Research paper

Title: Enological characterization of Spanish *Saccharomyces kudriavzevii* strains, one of the closest relatives to parental strains of winemaking and brewing *S. cerevisiae* × *S. kudriavzevii* hybrids.

Authors: Peris D.[1,2], Pérez-Través L.[3], Belloch C.[3], Querol A.[3]

[1] Biodiversity and Evolution of Eukaryotic Microorganisms. "Cavanilles" Institute of Biodiversity and Evolution, University of Valencia, Valencia, Spain.

[2] Laboratory of Genetics, DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI 53706, USA.

[3] Food Biotechnology Department. Institute of Agrochemistry and Food Technology (IATA), CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

Corresponding Author:

A. Querol, Food Biotechnology department. Institute of Agrochemistry and Food Technology (IATA, CSIC). P.O. Box 73. E-46100 Burjassot, Valencia, Spain.

E-mail: aquerol@iata.csic.es

Abstract

Wine fermentation and innovation have focused mostly on *Saccharomyces cerevisiae* strains. However, recent studies have shown that other *Saccharomyces* species can also be involved in wine fermentation or are useful for wine bouquet, such as *S. uvarum* and *S. paradoxus*. Many interspecies hybrids have also been isolated from wine fermentation, such as *S. cerevisiae* × *S. kudriavzevii* hybrids. In this study, we explored the genetic diversity and fermentation performance of Spanish *S. kudriavzevii* strains, which we compared to other *S. kudriavzevii* strains. Fermentations of red and white grape musts were performed, and the phenotypic differences between Spanish *S. kudriavzevii* strains under different temperature conditions were examined. An ANOVA analysis suggested striking similarity between strains for glycerol and ethanol production, although a high diversity of aromatic profiles among fermentations was found. The sources of these phenotypic differences are not well understood and require further investigation. Although the Spanish *S. kudriavzevii* strains showed desirable properties, particularly must fermentations, the quality of their wines was no better than those produced with a commercial *S. cerevisiae.* We suggest hybridization or directed evolution as methods to improve and innovate wine.

Keywords: Saccharomyces kudriavzevii, phylogenetics, must fermentation, low temperature, aroma, winemaking.

1. Introduction

Nowadays, wine fermentations are likely performed under sterile conditions with wine starter cultures, where *Saccharomyces cerevisiae* is the most frequently chosen one. In the last decade, other species of the genus *Saccharomyces* have been identified as being responsible for wine fermentations, such as *S. uvarum* in Tokaj and Alsatian wines (Naumov et al., 2002; Demuyter et al., 2004), where others, such as *S. paradoxus* found in Croatian vineyards, have been successfully tested (Redzepovic et al., 2002; Orlić et al., 2010). These previous studies have highlighted the importance of the genus *Saccharomyces* on the whole to winemaking, and have suggested that diversity might be useful for innovation in the wine industry.

The genetic characterization of *Saccharomyces* hybrids, isolated from wines of Central Europe and Northern Spain, which are regions characterized for having low temperatures, has demonstrated that they are likely generated by rare-mating between species *S. cerevisiae* and *S. kudriavzevii* (Peris et al., 2012a). In these hybrids, acquisition and maintenance of important genes from both parents seem to be adaptive traits for growth at low fermentation temperatures (González et al., 2007; Belloch et al., 2008). A molecular analysis of independently isolated *S. cerevisiae* × *S. kudriavzevii* hybrids has indicated that there were multiple independent hybridizations events (Erny et al., 2012; Peris et al., 2012b), which suggests that the hybridization process between *S. cerevisiae* and *S. kudriavzevii* was more successful under the aforementioned low temperatures of fermentative conditions. To the successful of these hybrids it must have been important that the properties of the new wine was of high quality to continue the domestication of hybrids by humans, a likely scenario in view of the high quality of hybrid-produced wines (González et al., 2007; Lopandic et al., 2007). The idea that natural hybridization occurs frequently in nature, and the high quality of the resulting wines, have meant that more interest is shown in developing methods to generate artificial hybrids (Pérez-Través et al., 2012) as an alternative to producing new wine products. The wine products of the artificial hybrids between strains *S. cerevisiae* and non *cerevisiae* have been shown to have good organoleptic properties (Bellon et al., 2011; Bellon et al., 2013).

Understanding the behavior of hybrid parental strains during must fermentation might influence either the generation of artificial hybrids or their application in the

wine industry. Until recently, the studies that have explained the adaptation of natural *S. cerevisiae* × *S. kudriavzevii* hybrids to the wine environment have used the Japanese *S. kudriavzevii* IFO1802 strain as the parental reference strain (Belloch et al., 2008; Tronchoni et al., 2009; Peris et al., 2012c, Gamero et al., 2013). The description and characterization of new *S. kudriavzevii* strains isolated from diverse *Quercus* species in Portugal (Sampaio and Gonçalves, 2008) and Spain (Lopes et al., 2010) have demonstrated the physiological differences between Iberian and Japanese strains (Hittinger et al., 2010; Lopes et al., 2010). Spanish and Portuguese *S. kudriavzevii* strains are closely related to the *S. kudriavzevii* subgenome from *S. cerevisiae* × *S. kudriavzevii* hybrids (Peris et al., 2012b), which means that they are closely related to the ancestral *S. kudriavzevii* parent of hybrids. However, strains from different countries have not been studied together in a phylogenetic context. In addition, very little is known about the performance of these Iberian/European *S. kudriavzevii* strains under wine fermentation conditions.

