability. After fermentation, ten colonies were isolated and characterized by mtDNA-RFLP, interdelta, RAPD and DNA content analyses. Only derived hybrids R2-original, R8-original and R8-A were shown to be unstable due to changes in DNA content (Table 3).

4. Discussion

Intraspecific hybrids—using two *S. cerevisiae* strains bearing interesting and complementary oenological features—as well as interspecific hybrids—using *S. cerevisiae* and *S. kudriavzevii*—were obtained

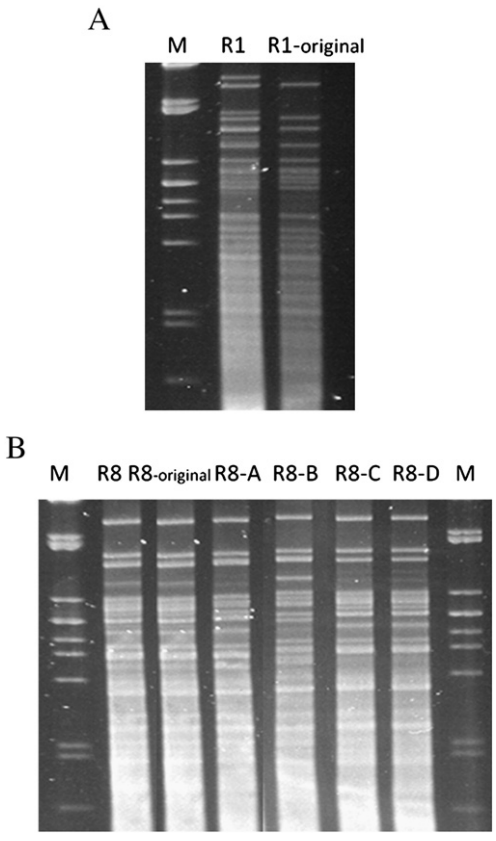

Fig. 2. mtDNA-RFLP analysis comparing the initial hybrid and the derived ones. A) R1 and B) R8. λ-PstI was used as the molecular weight marker (M).

by means of non GMOs generating techniques: rare-mating (R) and spore to spore mating (S) and compared with hybrids generated by protoplast fusion (P), a commonly used GMOs producing technique.

Selection procedures of hybrids based on complementation of auxotrophic parental strains are difficult because industrial strains are prototrophic (Akada, 2002; Nakazawa and Iwano, 2004). For this reason, spontaneous *ura3*$^{-}$ and *lys2*$^{-}$ auxotrophic mutants were selected in this work by the use of 5-FOA and α-AA agar plates (Boeke et al., 1987; Zaret and Sherman, 1985). The generation of auxotrophic strains has been reported to be difficult for industrial *S. cerevisiae* strains, because of their generally polyploid or aneuploid nature (Bell et al., 1998). However, we succeeded in the recovery of these natural mutants for both *S. cerevisiae* industrial as well as for the wild *S. kudriavzevii* parental strains. The fact that both 5-FOA and α-AA allow the identification of naturally occurring mutants among the yeast populations, makes it possible to use these compounds in non-GMO producing protocols. Artificially generated mutants could not be used with the same purpose.

The need for a confirmatory method for the evaluation of hybrids generation was the second important step in this study. Confirmatory methods must be able to differentiate the parental strains involved in hybridization. Different methodologies have been described in scientific literature for the differentiation of *S. cerevisiae* strains including mtDNA-RFLP, RAPD, interdelta, karyotyping, microsatellites, among others (Baleiras Couto et al., 1996; de Barros Lopes et al., 1996; Hennequin et al., 2001; Pérez et al., 2001; Querol et al., 1992; Vezinhet et al., 1992). Some of them were also useful to discriminate strains belonging to other species of *Saccharomyces* genus as *S. kudriavzevii* and *S. bayanus* (Demuytier et al., 2004; Lopes et al., 2010; Masneuf-Pomarède et al., 2007). Due to the fact that mitochondrial DNA could be inherited without changes from one of the parental strains involved in the hybridization event (Dujon et al., 1974), the use of mtDNA-RFLP analysis as a confirmatory method for hybrids generation is not adequate. Both microsatellite and PCR-RFLP analyses were selected in this work because these methods are fast and simple to interpret. Using these methods, reliable molecular patterns able to discriminate between parental and hybrid strains in interspecific and intraspecific hybridizations respectively were obtained (Tables 1 and 2).

