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Yeast Interspecies Hybrids

Saccharomyces cerevisiae × *Saccharomyces uvarum* hybrids generated under different conditions share similar winemaking features

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Abstract

Interspecific hybrids among species in the *Saccharomyces* genus are frequently detected in anthropic habitats and can also be obtained easily in the laboratory. This occurs because the most important genetic barriers among *Saccharomyces* species are post-zygotic. Depending on several factors, including the involved strains, the hybridization mechanism and stabilization conditions, hybrids that bear differential genomic constitutions, and hence phenotypic variability, can be obtained. In the present study, *Saccharomyces cerevisiae* × *Saccharomyces uvarum* hybrids were constructed using genetically and physiologically different *S. uvarum* parents at distinct temperatures (13 and 20°C). The effect of those variables on the main oenological features of the wines obtained with these hybrids was evaluated. Hybrids were successfully obtained in all cases. However, genetic stabilization based on successive fermentations in white wine at 13°C was significantly longer than that at 20°C. Our results demonstrated that, irrespective of the *S. uvarum* parent and temperature used for hybrid generation and stabilization, similar physicochemical and aromatic features were found in wines. The hybrids generated herein were characterized by low ethanol production, high glycerol synthesis and the capacity to grow at low temperature and to produce malic acid with particular aroma profiles. These features make these hybrids useful for the new winemaking industry within the climate change era frame. Copyright © 2017 John Wiley & Sons, Ltd.

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Introduction

Numerous *Saccharomyces* strains that possess chimerical genomes composed of portions from different species in the genus have been isolated from diverse fermented beverages, including wine, cider and beer (Morales and Dujon, 2012; Sipiczki, 2008). It is believed that most have arisen by natural hybridization, a phenomenon that is possible through the presence of weak pre-zygotic barriers among species that allow the generation of viable hybrid cells (Sipiczki, 2008). Although some genetic mechanisms (differences in genome architectures, incompatibility genes, mismatch repair) generally avoid sporulation or significantly reduce the spore viability of these chimeric strains, they

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can persist in nature by means of asexual mitotic divisions (Karanyicz *et al.,* 2017; Naumov, 1996).

The interesting physiological features frequently observed in chimerical strains, which are generally intermediate among their parental phenotypes (Belloch *et al.,* 2008; Bellon *et al.,* 2011; Bizaj *et al.,* 2012), have attracted the interest of both the scientific community and industry. Indeed different methodologies have appeared to mimic the natural hybridization phenomena under controlled laboratory conditions to obtain 'à la carte' yeast strains. These include sexual, i.e. crosses of individual spores (or direct mating), mass mating, rare mating, and asexual, i.e. cytoduction and protoplast fusion-hybridizations (Pérez-Través *et al.,* 2012; Steensels *et al.,* 2014) methodologies. The choice of one methodology or another to generate hybrids is related directly to the study aim. As a general rule for the food industry, and more specifically for winemaking, it is important to select non-GMO generation strategies for yeast strain development. These non-GMO-generating methods are associated mainly with sexual hybridization.

As a direct result of the selected hybridization methodology, genome stabilization of recently generated hybrids could be required to guarantee yeast culture soundness (Pérez-Través *et al.,* 2014). Regarding the hybridization of haploid strains, a generally stable diploid hybrid is formed and the stabilization process is quite simple. However, some interesting features present in generally diploid original parental strains could be lost during the sporulation mechanism, which could lead to haploid strains. Methods that involve diploid strains, like rare mating, have been demonstrated to be advantageous. Nevertheless, this methodology requires the generated hybrids to be subjected to a genetic stabilization process, which is generally associated with a reduction in their DNA content until stable values around diploidy are achieved (Pérez-Través *et al.,* 2012, 2014).

In particular, the hybrids between *Saccharomyces cerevisiae* and cryotolerant species *Saccharomyces uvarum* for winemaking have been generated in different laboratories (most have been recently summarized by Morales and Dujon, 2012; Pérez-Través *et al.,* 2012). Owing to the fact that *S. uvarum* was mis-synonymized with *Saccharomyces bayanus* in the past, hybrids between *S. cerevisiae* and *S. bayanus* var. *uvarum*, with similar characteristics, could be also found in the literature. In a recent work, Nguyen and Boeckhout (2017) have proposed the invalidation of the varietal designation for *S. bayanus* and *S. uvarum* based on the hybrid nature (*Saccharomyces eubayanus* × *S. uvarum*) of *S. bayanus* type strain.

The success of *S. cerevisiae* × *S. uvarum* hybrids is particularly due to the fact that the two species are largely syntenic, e.g. they differ in five reciprocal translocations (Kellis *et al.,* 2003), and they possess interesting complementary characteristics of relevance in this industry. Besides the well-known fermentative performance of *S. cerevisiae*, *S. uvarum* is also known for its capacity to ferment at low temperature and to produce particular aromatic profiles that come about in the special combination of secondary metabolites. *S. uvarum* has a particular fermentation profile characterized by the production of low levels of acetic acid and ethanol, and high concentrations of glycerol and both malic and succinic acids as regards *S. cerevisiae* (Bertolini *et al.,* 1996; Giudici *et al.,* 1995; Kishimoto, 1994). Among volatile compounds, this species has been associated with the production of high concentrations of higher alcohol 2-phenylethanol and its acetate (Masneuf-Pomarède *et al.,* 2010), and also with the production of volatile thiols.

S. uvarum has been isolated from both natural habitats (Almeida *et al.,* 2014; Libkind *et al.,* 2011; Masneuf-Pomarede *et al.,* 2016; Naumov *et al.,* 2011; Rodríguez *et al.,* 2014; Sampaio and Goncalves, 2008) and alcoholic beverages fermented at low temperature (Coton *et al.,* 2006; Demuyter *et al.,* 2004; Masneuf-Pomarede *et al.,* 2016; Masneuf-Pomarede *et al.,* 2016; Rodríguez *et al.,* 2017; Sipiczki, 2002; Suárez Valles *et al.,* 2007). The existence of at least three different clades has been recently proposed for *S. uvarum*: clade 'C' from Autralasia; clade 'B' corresponding to South American natural strains (South American population B or SA-B); and clade 'A' including both South American natural strains (South American population A or SA-A) and all *S. uvarum* Holartic strains (Almeida *et al.,* 2014). The recent discovery of *S. uvarum* strains in fermented beverages (apple *chichas*) in Patagonia (Rodríguez *et al.,* 2017) introduced a new factor not discussed by Almeida *et al*. (2014). In the work by Rodríguez *et al*. (2017), phylogenetic and population structure analyses based on multilocus sequences demonstrated that the *S. uvarum* strains

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from *chichas* were included in the clade A. That result suggested that *S. uvarum* strains from *chichas* from Patagonia could be originated from Holartic strains, introduced in South America together with the domestication of apple trees by Mapuche communities. Besides their genetic differences, we demonstrated that the strains from natural or fermentative environments in South America also possess distinct physiological features of relevance for cider making (González Flores *et al.,* 2017).

In this work, we evaluated the main oenological features of the hybrids generated by a common non-GMO producing technique for the first time with two genetically and physiologically different *S. uvarum* strains and the same wine *S. cerevisiae* strain. The effect of different temperatures (13 and 20°C) for hybrid generation was also evaluated to optimize and obtain the best candidates for white wine fermentation at low temperatures in North Patagonia.

Materials and methods

Yeasts strains

Nineteen Patagonian yeast strains, belonging to the *S. uvarum* species, were used in the present study: five strains from *Araucaria araucana* bark (Su^{a}) and 14 strains from artisanal apple *chichas* (Su^{ch}). All the strains have been genetically characterized in previous studies (Rodríguez *et al.,* 2014, 2017), and are deposited in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina. *S. uvarum* CBS 7001, and a commercial wine strain identified as *S. cerevisiae* were used for comparison purposes.

Generation of interspecific hybrid yeasts strains

A natural auxotrophic ($lys2^{-}$) strain of *S. cerevisiae* was generated by cultivating colonies in Minimum Medium (% p/v: 0.17 Yeast Nitrogen Base without amino acids, 2 glucose, 2 agar-agar), added with α-aminoadipic acid following the methodology proposed by Zaret and Sherman (1985), and partially modified by Pérez-Través *et al*. (2012).