In order to acquire better knowledge of wild *S. kudriavzevii* strains from Spain, must microvinifications were performed at 14 ºC and 22 ºC. These temperatures are expected to be more optimal for cryotolerant *S. kudriavzevii* than *S. cerevisiae*. Our results explored the phylogenetic relationship between the Spanish and Portuguese *S. kudriavzevii* strains, and whether a multilocus gene approach and an ecological environment would be useful for explaining and inferring the different patterns in must fermentation performance among Spanish strains. The results of this study suggest future applications of wild *S. kudriavzevii* strains in the wine industry.

2. Material and Methods

2.1 Yeast strains

The isolation source and geographical origin of the *S. kudriavzevii* strains used herein are shown in Table 1. Strains *S. kudriavzevii* CRs and CA111 have been previously identified and differentiated in Lopes et al. (2010). Strain ZP591 has been described in Sampaio et al. (2008) and reference strain IFO1802 has been obtained from Naumov et al. (2000). A commercial *S. cerevisiae* wine strain, Uvaferm VRB, was used as a control in the microvinification experiments. VRB was selected given its optimum temperature range of 15-30 ºC, high ethanol tolerance (>17%) and low

pH tolerance (pH=3.1), and also because it has been extensively used in the wine fermentations described by Lallemand: (see http://www.lallemandwine.com/products/catalogue/product-detail/?range=9&id=43).

2.2 Nucleotide diversity and phylogenetic tree reconstruction

The sequences of five nuclear genes (*BRE5, CAT8, CYC3, CYR1* and *EGT2*) from a previous study (Peris et al., 2012b), and the partial mitochondrial *COX2* gene from Peris et al. (2012a), were used to run a genetic diversity analysis and a phylogenetic tree reconstruction. The gene sequence accession numbers are found in Table S1. A trimmed concatenated alignment (~2.5 Kb) of the individual genes, obtained by FASCONCAT, v1.0 (Kück and Meusemann, 2010), was used for posterior analyses.

Genetic diversity data and genetic distance, corrected by Jukes and Cantor model, were calculated by DnaSP, v5 (Librado and Rozas, 2009), and MEGA, v5.2 (Tamura et al., 2011), respectively.

A Bayesian phylogenetic tree was reconstructed in BEAST, v1.7.5 (Drummond and Rambaut, 2007). Three independent runs of MCMC length 10,000,000 were performed with both a strict molecular clock and a Yule process as our tree prior. Sampling was done every 1000 steps. To assess convergence across separate runs, we analyzed the posterior distribution of the sampled parameters in TRACER, v1.5 (Rambaut and Drummond, 2001). Estimated sample size (ESS) values over 300 were considered to indicate good sampling convergence between independent runs. The final phylogenetic tree was obtained by TreeAnnotator (BEAST) after discarding 10% of the sample trees from each run as burn-in. The posterior distribution of each branch was displayed in FIGTREE, v1.3.1 (Rambaut and Drummond, 2010).

The maximum likelihood phylogenetic trees of the concatenated alignment and the partial mitochondrial *COX2* gene were reconstructed in MEGA 5.2 (Tamura et al., 2011) using the best BIC fitted model, TN93 with invariant sites and TN93 with gamma distribution, respectively.

2.3 Yeast growth with enological stress factors

Yeast cultures, dilutions and growth tests were performed as in Belloch et al. (2008) by measuring OD at A600 nm to adjust the initial cultures to 0.3 OD_{600} units. We tested the colony development of six serial dilutions. Colony development was checked daily. Key wine fermentation stresses were tested: osmotic stress (300g L^{-1}), low pH (3.0), ethanol (EtOH) content (5%, 10%, 12% and 15%), and low and high temperatures (10 ºC, 16 ºC, 30 ºC and 37 ºC). Growth was tested on YPD plates (2% glucose, 2% peptone, 1% yeast extract, 2% agar) supplemented with the adequate compound concentration or adjusted to the required pH. Ethanol tests were performed with freshly poured ethanol plates, which were sealed to prevent ethanol evaporation. Temperature was set at 30 ºC for all the growth conditions, except for the temperature stress test.