As a general rule, hybridization frequency obtained in this work after S was higher than the same for P and R (data not shown); however, a high number of hybrids were obtained in all cases even if R has been suggested as a difficult method to generate hybrids according to the literature. These results are in accordance with previous reports that showed a low hybridization frequency after P and R events (Bell et al., 1998; Gunge and Nakatomi, 1972; Spencer and Spencer, 1977).

Independently from the methodology used for hybridization, the frequency of hybrids was significantly improved when recently formed putative hybrid cultures were maintained in starvation medium for 1–2 h before seeding on MM agar plates. This procedure allowed us to diminish the recovery of parental strains growing in MM by means of their own nutritional reserves.

In the particular case of S due to the presence of individual spore auxotrophies, hybridization events were not observed when minimal medium was directly used for asci dissection and mating. To solve that problem, we proposed the use of a complete medium (GPY) to make the individual crosses prior to selection of hybrids in MM. Several S hybrids were obtained after using this additional step.

A high genetic variability in both nuclear and mitochondrial genomes was observed among hybrid colonies obtained from both intra- and interspecific crosses, according to their mtDNA-RFLP, interdelta and RAPD patterns. This variability indicates that different processes of lost and reorganization of genetic material from both parents are taking place during the early stages of hybrids generation.

With regards to mtDNA restriction patterns, most hybrid colonies showed mtDNA-RFLP patterns indistinguishable from those of the parental strains. This finding was previously described by Dujon et al. (1974), who demonstrated that different mitochondrial genomes brought together by mating, are rapidly segregated through subsequent mitotic divisions and new generated buds inherit only one kind of mtDNA. This uniparental inheritance of mitochondrial genome has been detected in *S. cerevisiae*×*S. pastorianus* hybrids obtained by R (de Barros Lopes et al., 2002), in *S. cerevisiae*×*S. uvarum* hybrids obtained by mass mating (Antunovics et al., 2005) and in natural hybrids isolated from wine and beer (González et al., 2006, 2008; Rainieri et al., 2008). Interestingly, a minority number of hybrid colonies obtained in this work exhibited mtDNA-RFLP patterns originated by putative recombinant events among parental mtDNA molecules (Tables 1 and 2). These new patterns were named r1, r2, etc. (Tables 1 and 2 and Fig. 1). In this sense, Berger and Yaffe (2000) showed that during the hybridization event, parental mtDNAs can recombine with high frequency and hybrid colonies could inherit either parental or recombinant mtDNA molecules. The fact that the same putative recombinant mtDNA pattern was detected in different hybrid colonies obtained by the same or different hybridization protocols (Tables 1 and 2) seems to indicate the potential presence of hot spots of recombination in the mtDNA, as it was suggested by other authors (Berger and Yaffe, 2000; Dujon et al., 1974; Piškur, 1994). The higher frequency of recombinant mtDNA molecules among intraspecific hybrids with regard to that in interspecific hybrids (26% and 13% on average respectively) can be explained by the higher genetic similarity among parental genomes in the former cross, which favors homologous recombination (Bernardi, 2005). Additionally, the possible incompatibility between the mitochondrion of one species and the nucleus of the other species—as it was demonstrated by Lee et al. (2008) for *S. cerevisiae* and *S. bayanus*—can be another explanation for the existence of recombinant mtDNA molecules. During genetic stabilization process, changes in mtDNA-RFLP patterns were observed, i.e. hybrids obtained after five fermentation steps inoculated with only one particular hybrid colony couldn't show the same mitochondrial pattern founded in the original (Table 3). This happened for the interspecific hybrid R1 and the intraspecific hybrid R8 (Fig. 2). It may be due to the DNA repair machinery, active during the stabilization process and responsible for the chromosomal rearrangement, that could act on the mtDNA allowing recombination between sister molecules. This process could be favored by the high homology between them (100% homology) (Bernardi, 2005).

Similar situations were also observed in nuclear genome of hybrid strains, and these differences were detected with interdelta and RAPD analyses. For both molecular markers, most hybrids showed patterns basically originated for the addition of bands from the patterns generated with both parental strains involved in the mating (Fig. 1); however, some hybrid strains also exhibited new bands probably originated from chromosomal rearrangements (Fig. 1). The existence of these rearrangements was also evident during the vegetative stabilization process, particularly for colonies derived from the intraspecific hybrids R2 and R8, which showed different interdelta or RAPD patterns from a single pattern in the originally inoculated strain (Table 3).

Both interdelta and RAPD analyses were demonstrated to be useful tools for the differentiation of recently generated hybrid strains; a total of 26 interdelta and 26 RAPD-R3 patterns were observed among the 69 confirmed hybrids analyzed; however, most molecular differences arising during stabilization process were only detected in interdelta analysis (Table 3).