Hybrids were generated by rare mating between two selected *S. uvarum* strains and the auxotrophic strain of *S. cerevisiae* (lys^{-}), according to Pérez-Través *et al*. (2012). Hybrid colonies were selected on Minimum Medium plates, incubated at 37°C (the *S. uvarum* strains were unable to grow at 37°C) for 4–5 days. The colonies that grew under these conditions were repitched in the same medium and were immediately conserved in 20% v/v glycerol at −80°C. Hybrid nature was confirmed by the PCR amplification of the *CBT2* and *GSY1* nuclear genes, and the subsequent restriction analysis with endonucleases *Hae* III and *EcoR* I, as described below.

Having confirmed the newly formed hybrid strain from each cross, it was genetically stabilized by five successive fermentations in glass flasks that contained 10 mL of sterilized (120°C, 20min) *Sauvignon blanc* grape must (13.8° Brix). Microfermentations were carried out with no shaking at two temperatures: 13 and 20°C. After each fermentation (20–25 days, depending on the incubation temperature), a 100 μL aliquot was used to inoculate the following flask that contained 10 mL of the same fresh must, and was incubated under the same conditions. This procedure was repeated for five fermentations, as suggested by Pérez-Través *et al*. (2012). An aliquot (100 μL) from the last (fifth) fermentation was then used for the hybrid colonies isolation on GPY-agar plates (% p/v: 0.5 peptone, 0.5 yeast extract, 2 glucose, 2 agar-agar) at the same temperature.

Ten putative stable hybrid colonies were randomly picked and characterized by RAPD-PCR and mtDNA-RFLP analyses, as proposed by Pérez-Través *et al*. (2012).

The hybrids randomly selected among those which exhibited different molecular combined patterns were individually inoculated in new sterile must and incubated under the same conditions. After these last fermentations, 10 colonies were isolated and molecularly analysed. Their molecular patterns were compared with that in the colony used for inoculation. Hybrids were considered genetically stable when this comparison showed the same combined molecular pattern.

Molecular and genetic analyses

PCR-RFLP analysis of nuclear genes

Total DNA isolation was performed according to Querol *et al*. (1992). The PCR amplifications of the 33 protein-encoding nuclear genes distributed along all of the 16 *S. cerevisiae* and 16 *S. uvarum* chromosomes were carried out with the DNA

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extracted from hybrids. The primers, amplification conditions and differential patterns for each species were obtained from Pérez-Través *et al*. (2014).

Sequencing analysis

Both nuclear gene *MNL1* and mitochondrial gene *COX2* were amplified and sequenced from genetically stable hybrids, as described by Pérez-Través *et al*. (2014) and by Belloch *et al*. (2000), respectively. PCR products were cleaned with the AccuPrep PCR purification kit (Bioneer, Inc, USA) and submitted to the sequencing service (Macrogen, Korea).

DNA content analysis

The total DNA content in the genetically stable hybrids was estimated by a flow cytometry analysis in a FACScan cytometer (Becton Dickinson Inmunocytometry System) following the SYTOX Green method described in Haase and Reed (2002). Ploidy levels were scored on the basis of florescence intensity compared with the haploid (S288c) and diploid (FY1679) reference *S. cerevisiae* strains. The values reported were the result of three independent measures.

Temperature growth profiles

Temperature growth profiles were evaluated following the methodology described by Belloch *et al*. (2008). The analysis was performed on the GPY-agar medium inoculated with drops of serial dilutions (1:5 each) of the respective yeast strain (six dilutions in all). Plates were incubated at the appropriate temperature (4, 8, 13, 20, 25, 30 and 37°C) until colonies appeared in all the dilutions on plates under no-stress condition (25°C).

Fermentations

Laboratory-scale fermentations were carried out in 50 mL flasks that contained 35 mL of sterilized (120°C, 15 min) synthetic must MS 300 (Rossignol *et al.,* 2003) and *Sauvignon blanc* must (13.8). Fermentations were inoculated individually with 2×10^6 CFU mL^{-1} of the respective yeast strain and were incubated at 13°C (MS 300), or at both 13 and 20°C (*Sauvignon blanc* must) without shaking. Fermentation evolution was followed daily by weight loss until constant weight during two consecutive measures. Experiments were carried out in triplicate.

Scaled-up fermentations (1 L flasks that contained 800 mL must) were carried out using the same *Sauvignon blanc*. Fermentations were inoculated with 2×10^6 cells/mL and incubated at 13 and 20°C. In this case, fermentation evolution was monitored by measuring °Brix on a daily basis. Fermentations were carried out in duplicate.

In all cases when alcoholic fermentations had been completed, the fermented products were centrifuged (4000 ***g*** for 5 min) to obtain the supernatants, and their oenological and kinetic parameters were determined as described below.

General physicochemical oenological parameters

Enzyme commercial kits were used to determine glycerol (Boehringer Mannheim) and residual sugars (glucose and fructose; Megazyme). In microfermentations (50 mL flasks), the ethanol concentration and volatile acidity were determined in an OenoFoss wine analyser Fourier-Transform Infrared Spectroscopy (FTIR). In the 1 L fermentations, ethanol concentration was determined by steam distillation, while volatile acidity was established by steam distillation followed by titration with NaOH 0.1 M, and was expressed as acetic acid (g L^{-1}), according to the methods proposed by Ribereau-Gayon *et al*. (2003).

Determinations of higher alcohols, esters, acetaldehyde and terpenes

Aliquots of the fermented young wines were analysed by headspace solid-phase microextraction sampling using 50/30 μm DVB/CAR/PDMS fibres (Sigma-Aldrich), and gas chromatography according to Rojas *et al*. (2001). Aliquots of 1.5 mL of samples were placed in 15 mL phials and 0.3 g of NaCl and 15 μL of 0.1% (v/v) 2-octanol in ethanol were added as an internal standard. Phials were closed with screwed caps and 3 mm-thick teflon septa. Fibres were injected through the phial septum and exposed to the headspace for 30 min, and were then desorbed for 10 min in an HP 7890 series II gas chromatograph equipped with an HP Innowax column (Hewlett-Packard; length, 60 m; inside diameter, 0.32 mm; film thickness,

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0.50 μm). The injection block and detector Flame Ionization Detector (FID) temperatures were kept constant at 220 and 250°C, respectively. The oven temperature was programmed as follows: 40 (7 min) to 180°C at 5°C min^{-1}, and 200–260°C at 20°C min^{-1}, and kept for 15 min at 260°C. The total running time was 75 min.

The following standards were purchased from Sigma Aldrich: isobutilic alcohol, isoamylic alcohol, 1-hexanol, bencylic alcohol, 2-phenyl ethanol, ethyl acetate, isobutyl acetate, ethyl lactate, isoamyl acetate, hexyl acetate, diethyl succinate, bencyl acetate, ethyl caprylate, ethyl 3-hydroxibutanoate, 2-penylethyl acetate, 4-terpineol, limonene, linalool, nerol and geraniol. All standards were of N99% purity. The values calculated for each different compound were the average of two independent assays.

Organic acid determinations

Organic acids were determined by HPLC using a liquid chromatograph with a UV–visible detector (Shimadzu), according to the Official Methods of Analysis of AOAC International. The equipment also contained a bomb (LC 20AT), a column oven (CTO 6A), a controller (CBM 20A), an autoinjector (SIL 10 A), a detector diode array (SPD-M10A) and computer software data acquisition (LC Solution). Samples were filtered through 0.45 μm nylon filters and were directly injected (10 μL) into the chromatographic column.

Standard solutions (malic acid, lactic acid, acetic acid and shikimic acid) were prepared by diluting individual compounds in ultrapure water.

Statistical analysis

Kinetic parameters were individually calculated from each fermentation. For microfermentations, the analysis was performed using the amount of CO_2 lost daily by the system and the reparametized Gompertz equation proposed by Zwietering *et al.* (1990):

$$y = A * \exp\left(-\exp\left(\left(\frac{\mu_{max} * 2.718282}{A}\right) * (\lambda - t) + 1\right)\right)$$

where $y = \ln(N_t/N_0)$, N_0 being the system's initial weight (g) and N_t the weight at time t; $A = \ln(N_\infty/N_0)$ is the maximum CO_2 production with N_∞ as the asymptotic maximum; μ_{max} is the maximum fermentation rate (h^{-1}); and λ is the period of time needed to start vigorous fermentation by minimizing the sum of the squares of the difference between the experimental data and the fitted model (observed – predicted)^2. This analysis was run using the nonlinear module of the Statistica 8.0 software package and its Quasi-Newton option.