2.4 Microvinification experiments and fermentation kinetic analyses

The musts obtained from the Tempranillo (red grapes) and Macabeo (white grapes) varieties were used for the microvinification assays. Dimethyl dicarbamate (DMDC) at 1ml L^{-1} was added for sterilization purposes. Tempranillo must content was 194.94 g L^{-1} fermentable sugar and nitrogen levels were below 200 mg L^{-1}. Sugars were measured by HPLC (see below) and yeast assimilable nitrogen was determined by the Formol Index Method (Aerny, 1997) for organic nitrogen, and by an ammonia assay kit (Boehringer Mannheim; Mannehim, Germany) for inorganic nitrogen. Nitrogen content was adjusted to 220mg L^{-1} by adding a nitrogen supplement, which consisted of NH_4Cl. The final pH was adjusted to 3.6. With the Macabeo must, the fermentable sugar content was 227.81g L^{-1} and nitrogen was 200 mg L^{-1}, with a final pH of 3.2. Microvinifications were done in 250-ml flasks and with 175 ml of must. Strain inoculation was done at 10^6 cells/ml. Microvinification was carried out at 22 ºC and 14 ºC for the Tempranillo and Macabeo musts, respectively. Flasks were closed with stoppers and airlocks to release CO_2. Fermentation was monitored by mass loss until a constant mass was reached, considered to be the end of fermentation. Experiments were carried out in duplicate. Yeast cells were removed by centrifugation and supernatants were stored at -20 ºC until further analysis. Potential contaminations were monitored with a negative control (must without cells).

The monitored mass loss was corrected to the percent of sugar consumed, as in Pérez-Través et al (2014):

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

where C is the percent of sugar consumed at each time point, m is the mass loss value at that sampling time (g), S is the initial sugar concentration in the must (g L^{-1}), R is the final sugar concentration in the fermented must (residual sugar, g L^{-1}) and mf is the total mass loss value at the end of fermentation (g). Residual sugar values were obtained by the HPLC analysis of the fermentation samples.

Curve fitting was carried out using the re-parametrized Gompertz equation proposed by Zwietering et al. (1990):

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

where y is the percent of consumed sugar, D is the maximum sugar consumption value (the asymptotic maximum, %), μmax is the maximum sugar consumption rate (h^{-1}) and λ is the lag phase period (h) where no sugar consumption was detected. The Gompetz equation was fitted to data points by the non linear regression module of the STATISTICA 7.0 software package (Hilbe, 2007), and by minimizing the sum of the squares of the difference between the experimental data and the fitted model. Fit adequacy was estimated by the proportion of variance explained by the model (R^2) compared to the experimental data. Other formulas, such as Logistic and Richards, were tested by GCAT (http://www.glbrc.org/gcat-vm/), but failed to fit our data.

2.5 HPLC and GC analyses

The residual sugars (glucose and fructose), glycerol and ethanol from the microvinification end point samples were determined by HPLC (Thermo Fisher Scientific, Waltham, MA) using a refraction index detector and a HyperREZTM XP Carbohydrate H+ 8μm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples were diluted 5-fold, filtered through a 0.22-μm nylon filter (Symta, Madrid, Spain) and injected in duplicate. The analysis conditions were: eluent, 1.5 mM of H2SO4; 0.6 ml min^{-1} flux and oven temperature of 50 °C.

Volatile compounds extraction and gas chromatography were performed following the protocol of Rojas et al. (2001). The concentrations of higher alcohols and esters were determined at the end of fermentation. Extraction was done using headspace solid phase-micro-extraction sampling (SPME) with poly-dimethylsiloxane (PDMS) fibers (Supelco, Sigma-Aldrich, Barcelona, Spain). Aroma compounds were separated by GC in a Thermo TRACE GC ULTRA chromatograph (Thermo Fisher Scientific, Waltham, MA) with a flame ionization detector (FID), using a HP-INNOWAX 30 m × 0.25 mm capillary column coated with a 0.25-µm layer of cross-linked polyethylene glycol (Agilent Technologies Inc.). Helium was the carrier gas used (flow 1 ml min^{-1}). The oven temperature program was: 5 min at 60 °C, 5 °C min^{-1} to 190 °C, 20 °C min^{-1} to 250 °C and 2 min at 250 °C. The temperature detector was 280 °C and the temperature injector was 220 °C under splitless conditions. A chromatography signal was recorded by a HP Vectra QS/16S detector. The internal standard was 2-heptanone (0.05% w/v). The retention times of the eluted peaks were compared to those of commercial higher alcohols and ester standards. Concentrations, in µg mL^{-1}, were quantified by the calibration graphs (R2 value > 0.99) of the standards.

2.6 Statistical and Principal Component Analyses

The kinetic parameters, HPLC and GC data were analyzed by the `STATISTICA 7.0` software package (Hilbe, 2007), and by a one-way ANOVA and a Tukey test for the means comparison. A Principal Component Analysis (PCA) was performed with the `prcomp` function and represented with `ggbiplot` package (https://github.com/vqv/ggbiplot) from the `R` statistical package (Adler and Murdoch, 2009).