As it was previously suggested, the genetic variability observed in nuclear molecular markers could be the consequence of chromosomal rearrangements or losses occurring among parental genomes during the early stages of hybrids generation. This hypothesis was partially confirmed with the results of microsatellite analysis in the recently generated intraspecific hybrids, where the loss of some parental alleles was evident (Table 1). The fact that parental *Sc1* was homozygous for both O and P microsatellite regions, made it difficult to totally interpret DNA losses; only loss of alleles from parental *Sc2*

was informative in this sense (Table 1). Loss of genetic material after hybridization was also demonstrated after DNA content evaluation. DNA content obtained for recently generated hybrids was generally lower than the values expected by the theoretical addition of DNA content from the respective parental strains in both inter and intraspecific crosses. This phenomenon was especially evident in hybrids generated by P and R (Tables 1 and 2), independently from the evaluated cross, and it seems to happen during the early stages immediately after hybrids generation. During the stabilization process, changes in DNA content were also evident particularly for intra- and interspecific R hybrids originally possessing a higher amount of DNA than S hybrids (Table 3). In a similar way, a DNA content reduction process was also evidenced by Antunovics et al. (2005) after stabilization of *S. cerevisiae*×*S. uvarum* hybrids by successive sporulation events and Marinoni et al. (1999) after interspecific hybridization by mass-mating. The infertility found in most vegetatively stable interspecific hybrids indicated that, even after DNA losses, they still maintained DNA from each parental strain. Only interspecific hybrid R3 showed a 50% spores viability; this behavior could be related to the fact that this strain probably conserved a complete diploid set of chromosomes from one of the two parental, as can be inferred from the high DNA content (3.6) (Table 3).

Polyploid genomes, as those obtained after hybridization in this work after R and P, are known to be unstable in *S. cerevisiae* (Gerstein et al., 2008; Storchova et al., 2006). Genome loss and rearrangement occurring during stabilization of newly formed hybrids have been reported (Gerstein et al., 2006). These phenomena might lead to loss of industrially important traits in hybrids. For that reason, the final crucial step in this study was the careful selection of stabilization conditions. The stabilization process proposed in this work, based on five consecutive fermentation steps, was successful in the generation of some vegetatively stable colonies from all analyzed original hybrids (Table 3). However, some unstable hybrid colonies, generally maintaining the same molecular patterns and DNA content than the original hybrids, were still detected after the five fermentation steps. This behavior was particularly detected after stabilization of intraspecific hybrids generated by R (Table 3). Our results suggest that stabilization of hybrids obtained by S is faster than stabilization of hybrids obtained by R, although in general both kinds of hybrids finally stabilize with values of DNA content close to those presented by the parents (close to diploidy). Gerstein et al. (2006) also observed the same behavior in which both triploid and tetraploid strains showed a reduction in their DNA content until a same value close to 2n. Additionally, the stabilization process in R hybrids generated by interspecific mating seems to have occurred faster than the same in intraspecific R hybrids (Table 3). In this case, genetic similarity among parental genomes in intraspecific mating—as it was mentioned for mtDNA—could favor homologous recombination (Bernardi, 2005). In fact, differences in molecular patterns probably arisen from homologous recombination were also observed in intraspecific R hybrids during stabilization, as previously mentioned. A difference in the moment in which different chromosomes are lost during both hybrids formation and stabilization in intra and interspecific hybrids can also be responsible for the differences detected in this work. It is important to remark that the particular vegetative stabilization process used in this work, however, does not guarantee that the hybrid will not change under different conditions. For that reason, as well as due to the differences detected among hybrids obtained from different methods and crosses, a more detailed study about the whole stabilization process in intra and interspecific hybrids is being carried out in our laboratory.

5. Conclusions

A high diversity of intra and interspecific hybrids was successfully obtained by three hybridization methodologies. Despite the low hybridization frequency obtained after protoplast fusion and rare-mating, hybrids generated by means of these methodologies have theoretically a more complete subset of genetic material inherited from each parental strain. Consequently, they possess an extremely high genetic plasticity which could render a potentially better adaption to the environment. Due to the fact that a loss of genetic material occurs during both hybrids generation and genetic stabilization, hybrids possessing a high amount of DNA became a better resource to obtain the best suitable hybrid strain for industrial purposes. The usefulness of rare-mating generated hybrids for industries in which GMO is a legal and public concern problem became evident. These hybrids were easily obtained and stabilized by the improved methodology described in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ijfoodmicro.2012.03.008.

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