For the 1 L fermentations, °Brix decrease was fitted to the previous equation, but some parameters presented different meanings: a dependent variable represents the concentration of total soluble solids (°Brix) and μ_{max} is the maximum consumption rate (°Brix h^{-1}).

ANOVA and Tukey honest significant difference tests, with $\alpha = 0.05$, were performed by comparing the kinetic and physiological analyses. The data normality and variance homogeneity in the residuals were verified by the Lilliefors test and by the Bartlet test, respectively. Principal component (PCA) and clusters (Unweighted Pair Group Method with Arithmetic Mean, UPGMA) analyses were performed on the kinetic and physicochemical parameters, and on the volatile compounds with the NTSYS programme (Numerical Taxonomic System version 2.11; Rohlf, 2000).

Results

Physiological characterization and selection of the *S. uvarum* parental strains

Nineteen *S. uvarum* strains isolated from natural habitats (five strains) and apple *chichas* (14 strains) were analysed for their main physiological features of relevance in oenology. For this purpose, microfermentations (50 mL flasks) containing synthetic must were carried out with cultures of each different strain. The kinetic and physicochemical parameters obtained from these fermentations are shown in Table S1. All of this information was used to perform a PCA, which allowed us to determine the putative relationships among products and hence yeast strains. A PCA plot was generated to group strains according to their features (Figure 1). The first two components explained 47.57% of the total variability in data. Three clusters were clearly separated in the PCA

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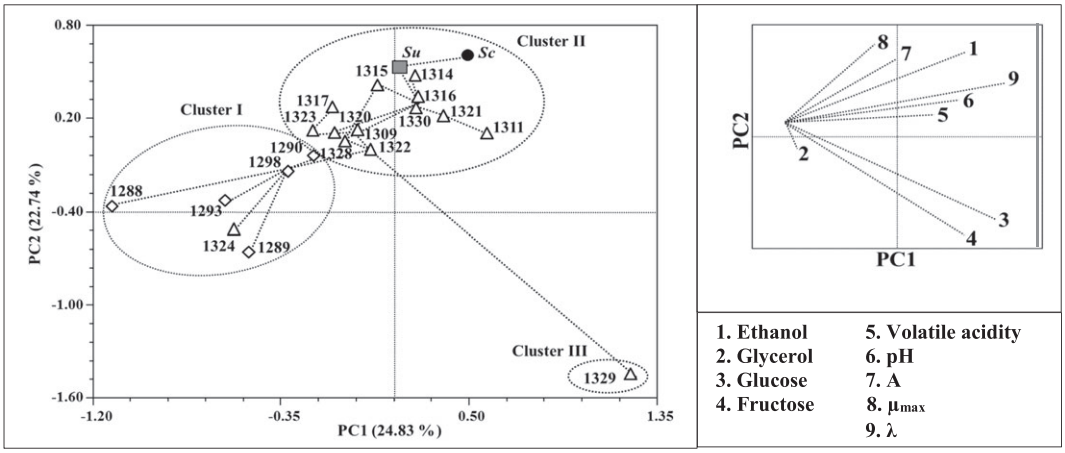

Yeast strains analysed: ■ *S. uvarum* CBS 7001 (*Su*); ● Commercial *S. cerevisiae* (*Sc*); ◇ *S. uvarum* from *Araucaria araucana* (Su^{a}); △ *S. uvarum* from *chicha* (Su^{ch}).

Figure 1. PCA (principal component analysis) generated from the analysis of physicochemical and kinetic parameters obtained in synthetic must fermentations for all yeast strains analysed

plot; cluster I grouped the products of all the *S. uvarum* strains isolated from natural habitats, as well as strain *S. uvarum* NPCC 1324 from apple *chicha*. This cluster was characterized mainly by the lowest values for most parameters, as indicated by the vectors in Figure 1. Cluster II included all the remaining strains from apple *chicha* (except strain *S. uvarum* NPCC 1329) and reference strains *S. uvarum* CBS 7001 and *S. cerevisiae*, and was characterized mainly by higher ethanol concentrations and the highest values for kinetic parameters μ_{max}, λ and *A*. Finally, Cluster III was composed only of the *S. uvarum* NPCC 1329 product from apple *chicha*, characterized by high residual sugars (Figure 1 and Table S1). The glycerol concentration was variable, with strains exhibiting high and low levels inside Clusters I and II, However, *S. cerevisiae* produced the lowest level of this metabolite (Table S1).

In light of these results, we decided to select one *S. uvarum* strain that represented each cluster (two clusters) associated with different origins. Strain NPCC 1290 was selected among the strains from *A. araucana* given its high glycerol production. Strain *S. uvarum* NPCC 1314 from apple *chicha* was selected for its capacity to produce high glycerol and low ethanol concentrations, as well as the low levels of residual sugars in the final products (Table S1). These two strains also showed interesting fermentative behaviour in apple must and enzymatic activities of interest in oenology according to a previous study carried out in our laboratory (González Flores *et al.,* 2017). The selected strains were used as parental strains for hybrid generation, together with a commercial wine strain.

Hybridization and genetic stabilization under oenological conditions

A natural lys^{-} auxotrophic mutant of the *S. cerevisiae* wine parental strain, obtained in α-amonoadipic acid agar plates, was used to generate the interspecific hybrids with the cultures of both wild strains *S. uvarum* NPCC 1290 and 1314. The hybrids generated by the rare-mating method were selected on minimum media agar plates incubated at 37°C and confirmed for nuclear genes *CBT2* and *GSY1* by PCR-RFLP (Figure 2). After generation, hybrids were subjected to genetic stabilization based on consecutive fermentations in *Sauvignon blanc* at both 13 and 20°C.

The carbon dioxide release data obtained during all of the successive fermentations were fitted to a decay model, and kinetic parameters were obtained and compared throughout the process at the two analysed temperatures (Table S2). Significant differences in the total time required for completing fermentations were observed at the two temperatures (3800 vs. 1900 h for *S. cerevisiae* × *S. uvarum*^{a} and 3100 vs. 1900 h for *S. cerevisiae* ×

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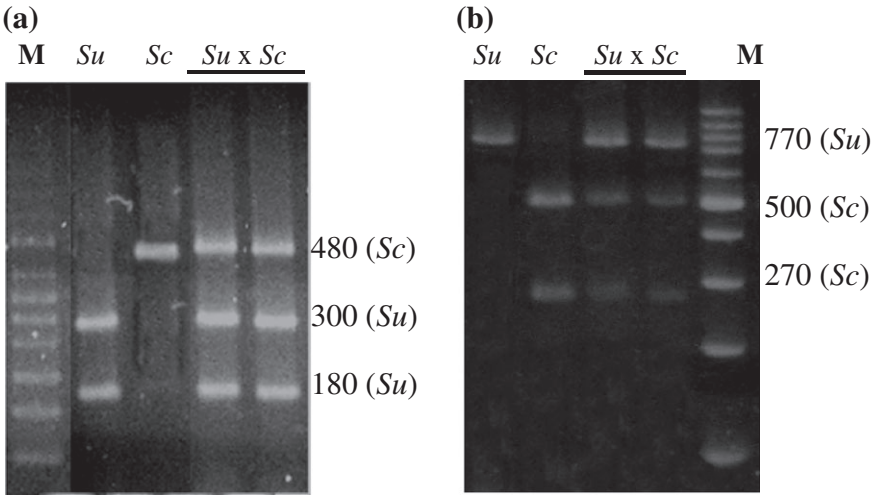

Figure 2. Confirmation of interespecific hybrids by PCR-RFLP of (a) *CBT1* nuclear gene with *Hae* III and (b) *GSY1* nuclear gene with *EcoR* I. M, molecular ladder (100 and 50 bp). Values on the right indicate molecular weight (base pairs) of restriction bands obtained for parental and hybrid strains. *Sc*, *Saccharomyces cerevisiae*; *Su*, *Saccharomyces uvarum*. *Su* × *Sc*, hybrid strains. M, molecular marker (50 bp and 100 bp DNA ladder)

S. uvarum^{ch} at 13 and 20°C, respectively) and independently of the *S. uvarum* parental strain involved in hybrid generation (Table S2). Similar differences were also observed during successive fermentations, from 405 h in the first step for the two crosses, to 950–1400 h in the fifth step (Table S2). Interestingly, the λ value decreased during the last two fermentations (the fourth and fifth fermentations) of the two crosses (Table S2). When the complete process ended, four stable colonies were confirmed from the cross *S. cerevisiae* × *S. uvarum*^{a} at 13°C, as were four colonies at 20°C. Similarly, five stable colonies were confirmed from the cross *S. cerevisiae* × *S. uvarum*^{ch} at each temperature (13 and 20°C). Genetic stability was demonstrated by the invariability of RAPD-PCR (Figure S1 in the Supporting Information) and mtDNA-RFLP patterns, as proposed by Pérez-Través *et al*. (2014) as well as because all of the stable hybrids obtained in this work showed a total DNA content between 1.85*n* and 2.04*n* (Table 1). Additionally, the PCR-RFLP analysis of 33 genes (including the sequencing analysis of *MNL1* for which no discriminative restriction patterns have been proposed) distributed along the 16 chromosomes suggested that hybrids retained a complete subgenome of each parental species (data not shown), while the mtDNA-RFLP analysis with endonuclease *Hinf* I and *COX2* sequencing demonstrated the monoparental inheritance of *S. cerevisiae* mtDNA in all the stable hybrids (Table 1).