3. Results

3.1 Portuguese and Spanish *S. kudriavzevii* are closely related

To quantify the levels of genetic diversity between the *S. kudriavzevii* strains, we inferred nucleotide diversity by concatenated alignment (2.5kbp). The level of nucleotide diversity in the Spanish *S. kudriavzevii* strains was 0.21% and no increase in this value was observed when ZP541 was included. However, nucleotide diversity

increased to 0.51% when IFO1802 was included (Table 2). The average pairwise genetic distance between *S. kudriavzevii* from Portugal and Spain was 0.22%, Portugal-Japan 1.23% and Spain-Japan 1.25%. Based on the nucleotide diversity and phylogenetic analysis, the Spanish and Portuguese strains could belong to a unique group, which we termed the Iberian/European population.

The reconstructed phylogenetic tree, with concatenated alignment, indicated no cluster differentiation based on country of isolation, with CR89 and CR90 being the only strains that clustered together with high bootstrap and posterior probability (PP) values (Figure 1A). Although CR85, CR91 and ZP591 clustered together, the group was not supported by the PP and bootstrap values. The topology of the *COX2* phylogenetic tree differed slightly from the nuclear phylogenetic tree (Figure 1B). After taking into account the isolation source, the *S. kudriavzevii* strains were independently clustered using the concatenated nuclear genes (Figure 1A). However, the reconstructed tree based on the mitochondrial gene showed a correlation between the isolation source and the strain clusters (Figure 1B). The sugar consumption data from Sampaio *et al.* (2008) and Lopes *et al.* (2010) did not correlate with our multilocus and *COX2* phylogenetic trees (Figure S1). For the supported clusters, CA111, CR89 and CR90, strain CA111 was unable to consume maltose. The spore viability for our Spanish strains was low compared to the Portuguese strains, a result which might indicate a mosaic genome for the Spanish strains.

3.2 Some Spanish *S. kudriavzevii* strains are more tolerant to osmotic and ethanol stress than the Japanese strain

The ability of the *S. kudriavzevii* strains to grow under the stressful conditions that occurred during fermentation was assayed in order to infer the potential application of the wild *S. kudriavzevii* strains to microvinification. We were unable to explore ZP591 because it was not in our strain collection and we had no access to it. The stress assay results indicated no differences between the Japanese and Spanish *S. kudriavzevii* strains in terms of temperature and pH (Table 3). However, clear differences between the strains were observed for the ethanol and osmotic stress conditions. Strains CR89, CR90 and CR91, isolated from *Q. faginea*, were

better able to grow at a high ethanol concentration. They were also able to grow on 15% ethanol medium, but IFO1802 was unable to grow at 10% ethanol, or higher. In osmotic stress, all the strains were able to grow at 300 gL^{-1} of glucose; although the CR strains isolated from *Q. faginea* and Japanese strain IFO1802 showed a better growth profile (Table 3). Strains CA111 and CR85, isolated from *Q. ilex*, were slightly more ethanol-tolerant than IFO1802, but were somewhat more affected by osmotic stress (Table 3). All the strains were able to grow well at pH 3 and within temperature ranges of 10-30 ºC; however, none of the *S. kudriavzevii* strains was able to grow at 37 ºC.

3.3 Must fermentation performance

3.3.1 Spanish *S. kudriavzevii* showed better kinetic parameters in Tempranillo at 22 ºC than Macabeo at 14 ºC

The kinetic growth curves from must fermentations Tempranillo (red variety) and Macabeo (white variety) were inferred by following mass loss (Figure 2). Growth curves were used to extract the kinetic parameters of each strain (Table 4). The Tempranillo fermentations performed by *S. kudriavzevii* at 22 ºC ended before 13 days, except for CR89 and CA111, which required 17 and 20 days, respectively (Figure 2A). The *S. cerevisiae* control strain finished fermentation at a similar time to one of the best performing *S. kudriavzevii* strains, CR85. For all the *S. kudriavzevii* strains, the lag phase was longer than the commercial wine strain of *S. cerevisiae* (Table 4A). IFO1802 required a longer time to start fermentation, close to 1 day. According to the maximum consumption rate parameter (Table 4A), the fastest strain was CR85, followed by the commercial *S. cerevisiae* strain and IFO1802, and the slowest strain of *S. kudriavzevii* was CA111. The maximum sugar consumption rate (m) was 3.5 times higher for the fastest strain (CR85) than for the slowest strain (CA111). The *S. cerevisiae* control strain and CR85 were the most efficient ones to consume 50% and 75% of the sugars contained in must. IFO1802, followed by CR91, were the next fastest strains to consume 50% of sugars, but they obtained similar consumption times to consume 75% of sugars.