Characterization and selection of hybrids

Fermentative behaviour

All of the stable colonies and their respective parental strains were subsequently used to perform individual fermentations in the same grape must at the same temperature used to produce them (13 and 20°C). The physicochemical and kinetic data obtained from fermentations demonstrated that, regardless of the *S. uvarum* parental strains employed for hybrid generation, hybrids displayed similar features. This phenomenon was observed at both of the analysed temperatures (13 and 20°C; Table 2). The PCA analyses of these data demonstrated this homogeneity; at both 13 and 20°C, all of the hybrids grouped in the same cluster together (20°C), or were closely related (13°C) to the parental *S. uvarum*^{ch} (cluster I), while *S. cerevisiae* and *S. uvarum*^{a} were respectively located in the separate clusters II and III (Figure 3).

As a general rule, hybrids produced the highest glycerol levels, which were particularly high at

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Table 1. Genetic characterization of stable hybrids

Temperature	Cross*	Hybrid	RAPD-PCR molecular patterns**			$_{mt}$DNA†	DNA content‡
			p24	p28	Comb		
13 °C	*S. cerevisiae* × *S. uvarum*[a]	H1	B	1	B1	*S. cerevisiae*	1.80 ± 0.02
		H2	A	1	A1	*S. cerevisiae*	2.04 ± 0.01
		H3	A	3	A3	*S. cerevisiae*	1.87 ± 0.00
		H4	C	1	C1	*S. cerevisiae*	1.79 ± 0.02
	S. cerevisiae × *S. uvarum*[ch]	H5	B	2	B2	*S. cerevisiae*	1.92 ± 0.06
		H6	A	2	A2	*S. cerevisiae*	1.85 ± 0.01
		H7	B	3	B3	*S. cerevisiae*	1.89 ± 0.12
		H8	D	2	D2	*S. cerevisiae*	1.85 ± 0.00
		H9	A	3	A3	*S. cerevisiae*	1.91 ± 0.01
20 °C	*S. cerevisiae* × *S. uvarum*[a]	H10	A	1	A1	*S. cerevisiae*	1.99 ± 0.00
		H11	C	2	B2	*S. cerevisiae*	1.63 ± 0.01
		H12	C	4	C4	*S. cerevisiae*	2.03 ± 0.01
		H13	C	1	C1	*S. cerevisiae*	1.87 ± 0.04
	S. cerevisiae × *S. uvarum*[ch]	H14	C	1	C1	*S. cerevisiae*	1.99 ± 0.03
		H15	A	1	A1	*S. cerevisiae*	1.86 ± 0.03
		H16	D	3	D3	*S. cerevisiae*	1.77 ± 0.02
		H17	D	1	D1	*S. cerevisiae*	1.94 ± 0.04
		H18	B	1	B1	*S. cerevisiae*	1.89 ± 0.00

*Superscript letters in *S. uvarum* indicate origin ([a] *A. araucana*; [ch] *chicha*).
**Molecular patterns obtained by p24 (primer p24), p28 (primer p28) and Comb (combination of patterns obtained with the two primers).
†Pattern obtained by mtDNA-RFLP and sequencing of *COX2* gene. *S. cerevisiae* indicates that the hybrids exhibited the mtDNA-RFLP pattern found in the *S. cerevisiae* parental strain and also the same sequence for gene *COX2*.
‡Measured by flow cytometry.

20°C, and the parental *S. cerevisiae* generated the highest ethanol and volatile acidity concentrations at both of the analysed temperatures.

Temperature growth profiles

As an additional characterization of hybrids, we evaluated the effect of temperature on yeast growth. Serial dilutions of each different strain (both hybrids and parents) were dropped into GPY agar plates and incubated at different temperatures (4, 8, 13, 20, 25, 30 and 37°C). All the analysed yeasts grew well at temperatures between 20 and 30°C, but were unable to grow at 4°C after a 15 day incubation (Table 3). None of the *S. uvarum* strains was able to grow at 37°C, but both the *S. cerevisiae* parental strain and all of the hybrids grew at this temperature in most of the tested dilutions. In contrast, at 8°C, the *S. uvarum* parental strains grew until dilution 5 and hybrids grew until dilutions 1–3, while *S. cerevisiae* was unable to grow (Table 3).

Selection of hybrids and production of aroma compounds in wine

Owing to the homogeneity of the physiological parameters observed for all of the hybrids, one that was representative of each cross and temperature was selected to perform the 1 L fermentations in the same must to obtain data about the production of their differential volatile compounds. All of the chemical compounds measured in the young wines fermented with both the selected hybrids and parental strains are shown in Table S3. Once again, a PCA was carried out with these data, and the plot obtained from PC 1 and PC 2, which explained 66.85% of total variability, is shown in Figure 4. This analysis allowed us to differentiate three different clusters associated with the yeast used for fermentation rather than with the fermentation temperature. Cluster I was composed of the fermented products obtained with the parental *S. cerevisiae* at both temperatures; Cluster II comprised the wines produced by the two *S. uvarum* parental strains; Cluster III grouped the wines fermented with all four hybrids. The wines

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Table 2. Physicochemical and kinetic parameters obtained from microfermentations of interspecific hybrids and parental strains in *Sauvignon blanc* must at 13 and 20°C

Temperature	Yeast species*	Strain*	Physicochemical parameters						Kinetic parameters		
			Ethanol (%v/v)	Glycerol (g/L)	Glucose (g/L)	Fructose (g/L)	Volatile acidity (g/L)	pH	*A* (g)	μ (g/h)	λ (h)
13 °C	*Saccharomyces cerevisiae*	Commercial	9.83 ± 0.64^{b}	4.04 ± 0.04^{a}	0.005 ± 0.002^{a}	0.014 ± 0.004^{a}	0.67 ± 0.06^{c}	3.29 ± 0.02^{abc}	2.867 ± 0.169^{b}	0.017 ± 0.001^{c}	64.495 ± 2.340^{a}
	Saccharomyces uvarum	NPCC 1290^{a}	9.09 ± 0.02^{ab}	5.43 ± 0.10^{f}	0.008 ± 0.006^{a}	0.026 ± 0.000^{a}	0.30 ± 0.01^{a}	3.34 ± 0.00^{bc}	2.619 ± 0.097^{ab}	0.018 ± 0.001^{c}	64.732 ± 2.112^{a}
		NPCC 1314^{ch}	9.00 ± 0.00^{ab}	4.35 ± 0.07^{ab}	0.009 ± 0.001^{a}	0.046 ± 0.014^{a}	0.45 ± 0.03^{b}	3.37 ± 0.01^{c}	2.764 ± 0.069^{ab}	0.014 ± 0.001^{bc}	104.806 ± 2.672^{e}
	S. cerevisiae × *S. uvarum*^{a}	H1	9.32 ± 0.28^{ab}	4.56 ± 0.33^{abc}	0.016 ± 0.007^{a}	0.105 ± 0.071^{a}	0.25 ± 0.03^{a}	3.32 ± 0.04^{abc}	2.436 ± 0.169^{a}	0.010 ± 0.000^{ab}	74.461 ± 4.757^{abc}
		H2	9.33 ± 0.06^{ab}	4.92 ± 0.04^{cdef}	0.000 ± 0.000^{a}	0.620 ± 0.076^{b}	0.27 ± 0.02^{a}	3.33 ± 0.02^{bc}	2.467 ± 0.130^{a}	0.010 ± 0.001^{ab}	80.992 ± 0.243^{bcd}
		H3	9.00 ± 0.42^{ab}	4.50 ± 0.20^{abc}	0.030 ± 0.040^{a}	0.083 ± 0.026^{a}	0.24 ± 0.00^{a}	3.30 ± 0.01^{abc}	2.589 ± 0.043^{ab}	0.010 ± 0.002^{ab}	65.361 ± 5.083^{a}
		H4	9.00 ± 0.13^{ab}	4.62 ± 0.19^{bcd}	0.003 ± 0.004^{a}	0.048 ± 0.001^{a}	0.20 ± 0.01^{a}	3.24 ± 0.04^{ab}	2.637 ± 0.050^{ab}	0.011 ± 0.001^{ab}	77.349 ± 4.021^{bcd}
	S. cerevisiae × *S. uvarum*^{ch}	H5	8.70 ± 0.14^{a}	5.04 ± 0.01^{cdef}	0.002 ± 0.003^{a}	0.043 ± 0.007^{a}	0.22 ± 0.01^{a}	3.33 ± 0.01^{bc}	2.687 ± 0.034^{ab}	0.009 ± 0.000^{a}	70.542 ± 2.756^{ab}
		H6	8.90 ± 0.14^{ab}	5.14 ± 0.00^{def}	0.008 ± 0.007^{a}	0.038 ± 0.009^{a}	0.21 ± 0.01^{a}	3.22 ± 0.01^{a}	2.504 ± 0.040^{ab}	0.011 ± 0.000^{ab}	76.891 ± 0.810^{bcd}
		H7	8.60 ± 0.00^{a}	5.17 ± 0.03^{ef}	0.006 ± 0.000^{a}	0.031 ± 0.003^{a}	0.21 ± 0.00^{a}	3.29 ± 0.02^{abc}	2.627 ± 0.046^{ab}	0.011 ± 0.001^{ab}	71.545 ± 0.058^{ab}
		H8	8.70 ± 0.00^{a}	4.81 ± 0.12^{bcde}	0.007 ± 0.001^{a}	0.046 ± 0.026^{a}	0.20 ± 0.03^{a}	3.27 ± 0.01^{abc}	2.652 ± 0.137^{ab}	0.010 ± 0.002^{ab}	82.562 ± 0.413^{cd}
		H9	9.08 ± 0.03^{ab}	4.65 ± 0.08^{bcde}	0.035 ± 0.034^{a}	0.084 ± 0.044^{a}	0.23 ± 0.04^{a}	3.27 ± 0.06^{abc}	2.492 ± 0.041^{ab}	0.010 ± 0.001^{ab}	85.782 ± 0.467^{d}
20 °C	*S. cerevisiae*	Commercial	10.57 ± 0.82^{a}	3.89 ± 0.25^{a}	0.008 ± 0.006^{a}	0.029 ± 0.002^{a}	1.53 ± 0.05^{b}	3.35 ± 0.01^{a}	3.042 ± 0.218^{a}	0.022 ± 0.002^{a}	22.112 ± 5.583^{ab}
	S. uvarum	NPCC 1290^{a}	9.56 ± 0.27^{a}	5.04 ± 0.71^{ab}	0.069 ± 0.013^{b}	0.113 ± 0.039^{b}	0.27 ± 0.08^{a}	3.37 ± 0.11^{a}	2.791 ± 0.234^{a}	0.023 ± 0.001^{a}	12.814 ± 12.676^{a}
		NPCC 1314^{ch}	9.42 ± 0.59^{a}	4.91 ± 0.57^{ab}	0.029 ± 0.017^{ab}	0.050 ± 0.014^{ab}	0.48 ± 0.29^{a}	3.37 ± 0.01^{a}	2.806 ± 0.277^{a}	0.028 ± 0.001^{a}	44.173 ± 9.314^{b}
	S. cerevisiae × *S. uvarum*^{a}	H10	9.41 ± 0.12^{a}	5.21 ± 0.16^{ab}	0.013 ± 0.003^{a}	0.029 ± 0.002^{a}	0.27 ± 0.05^{a}	3.29 ± 0.13^{a}	2.820 ± 0.107^{a}	0.023 ± 0.001^{a}	37.459 ± 5.353^{ab}
		H11	9.52 ± 0.02^{a}	5.54 ± 0.06^{ab}	0.020 ± 0.003^{a}	0.033 ± 0.006^{a}	0.18 ± 0.08^{a}	3.24 ± 0.03^{a}	2.727 ± 0.096^{a}	0.024 ± 0.000^{a}	38.251 ± 0.346^{ab}
		H12	9.42 ± 0.19^{a}	4.73 ± 0.04^{ab}	0.009 ± 0.004^{a}	0.024 ± 0.005^{a}	0.24 ± 0.01^{a}	3.26 ± 0.06^{a}	2.901 ± 0.534^{a}	0.023 ± 0.004^{a}	21.926 ± 7.201^{ab}
		H13	9.51 ± 0.04^{a}	5.98 ± 0.36^{b}	0.017 ± 0.002^{a}	0.028 ± 0.005^{a}	0.23 ± 0.01^{a}	3.24 ± 0.04^{a}	2.555 ± 0.047^{a}	0.024 ± 0.001^{a}	45.171 ± 2.298^{b}
	S. cerevisiae × *S. uvarum*^{ch}	H14	9.40 ± 0.11^{a}	5.94 ± 0.43^{b}	0.010 ± 0.002^{a}	0.036 ± 0.004^{ab}	0.26 ± 0.02^{a}	3.30 ± 0.07^{a}	2.532 ± 0.006^{a}	0.027 ± 0.002^{a}	38.775 ± 3.566^{ab}
		H15	9.41 ± 0.25^{a}	5.65 ± 0.09^{b}	0.007 ± 0.000^{a}	0.033 ± 0.007^{a}	0.25 ± 0.04^{a}	3.21 ± 0.04^{a}	2.615 ± 0.023^{a}	0.029 ± 0.005^{a}	25.917 ± 0.569^{ab}
		H16	9.54 ± 0.11^{a}	5.54 ± 0.44^{b}	0.003 ± 0.001^{a}	0.023 ± 0.007^{a}	0.24 ± 0.01^{a}	3.25 ± 0.03^{a}	2.674 ± 0.177^{a}	0.023 ± 0.002^{a}	37.710 ± 7.328^{ab}
		H17	9.45 ± 0.08^{a}	6.20 ± 0.31^{b}	0.015 ± 0.015^{a}	0.037 ± 0.023^{ab}	0.24 ± 0.01^{a}	3.22 ± 0.01^{a}	2.669 ± 0.083^{a}	0.022 ± 0.001^{a}	38.976 ± 4.566^{ab}
		H18	9.34 ± 0.26^{a}	4.92 ± 0.74^{ab}	0.018 ± 0.024^{a}	0.054 ± 0.048^{ab}	0.26 ± 0.05^{a}	3.28 ± 0.07^{a}	2.943 ± 0.542^{a}	0.022 ± 0.003^{a}	21.423 ± 11.016^{ab}

Different superscript letters in the same column indicate significant differences between values obtained from fermentations at the same temperature (ANOVA and Tukey test $n = 2$).
*Superscripts a and ch correspond to strains isolated from *A. araucana* and *chicha*, respectively.

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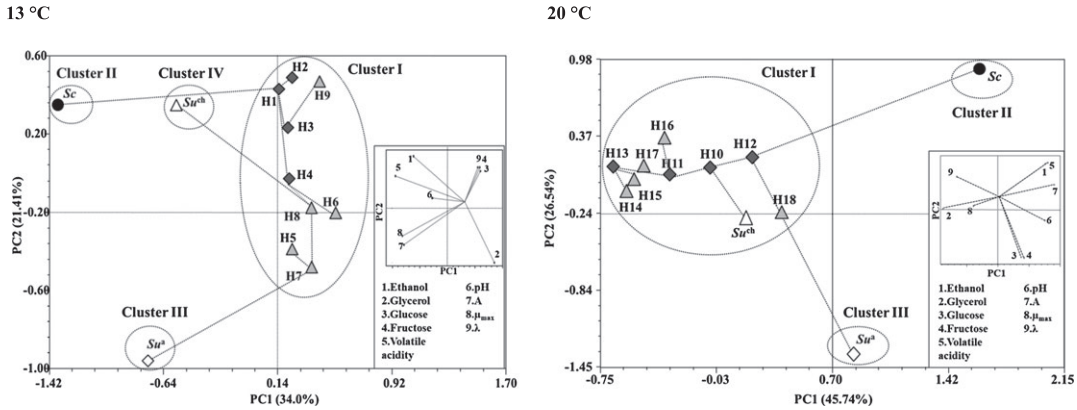

Parental strains: ● Commercial *Sc*; ◇ *Su* from *A. araucana* (Su^a); △ *Su* from chicha (Su^{ch}). Hybrid strains: ◆ Su^a x *Sc*; △ Su^{ch} x *Sc*.