Macabeo fermentations at 14 ºC took much longer than Tempranillo fermentations. The fermentation performed by the *S. cerevisiae* control strain was

completed after 45 days (Figure 2B). *S. cerevisiae* was able to consume 95% of must sugars. *S. kudriavzevii* strains required a time of between 35 and 65 days, but they consumed between 75% and 85% of sugars. The low percent of sugar consumption by the *S. kudriavzevii* strains indicated halted fermentation. One of the fermentation replicates of CR89 was unable to start fermentation. For this reason CR89 was removed from the analysis. According to the kinetic parameters analysis (Table 4B), strain CR90 showed the longest lag phase time, followed by CR85 and CR91. It should be noted that the high standard deviation values of CR90 and CR91 were indicative of adaptation issues to these must fermentation conditions. The higher values of these standard deviations masked the significant differences between the other strains. For this reason, we decided to perform a new ANOVA analysis for the lag phase parameter, from which CR90 and CR91 were removed (Table 4B). The new analysis showed that all the *S. kudriavzevii* strains had longer lag phase times than the *S. cerevisiae* control strain. CA111 was the *S. kudriavzevii* strain with the lowest lag phase value, followed by Japanese strain IFO1802. For the maximum sugar consumption rate, the fastest consumer was CR85, followed by IFO1802. The slowest strains were CA111 and the winemaking *S. cerevisiae*. Despite its low sugar consumption rate, CA111 consumed 50% of fermentable sugars, similarly to IFO1802, and no significant differences were found when compared with *S. cerevisiae* (Table 4B). CR90 took longer to consume 50% of sugars, probably due to the long lag phase. The first strains to reach the consumption of 75% sugars were *S. cerevisiae* and IFO1802. CR90 was the last strain to consume 75% of sugars, while the other Spanish *S. kudriavzevii* strains showed intermediate values between CR90 and the lowest strains IFO1802 and VRB.

3.3.2 Some Spanish *S. kudriavzevii* are characterized for producing more glycerol and less ethanol in both musts than *S. cerevisiae*

The concentrations of glucose, fructose, glycerol and ethanol were measured by HPLC (Table 4). At the end of the Tempranillo must fermentation (Table 4A), glucose levels were lower than or equal to 1 gL^{-1} in all cases. CA111 and *S. cerevisiae* completely depleted glucose. Residual fructose was found at the end of all fermentations, except for *S. cerevisiae*. Fructose content was between 4 g L^{-1} and 16

g L^{-1}, while CA111 had the lowest levels and IFO1802 the highest. The final glycerol content was around 8g L^{-1} for CR85, CR90, CR91 and IFO1802, and 9.32 gL^{-1} and 9.11 g L^{-1} for CA111 and CR89, respectively. Although *S. cerevisiae* consumed all sugars, it produced around 6g L^{-1} of glycerol. Ethanol content was higher than 12.4% in all cases, and CR85 was that with the least ethanol production. *S. cerevisiae* produced the highest ethanol levels (13.8%). CA111 was the *S. kudriavzevii* strain that consumed the most sugars and produced the most glycerol.

In the Macabeo fermentations (Table 4B), *S. cerevisiae* consumed all the glucose, although 5 g L^{-1} of fructose were not consumed. The residual sugar levels of the *S. kudriavzevii* fermentations were over 20 g L^{-1}, with CR85 exhibiting less residual sugars. In contrast to its Tempranillo fermentation, CA111 exhibited the most residual sugars, around 50 g L^{-1} which corresponded to 23% of the total available sugar in the must fermentation. The glycerol levels for *S. cerevisiae* were around 7.6g L^{-1}, while *S. kudriavzevii* strains produced more glycerol, around 10g L^{-1}. Ethanol content was below 12.7% for all the fermentations performed by the *S. kudriavzevii* strains. The commercial *S. cerevisiae* strain value was below 14.2%. Although the *S. kudriavzevii* strains produced less ethanol, these values could be due to lower sugar consumption.

3.3.3 High ethyl acetate levels might compromise the final wine product of Spanish *S.kudriavzevii*

The aromatic profile that consisted in higher alcohols and esters (Table 5) was measured for each microvinification by gas chromatography. The concentration of higher alcohols was around 100 mg L^{-1} in the must microvinifications, except for strains CA111 and CR89, which produced as many higher alcohols in the Tempranillo fermentation compared to the other strains and the Macabeo fermentation. However in both cases, the higher alcohol values were below the threshold associated with off-odors (Table S2). This was not the case for esters, which were over the off-odor threshold in the Tempranillo fermentations (Table 5, Table S2).

The Tempranillo must fermentation was characterized by the presence of remarkable amounts of isobutanol, isoamyl alcohol, 2-phenylethanol and esters ethyl