Figure 3. Analysis of physicochemical and kinetic parameters from microfermentations of interspecific hybrids and parental strains in *Sauvignon blanc* at 13 and 20°C

fermented with *S. cerevisiae* (Cluster I) gave the highest ethanol concentrations, higher alcohols isoamylic alcohol, bencylic alcohol and 1-hexanol and most ethyl esters, including ethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, and acetate esters (isoamyl acetate and isobutyl acetate). The fermented products obtained with the two *S. uvarum* (Cluster II) strains showed the highest glycerol levels, organic acids lactic and shikimic, higher alcohol 2-phenylethanol, and esters 2-phenylethyl acetate and ethyl lactate. Finally, all of the hybrids produced wines with the highest levels of malic acid, isobutyl alcohol and 1-propanol (Figure 4).

Irrespective of the differential chemical profiles that characterized the three clusters, it was noteworthy that hybrid H17 (*S. cerevisiae* × *S. uvarum*^{ch}) produced the highest levels of the total higher alcohols and the lowest levels of acetaldehyde (Table S3).

Table 3. Temperature stress response of parental and hybrid strains

Yeast strains		Temperature growth (°C)						
		4	8	13	20	25	30	37
Parental	*S. cerevisiae*	0	0	5	6	6	6	6
	S. uvarum^a	0	5	6	5	6	5	0
	S. uvarum^{ch}	0	5	6	6	6	5	0
Hybrids 13°C	H1	0	3	5	6	6	5	5
	H2	0	3	4	6	6	6	6
	H3	0	3	4	6	6	6	6
	H4	0	3	4	6	6	6	6
	H5	0	3	5	6	6	6	5
	H6	0	3	5	6	6	6	5
	H7	0	3	5	6	6	6	6
	H8	0	3	4	6	6	6	6
	H9	0	3	5	6	6	6	6
Hybrids 20 °C	H10	0	3	4	6	6	5	5
	H11	0	3	4	6	6	6	5
	H12	0	1	4	6	6	5	5
	H13	0	2	4	6	6	6	5
	H14	0	2	5	6	6	5	5
	H15	0	3	5	6	6	5	4
	H16	0	3	5	6	6	6	5
	H17	0	3	5	6	6	6	5
	H18	0	3	5	6	6	5	5

Numbers from 1 to 6 indicate the dilutions at which colony development was observed (0 idicates absence of growth and 6 indicates growth at the highest dilution). Shadowed rows indicate the selected hybrids used in aroma compound production evaluation.

Discussion

Based on the hypothesis that both different genetic backgrounds in the parents and the different selective pressures on the newly formed hybrids would lead to different genomic and, consequently, different phenotypic outcomes, we developed a strategy by varying both the *S. uvarum* parental strain and temperature for hybrid generation using the rare-mating hybridization method.

To date all *S. cerevisiae* × *S. uvarum* hybrids have been obtained from *S. uvarum* strains isolated from wines, which represent only one part of the genetic complexity of the *S. uvarum* species. In the present work, we used both strains belonging

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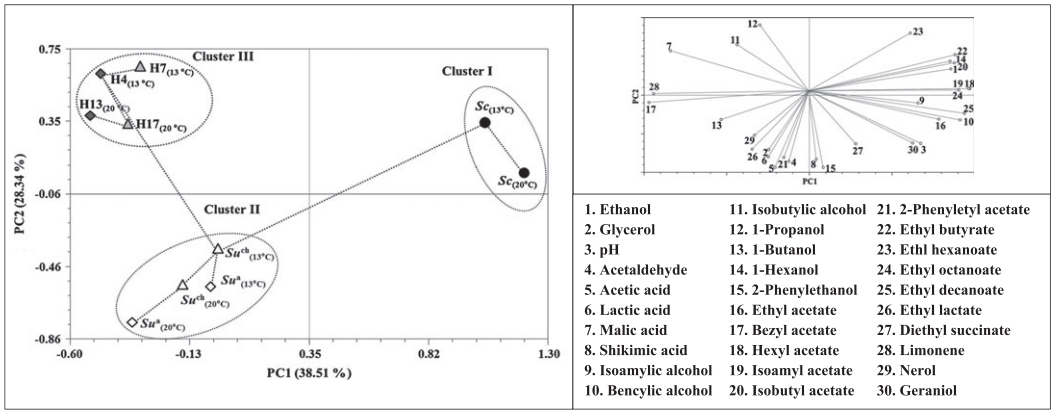

The total yeast strains analysed were fermented in 1 litre Sauvingnon blanc must at 13°C and 20°C. Parental strains: ● Commercial *Sc*; ◇ *Su* from *A. araucana* (Su^{a}); △ *Su* from chicha (Su^{ch}). Hybrid strains: ◆ Su^{a} x *Sc*; △ Su^{ch} x *Sc*.

Figure 4. PCA obtained from physicochemical composition of *Sauvignon blanc* wines obtained with interspecific hybrids and parental strains at 13 and 20°C

to the *S. uvarum* clade 'B' isolated from *A. araucana* trees and strains belonging to the clade 'A' isolated from Patagonian apple *chichas*. As a general rule, the physicochemical and kinetic parameters obtained in microfermentations carried out with the analysed strains show differences according to isolation origin. The only exception was the strain NPCC 1324 from apple *chicha*, which produced fermented products in synthetic must that was grouped with those generated with the South American strains isolated from natural habitats. Interestingly, strain NPCC 1324 was not genetically characterized in our previous work (Rodríguez *et al*., 2017), hence it could belong to a South American native population (population B according to Almeida *et al.,* 2014) even if it was isolated from apple *chicha*. Likewise, *S. uvarum* strain NPCC 1289, which had been isolated from an *A. araucana* tree bark sample, was genetically characterized in our laboratory as belonging to the clade 'A' (Rodríguez *et al.,* 2017). This fact, plus the presence of admixture strains that contained mosaic genomes containing gene regions from both 'A' and 'B' clades, demonstrated the genetic exchange among populations of this species in South America (Almeida *et al.,* 2014; Rodríguez *et al.,* 2017). Our analysis also demonstrated that the two *S. uvarum* clusters of strains, which are associated with two populations or origins, separated mainly according to the total amount of ethanol produced during fermentation, where the strains from *chicha* were the best ethanol producers. Differences in the physiological traits among the yeast strains from one same species, but obtained from different sources, have been described in *S. cerevisiae* species (Diezmann and Dietrich, 2009). Conversely, very little information about this variability among populations is available for *S. uvarum*. In a previous work carried out in our laboratory using apple must, we also demonstrated a clear physiological difference between the strains from natural habitats and apple *chichas* (González Flores *et al.,* 2017). Almeida *et al*. (2014) proposed a relationship between the physiological features of a large set of *S. uvarum* strains and their origin for the first time, and these authors attributed such differences to a domestication phenomenon.

The differences observed in the ethanol content among the fermented products obtained herein were not clearly associated with variations in sugar consumption and glycerol or acetic acid production. Glycerol, volatile acidity (acetic acid) and residual glucose and fructose levels were variable within clusters, and these features allowed us to select the most interesting strains from each origin/population for hybridization. In our previous work, interesting metabolic features for cider elaboration were also demonstrated for the same two strains *S. uvarum* NPCC 1290 and NPCC 1314 selected in this work (González Flores *et al.,* 2017).

In order to introduce more variability, and to also use two genetically distant strains, we

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employed the rare-mating methodology for hybridization. This methodology is based on the infrequent event of mating type switching which occurs in natural yeast populations (Spencer and Spencer, 1996). It has been used in only a few studies of *S. cerevisiae* × *S. uvarum* hybrid generation (Bellon *et al.,* 2015). In contrast, many studies have employed this methodology based on spore-to-spore crosses (for a summary see Pérez-Través *et al.,* 2012 and Morales and Dujon, 2012), which results in lower initial genetic plasticity in the generated hybrids.