359 acetate, ethyl octanoate, ethyl decanoate and isoamyl acetate (above the odor
360 thresholds (Table S2)). We found significant differences among the isobutanol,
361 isoamyl alcohol, ethyl acetate, ethyl octanoate and ethyl decanoate concentrations in
362 our *S. kudriavzevii* fermentations (Table 5). These higher levels were found for
363 CA111, CR89, CR90 and CR91. The PCA analysis suggested that CA111 and CR89
364 had a similar aromatic profile (Figure 3A), and higher isobutanol, isoamyl alcohol and
365 isoamyl acetate concentrations were shown (Table 5, Figure S2A). *S. cerevisiae*
366 produced more total higher alcohols than most *S. kudriavzevii* strains, except for
367 CA111 and CR89. VRB produced significantly higher diethyl succinate levels than *S.*
368 *kudriavzevii,* but the levels of esters were lower than the Spanish *S. kudriavzevii*
369 strains. When we analyzed the yield aroma production (g of aroma produced / g of
370 sugar consumed), the most productive strains were CA111 and CR89 ($2*10^{-3}$), and
371 the least productive one was IFO 1802 ($1.06*10^{-3}$). High ethyl acetate levels were
372 obtained in Tempranillo, this being the main compound, and the levels of the esters
373 in CA111, CR89, CR90 and CR91 increased compared to CR85, and IFO1802 and
374 VRB.
375 During the Macabeo fermentation, CA111 produced more higher alcohols than
376 the other *S. kudriavzevii* strains. CR85 and CR90 generated higher ester levels. Most
377 of our strains produced aromatic compounds above their odor thresholds (Table S2),
378 such as isoamyl alcohol, 2-phenylethanol, ethyl acetate, ethyl octanoate, ethyl
379 decanoate and isoamyl acetate. With isobutanol, only CR85 was able to produce
380 levels above the threshold, and VRB produced detectable odor levels for 2-
381 methylpropyl ethanoate. Considering aromatic production, the PCA showed how
382 CR85, CR90 and CR91 were closely related, and how *S. cerevisiae* was well
383 differentiated from the *S. kudriavzevii* strains (Figure 3B). The commercial *S.*
384 *cerevisiae* was differentiated from the *S. kudriavzevii* strains by the lower production
385 of higher alcohols and the higher production of esters (Table 5, Figure S2B). Yield
386 aroma production showed CA111 ($0.84*10^{-3}$) to be the most productive strain, and
387 CR85, CR90, CR91 and VRB ($0.64\text{-}0.67*10^{-3}$) to be the least productive ones.
388
389 *4. Discussion*

Phylogenetic studies and fermentation/stress performance analyses were used to elucidate the relationship between the *S. kudriavzevii* strains from different host sources and to explore the potential application of the Spanish strains in the wine industry. By a multilocus phylogenetic approach, with previously sequenced nuclear and mitochondrial genes (Peris et al, 2012b), we reconstructed the *S. kudriavzevii* phylogenetic tree and compared clades on the basis of an ecological niche and phenotypes to infer a potential correlation among genome, ecology and fermentation/stress performance.

4.1 *The Iberian/European population*

Previous studies have revealed the close relationship between the *S. kudriavzevii* strains isolated in Portugal and the natural *S. cerevisiae* × *S. kudriavzevii* double hybrids isolated from wine fermentation in central Europe (Sampaio and Gonçalves, 2008; Erny et al., 2012; Peris et al., 2012b). Recently, new *S. kudriavzevii* strains isolated in Spain, and characterized by both RFLPs (Lopes et al., 2010) and a multilocus phylogenetic network approach (Peris et al., 2012b), have also been demonstrated to be closely related to hybrids. These results indicate that the Iberian and Portuguese *S. kudriavzevii* strains might potentially be the closest relatives of the parents that generated the double *S. cerevisiae* × *S. kudriavzevii* and the triple *S. cerevisiae* × *S. kudriavzevii* x *S. uvarum* hybrids (Sampaio and Gonçalves, 2008; Lopes et al., 2010; Erny et al., 2012; Peris et al., 2012b). However, the relationship between the Iberian and Japanese *S. kudriavzevii* strains has not been profoundly analyzed, but has led to confusion about the number of populations in the Iberian Peninsula and their relationship with the Japanese strains. Sampaio and Gonçalves (2008) described those strains found in Portugal as a European population. The low nucleotide divergence (0.22%) between the Portuguese and Spanish *S. kudriavzevii* strains are much lower than the values shown for the two recently described *S. eubayanus* populations, Patagonia A and Patagonia B (Peris et al., 2014), and suggests that the Iberian *S. kudriavzevii* strains belong to a unique population. The proposed Iberian/European population may enclose the *S. kudriavzevii* strains actually isolated from the Iberian Peninsula.

4.2 Potential *S. kudriavzevii* wine applications

The results of the wine must fermentations showed that our strains are able to consume most sugars during the Tempranillo fermentation. However, during the Macabeo fermentation at 14 ºC, more than 20 g L^{-1} of sugars remained, which is a clear difference compared with the *S. cerevisiae* VRB strain that was able to consume all glucose and most fructose. Fermentative capabilities at low temperature are likely a general ability among the *S. kudriavzevii* strains from both the Iberian/European and Japanese populations. The *S. kudriavzevii* strains have shown a better growth profile at low temperatures than *S. cerevisiae* (Salvadó et al., 2011), mainly due to the development of a metabolic strategy for cold adaptation (López-Malo et al., 2013). Most *S. kudriavzevii* strains have obtained a better growth rate during Macabeo fermentation than *S. cerevisiae*. However, the synergistic effect of low temperature and an increase in the ethanol concentration might be the reasons for the interruption observed during *S. kudriavzevii* fermentation compared with *S. cerevisiae*. This stronger inhibition effect in *S. kudriavzevii* explains why *S. cerevisiae* outcompetes *S. kudriavzevii* during co-culture fermentations at 17 ºC, 24 ºC and 31 ºC (Arroyo-López et al., 2011).

Glycerol is a positive contributor to wine quality as it reduces wine astringency (Ishikawa and Noble, 1995; Remize et al., 2000). *In vivo*, glycerol is involved in adaptation of yeast to low temperature (Izawa et al., 2004), and it is important for osmoregulation (Ansell et al., 1997; Nevoigt and Stahl, 1997). Our *S. kudriavzevii* strains produced more glycerol than *S. cerevisiae,* which coincides with previous works (González et al., 2007; Arroyo-López et al., 2010; Oliveira et al., 2014), except for CR85 and CR90. The glycerol production differences between temperature conditions coincides with previous results (González et al., 2007; Oliveira et al., 2014). This glycerol production difference might be due to a redox imbalance induced during low-temperature fermentation (Paget et al., 2014).

Both wine quality and final composition are directly affected by low temperature fermentations (Llauradó et al., 2005; Beltran et al., 2008). The effects of temperature in Tempranillo must fermentation using non *cerevisiae* strains has been shown to follow neither the reduction described in higher alcohols nor the increase in acetate and ethyl esters, as in *S. cerevisiae* (Gamero et al., 2013), which has been

suggested to improve wine quality (Llauradó et al., 2005). We observed a lower production of higher alcohols and esters among our strains during low temperature fermentations using Macabeo must. The observed results, as well as the reduction in aroma yields, suggest that grape varietal, together with the temperature tested, influenced our final compound outcome.

The volatile compounds with values above the odor threshold, influencing in fruity and floral aromas, were 2-phenylethanol, ethyl acetate, ethyl octanoate, ethyl decanoate, isoamyl acetate and 2-methylpropyl ethanoate. Previous studies have shown that one of the highest producers of 2-phenylethanol, during Tempranillo must fermentation at 12 ºC and 28 ºC, was *S. kudriavzevii* IFO1802, compared with other *Saccharomyces* strains (Gamero et al., 2013). In our survey, we observed no significant differences related to the levels of those aromatic compounds among *S. kudriavzevii* strains. The only exceptions found were ethyl acetate, in Tempranillo where CR89, CR90 and CR91 produced more than the other strains, and isoamyl acetate in Macabeo, where the Spanish *S. kudriavzevii* strains produced less than IFO1802 and VRB. In general, the aroma yields in Tempranillo were higher than in Macabeo, and most of the *S. kudriavzevii* strains outperformed the *S. cerevisiae* aromatic yields under both conditions. Although production of higher alcohols and esters is desired to confer complexity to wine, levels must be below certain thresholds to avoid off-odors (Jackson, 2009). In most of the Spanish *S. kudriavzevii* strains, ethyl acetate levels were above the off-odor threshold, which might negatively influence the final Tempranillo aroma compared to *S. cerevisiae* and IFO1802. In Macabeo, these levels were below the off-odor thresholds.

An aroma profile is determined by the interaction of different factors, such as glycerol, ethanol, residual sugars, temperature and the production or inheritance from grapes of volatile compounds, among others (Jackson, 2009). The quality of this profile is better assayed by a sensory analysis, which was not done in our study. Our results showed how the Spanish *S. kudriavzevii* strains are good producers of glycerol and some aromatic compounds. However, the high levels of esters and fermentative kinetics under our conditions might limit the direct application of our strains to wine fermentation, which must be the reason why *S. kudriavzevii* was not isolated from wine fermentations. Notwithstanding, the interesting properties of *S.*

kudriavzevii make them potentially applicable for breeding with commercially available *S. cerevisiae* to generate new hybrids for wine production.

4.3 *Diversity and biotechnological applications*

Environmental variables, such as niche chemical composition, are important for maintaining specific yeast strains (Starmer and Lachance, 2011) and contribute to the diversification of microorganisms (Kassen, 2002; MacLean and Bell, 2002). The Iberian *S. kudriavzevii* strains have been isolated from different species of *Quercus* trees (Sampaio and Gonçalves, 2008; Lopes et al., 2010). Sampaio and Gonçalves (2008) investigated the distinct sugar compositions among *Quercus* trees, a factor that might influence the diversification of *S. kudriavzevii* strains. We used a multilocus phylogenetic approach to infer a correlation between genome and phenotype, which might be translated to similar phenotypes.