Finally, and based on the fact that different selective pressures which act on recently formed artificial hybrids determine the genomic fate of hybrids, we employed two stabilization temperatures (13 and 20°C) for hybrid generation and stabilization. In line with this, Piotrowski *et al*. (2012) demonstrated that the genomic fate of *S. cerevisiae* × *S. uvarum* hybrids strongly depended on the selective pressure that they were subjected to during their evolution. These authors also demonstrated that high temperatures with *S. cerevisiae* × *S. uvarum* newly formed hybrids favoured *S. uvarum* genome loss, while the effect of ethanol on the same hybrids favoured the euploid hybrids that possessed the two genomes. The strategy that we adopted with low temperature allowed us to succeed in conserving the *S. uvarum* subgenome, as shown in the PCR-RFLP of all 33 nuclear genes. Neither physiological nor genomic differences were observed in the present work among the hybrids obtained at both tested temperatures. All of the hybrids showed an additive phenotype among their parental strains, and grew at both low and high temperatures. All of the hybrids had the same nuclear genomic composition, showed the same mtDNA and possessed a similar total DNA content. The main difference between the two processes was the total time required for stabilization to be carried out by successive fermentations. Pérez-Través *et al*. (2014) demonstrated that five successive fermentations sufficed to obtain genetically stable intra- and interspecific hybrids. After running the rare-mating methodology for hybridization, these authors demonstrated a lower total DNA content during stabilization, which went from values ~4*n* to some ~2*n*. All of the stable hybrids obtained in this work following the same methodology demonstrated a total DNA content of around 2*n* (1.85–2.04). Similar genome reduction processes in recently formed hybrids have also been demonstrated by Marinoni *et al*. (1999), although these hybrids were generated by mass mating. Similarly, Gerstein *et al*. (2006) observed in evolution studies a lower DNA content for the polyploidy cultures of *S. cerevisiae* during first generations, and a tendency to stabilize in ploidy values that came close to 2*n*.

In contrast to what happened with the nuclear genome, the mtDNA of *S. cerevisiae* and *S. uvarum* were quite different in both size and gene order terms, the *S. uvarum* mtDNA being smaller that the same in *S. cerevisiae* (Cardazzo *et al.,* 1998). In the *Saccharomyces* hybrids, the zygote is heteroplasmic; i.e. it contains mtDNA from the two parents. However after mitotic divisions, only one mtDNA was conserved (homoplasmy; Piškur, 1994; Berger and Yaffe, 2000). All of the hybrids obtained in this work retained the mtDNA from the *S. cerevisiae* parent irrespective of the conditions under which they were generated. This fact could be, at least in part, responsible for the physiological homogeneity of the hybrids that bore different *S. uvarum* subgenomes, but the same *S. cerevisiae* nuclear subgenome and mtDNA. Marinoni *et al*. (1999) also observed that *S. cerevisiae* × *S. uvarum* hybrids preferentially inherited *S. cerevisiae* mtDNA, while other authors did not report this (De Vero *et al.,* 2003; Pulvirenti *et al.,* 2000; Solieri *et al.,* 2008). In their work, Solieri *et al*. (2008) suggested that the hybrids with *S. uvarum* mtDNA displayed a marked tendency to ferment and a lesser tendency to respire than those with *S. cerevisiae* mtDNA. Our hybrids, however, which were stabilized under non-aerobic conditions (successive fermentations), conserved only *S. cerevisiae* mtDNA.

Even when considering different *S. uvarum* parental strains, distinct stabilization temperatures and rare mating being used as a hybridization methodology, all of the stable hybrids generated in this work demonstrated similar oenologically relevant features. Solieri *et al*. (2005) obtained *S. cerevisiae* × *S. uvarum* hybrids by a spore-to-spore methodology. As exhibited in our work, these authors also observed uniform physiological and transcriptional profiles in all of the generated hybrids. Nevertheless, only a few metabolites produced by yeast strains were evaluated in their work. In the two cases, hybrids exhibited intermediate concentrations of ethanol and glycerol with

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total sugar consumption. We also observed that hybrids produced wines with a particular combination of aroma compounds in the *Sauvignon blanc* must, which differed from that found in the parental strains. Hence we were able to obtain wines with particular characteristics. Indeed it has been proposed that high production of 2-phenylethanol and its acetate, typical of *S. uvarum*, could not be beneficial in such wines (*Sauvignon blanc*) because they can mask the characteristic aroma of this variety (Serra *et al.,* 2005). We successfully obtained hybrids that lacked this metabolic feature from *S. uvarum*, but we produced the highest levels of other higher alcohols, including 1-propanol and 1-butanol. High concentrations of 1-butanol were also shown in other *S. cerevisiae* × *S. bayanus* (= *S. uvarum*) hybrids by fermenting Riesling wines (Bellon *et al.,* 2015), which could be a feature that characterizes such hybrids. Other typical features from *S. uvarum,* such as the capacity to not only grow at low temperature (Sipiczki, 2002), but to also synthesize malic acid (Rainieri *et al.,* 1998), were also observed in all the hybrids generated in this study.

In short, the low ethanol production with a strong glycerol synthesis, the capacity to grow at low temperature and malic acid production make these hybrids a potential biotechnological tool for the new winemaking industry within the emergence markets.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

Almeida P, Gonçalves C, Teixeira S, *et al*. 2014. A Gondwanan imprint on global diversity and domestication of wine and cider yeast *Saccharomyces uvarum*. *Nat Commun* **5**: 4044.

Belloch C, Orlic S, Barrio E, Querol A. 2008. Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. *Int J Food Microbiol* **122**: 188–195.

Belloch C, Querol A, García MD, Barrio E. 2000. Phylogeny of the genus *Kluyverimyces* inferred from the mitochondrial cytochrome-C oxidase II gene. *Int J Syst Evol Microbiol* **50**: 405–416.

Bellon JR, Eglinton JM, Siebert TE, *et al*. 2011. Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. *Appl Microbiol Biotechnol* **91**(3): 603–612.

Bellon JR, Yang F, Day MP, Inglis DL, Chambers PJ. 2015. Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes. *Appl Microbiol Biotechnol* **99**: 8597–8609.

Berger KH, Yaffe MP. 2000. Mitochondrial DNA inheritance in *Saccharomyces cerevisiae*. *Trends Microbiol* **8**(11): 508–513.

Bertolini L, Zambonelli C, Giudici P, Castellari L. 1996. Higher alcohol production by cryotolerant *Saccharomyces* strains. *Am J Enol Vitic* **47**: 343–345.

Bizaj E, Cordente AG, Bellon JR, Raspor P, Curtin CD, Pretorius IS. 2012. A breeding strategy to harness flavour diversity of *Saccharomyces* interspecific hybrids and minimize hydrogen sulphide production. *FEMS Yeast Res* **12**(4): 456–465.

Cardazzo B, Minuzzo S, Sartori G, Grapputo A, Carignani G. 1998. Evolution of mitochondrial DNA in yeast: Gene order and structural organization of the mitochondrial genome of *Saccharomyces uvarum*. *Curr Genet* **33**(1): 52–59.

Coton E, Coton M, Levert D, Casaregola S, Shohier D. 2006. Yeast ecology in French cider and black olive natural fermentations. *Int J Food Microbiol* **108**: 130–135.

De Vero L, Pulvirenti A, Gullo M, Medeghini Bonatti P, Giudici P. 2003. Sorting of mitochondrial DNA and proteins in the progeny of *Saccharomyces* interspecific hybrids. *Ann Microbiol* **53**: 219–231.

Demuyter C, Lollier M, Legras JL, Le Jeune C. 2004. Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. *J Appl Microbiol* **97**: 1140–1148.

Diezmann S, Dietrich FS. 2009. *Saccharomyces cerevisiae*: Population divergence and resistance to oxidative stress in clinical, domesticates and wild isolates. *PLoS One* **4**(4): e5317.

Gerstein AC, Chun HJ, Grant A, Otto SP. 2006. Genomic convergence toward diploidy in *Saccharomyces cerevisiae*. *PLoS Genet* **2**: 1396–1401.

Giudici P, Zambonelli C, Passarelli P, Castellari L. 1995. Improvement of wine composition with cryotolerant *Saccharomyces* strains. *Am J Enol Vitic* **46**: 143–147.

González Flores M, Rodríguez ME, Oteiza JM, Barbagelata RJ, Lopes CA. 2017. Physiological characterization of *Saccharomyces uvarum* and *Saccharomyces eubayanus* from Patagonia and their potential for cidermaking. *Int J Food Microbiol* **249**: 9–17.

Haase SB, Reed SI. 2002. Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle* **1**(2): 132–136.

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DOI: 10.1002/yea

Karanyicz E, Antunovics Z, Kallai Z, Sipiczki M. 2017. Non-introgressive genome chimerization by malsegregation in autodiploidised allotetraploids during meiosis of *Saccharomyces kudriavzevii* × *Saccharomyces uvarum* hybrids. *Appl Microbiol Biotechnol* **101**(11): 4617–4633.

Kellis M, Patterson N, Endrizzi M, Birren BW, Lander ES. 2003. Sequencing and comparison of yeast species to identify gees and regulatory elements. *Nature* **423**: 241–254.

Kishimoto M. 1994. Fermentation characteristics of hybrids between the cryophilic wine yeast *Saccharomyces bayanus* and the mesophilic wine yeast *Saccharomyces cerevisiae*. *J Ferment Bioeng* **77**(4): 432–435.

Libkind D, Hittinger CT, Valerio E, *et al*. 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast.. *PNAS Early EDN* **108**: 1–6.

Marinoni G, Manuel M, Petersen RF, Hvidtfeldt J, Sulo P, Piskur J. 1999. Horizontal transfer of genetic material among *Saccharomyces* yeasts. *J Bacteriol* **181**: 6488–6496.

Masneuf-Pomarede I, Bely M, Marullo P, Albertin W. 2016. The genetics of non-conventional wine yeasts: Current knowledge and future challenges. *Front Microbiol* **6**: 1563.

Masneuf-Pomarède I, Bely M, Marullo P, Lonvaud-Funel A, Dubourdieu D. 2010. Reassessment of phenotypic traits for *Saccharomyces bayanus* Var. *uvarum* wine yeast strains. *Int J Food Microbiol* **139**: 79–86.

Masneuf-Pomarede I, Salin F, *et al*. 2016. Microsatellite analysis of *Saccharomyces uvarum* diversity. *FEMS Yeast Res* **16**: fow002.

Morales L, Dujon B. 2012. Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. *Microbiol Mol Biol R* **76**(4): 721–739.

Naumov GI. 1996. Genetic identification of biological species in the *Saccharomyces sensu stricto* complex. *J Ind Microbiol* **17**: 295–302.

Naumov GI, Naumova ES, Martynenko NN, Masneuf-Pomaréde I. 2011. Taxonomy, ecology and genetics of the yeast *Saccharomyces bayanus*: A new object for science and practice. *Microbiology* **80**(6): 735–742.

Nguyen HV, Boeckhout T. 2017. Characterization of *Saccharomyces uvarum* (Beijerinck, 1898) and related hybrids: Assessment of molecular markers that predict the parent and hybrid genomes and proposal ton ame yeast hybrids. *FEMS Yeast Res* **17**: fox014.

Pérez-Través L, Lopes CA, Barrio E, Querol A. 2012. Evaluation of different genetic procedures for the generation of artificial hybrids on *Saccharomyces* genus for winemaking. *Int J Food Microbiol* **156**: 102–111.

Pérez-Través L, Lopes CA, Barrio E, Querol A. 2014. Stabilization process in *Saccharomyces* intra- and interspecific hybrids in fermentative conditions. *Int Microbiol* **17**: 213–224.

Piotrowski JS, Nagarajan S, Kroll E, *et al*. 2012. Different selective pressures lead to different genomic outcomes as newly-formed hybrids yeasts evolve. *BMC Evol Biol* **12**(1): 46.

Piškur J. 1994. Inheritance of the yeast mitochondrial genome. *Plasmid* **31**: 229–241.

Pulvirenti A, Nguyen H, Caggia C, Giudici P, Rainieri S, Zambonelli C. 2000. *Saccharomyces uvarum*, a proper species within *Saccharomyces* Sensu stricto. *FEMS Microbiol Lett* **192**: 191–196.

Querol A, Barrio E, Ramón D. 1992. Molecular monitoring of wine fermentations conduced by active dry yeast strains. *Appl Environ Microbiol* **58**: 2948–2953.

Rainieri S, Zambonelli C, Giudici P, Castellari L. 1998. Characterization of thermotolerant *Saccharomyces cerevisiae* hybrids. *Biotechnol Lett* **20**(6): 543–547.

Ribereau-Gayon P, Dubourdieu D, Doneche B, Lonvaud A. 2003. *Tratado de Enología. 1. Microbiología del vino*. *Vinificaciones*. Ed. Hemisferio Sur: Buenos Aires; 515–583.

Rodríguez ME, Pérez-Través L, Sangorrín MP, Barrio E, Lopes CA. 2014. *Saccharomyces eubayanus* and *Saccharomyces uvarum* associated with the fermentation of *Araucaria araucana* seds in Patagonia. *FEMS Yeast Res* **1**: 1–18.

Rodríguez ME, Pérez-Través L, Sangorrín MP, Barrio E, Querol A, Lopes CA. 2017. *Saccharomyces uvarum* is responsible for the traditional fermentation of apple *chicha* in Patagonia. *FEMS Yeast Res* **17**: fow109.

Rohlf FJ. 2000. NTSYS-PC: numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software: Setauket, New York.

Rojas V, Gil JV, Piñaga F, Manzanares P. 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. *Int J Food Microbiol* **70**: 283–289.

Rossignol T, Dalau L, Julien A, Blondin B. 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **20**: 1369–1385.

Sampaio JP, Goncalves P. 2008. Natural population of *Saccharomyces kydriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae* and *paradoxus*. *Appl Environ Microbiol* **74**(7): 2144–2152.

Serra A, Strehaiano P, Taillandier P. 2005. Influence of temperature and pH on *Saccharomyces bayanus* Var. *uvarum* growth; impact of a wine yeast interspecific hybridization on these parameters. *Int J Food Microbiol* **104**: 257–265.

Sipiczki M. 2002. Taxonomic and physiological diversity of *Saccharomyces bayanus*. In Biodiversity and Biotechnology of Wine Yeasts, Ciani M (ed). Research Signpost: Kerala; 53–69.

Sipiczki M. 2008. Interspecies hybridization and recombination in *Saccharomyces* wine yeasts. *FEMS Yeast Res* **8**: 996–1007.

Solieri L, Antúnez O, Pérez-Ortín JE, Barrio E, Giudici P. 2008. Mitochondrial inheritance and fermentative: Oxidative balancein hybrids between *Saccharomyces cerevisiae* and *Saccharomyces uvarum*. *Yeast* **25**: 485–500.

Solieri L, Gullo M, De Vero L, Antúnez O, Pérez-Ortín JE, Giudici P. 2005. Homogeneity of interspecific hybrids between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* by phenotypic and transcriptional analysis. *Int J Biotechnol Biochem* **1**(1): 11–21.

Spencer JFT, Spencer DM. 1996. Rare-mating and cytoduction in *Saccharomyces cerevisiae*. In Methods in Molecular Biology: Yeast Protocols, Evans I (ed). Humana: Totowa, NJ; 39–44.

Steensels J, Snoek T, Meersman E, Nicolino MP, Voordeckers K, Verstrepen KJ. 2014. Improving industrial yeast strains: Exploiting natural and artificial diversity. *FEMS Miocrobiol Rev* **38**: 947–995.

Suárez Valles B, Pando Bedriñana R, *et al*. 2007. Yeast species associated with the spontaneous fermentation of cider. *Food Microbiol* **24**: 25–31.

Zaret KS, Sherman F. 1985. α-Aminoadipato as a primary nitrogen source for *Saccharomyces cerevisiae* mutants. *J Bacteriol* **162**(2): 579–583.

Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet K. 1990. Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**(6): 1875–1881.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1: RAPD-PCR patterns obtained with primers p24 and p28 for some analysed stable hybrid strains. **M**: molecular ladder 100 pb.

Table S1: Physicochemical and growth characterization of fermentations in synthetic must with indigenous yeast strains

Table S2: Kinetic parameters obtained for all successive fermentations.

Table S3: Physicochemical composition of Sauvignon blanc wines obtained with interspecific hybrids and parental strains at 13°C and 20°C.

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