Following the above principle, our aim was to cluster strains by their aromatic profile or other phenotypes based on the multilocus phylogenetic tree. Despite the low nucleotide diversity found among the *S. kudriavzevii* strains, aromatic profiles were diverse and did not correlate with nuclear and mitochondrial gene phylogenies. Similar results about glycerol production have been found between the Spanish *S. kudriavzevii* CR85 strain and Japanese IFO1802 compared with other European strains (Oliveira et al., 2014). Moreover, *GPD1* gene sequence comparisons, the most important gene in the glycerol pathway, did not reveal intraspecies nucleotide differences (Oliveira et al., 2014). The observable activity of an enzyme will depend on gene expression, protein levels, metabolic regulation and protein sequence (Rossouw et al., 2012). Most differences between species like *S. cerevisiae* and *S. mikatae* are not found at the gene sequence level. but in cis (promoters, UTRs) and trans-regulatory (transcription factors) elements (Borneman et al., 2007). A recent work has demonstrated how different *S. cerevisiae* strains with similar aromatic profiles also presented similar expression levels of the most important transcription factors (trans elements) involved in aroma metabolic pathways (Rossouw et al., 2012). For this reason, lack of correlation between gene sequences and the aromatic profile, in this study, or between gene sequences and glycerol

production from Oliveira et al. (2014), indicates that the major nucleotide changes which affect the phenotype of *S. kudriavzevii* might be in cis and trans elements.

When we explored other phenotypes, the *S. kudriavzevii* strains clustered together based on the isolation source, when mitochondrial gene *COX2* was used, suggesting that mitochondrial genome better explains the story of these strains; in fact, the mitochondrial genome has been used to explain the diversification time in the yeast, *Schizosaccharomyces pombe* (Jeffares *et al.,* 2015). The mitochondrial phylogenetic tree also correlated with the stress response, where CR85 and CA111, isolated from *Q. ilex,* were more sensitive to ethanol and osmotic stress. Instead, sugar uptake did not correlate with the mitochondrial and nuclear genome. Sugar uptake has been demonstrated to be strain specific than being specific of a population or species because it usually depends on some subtelomeric gene gain/losses (Borneman and Pretorius, 2015). Another phenotype, spore viability, was shown low for our Spanish *S. kudriavzevii* than for Portuguese strains (Sampaio and Gonçalves, 2008; Lopes *et al.* 2010 and this study). Strains generated by crosses among different lineages of *S. cerevisiae* have displayed low spore viability (Wang *et al.* 2012), which suggests that our Spanish *S. kudriavzevii* strains might be mosaics (admixture strains), with contributions from different *S .kudriavzevii* lineages. This new mixture of alleles can rewire the genotypic landscape by the new interactions of cis and trans-elements.

Whole genome sequence projects and a system biology approach will help us to better understand how cis and trans regulatory elements and gene differences in *S. kudriavzevii* have been modulated in nature and how we can use this information in winemaking.

5. *Conclusions*

Our study indicates how minor genetic differences have produced diverse phenotypes among the Spanish *S. kudriavzevii* strains from the Iberian/European population during must fermentation. Understanding the selection mechanisms or the genomic traits of a strain/species might help to better predict their performance in industrial processes. Our results also reveal that the relationship between genotype and phenotype in *S. kudriavzevii* is still unclear and needs further investigation. The

interest shown in *S. kudriavzevii* biodiversity is grounded in the potential applications of this species to the wine industry. Strain selection will depend on must type, the fermentation conditions to be used, and the desired final wine characteristics. This study suggests how *S. kudriavzevii* strains will be valuable through the generation of artificial hybrids to explore phenotypic landscapes that better fit the interests of wine industries by combining desirable *S. kudriavzevii* properties with other *Saccharomyces* strains, such as commercial *S. cerevisiae*. We expect these genome combinations to benefit from the higher glycerol production and reduced ethanol content inherited from the *S. kudriavzevii* subgenome, better fermentative kinetics and sugar consumption inherited from the *S. cerevisiae* subgenome, and a new aromatic profile from rewiring the gene networks in the hybrid.

Acknowledgments

We thank Emily Baker, Kayla Sylvester, Quinn Langdon and William G. Alexander for proofreading and making comments on the manuscript. D.P. acknowledges the Spanish Government for its Ministry of Science and Innovation (FPI) fellowship. L.P. acknowledges the CSIC and the Spanish Ministry of Education and Science (MEC) for an I3P fellowship. This work has been supported by grants AGL2012-39937-C02-01 from the Spanish Government, FEDER and Generalitat Valenciana PROMETEOII/2014/042 to AQ.

Author's contributions

D.P., L.P., A.Q. conceived and designed this study. D.P. and L.P. contributed equally to this work. D.P. performed the drop test, and the phylogenetic and statistical analyses. D.P. and L.P. performed the fermentations and the growth curve data analyses. L.P. and C.B. performed the HPLC runs. LP performed the GC runs for the compound concentration descriptions and the statistical analysis of the HPLC and GC data. D.P., L.P., C.B. and A.Q. were involved in preparing the manuscript.

References

Adler, D. and Murdoch D (2009). rgl: 3D visualization device system (Open GL) 2009.

Aerny,J. (1997). Composés azotes des moûts et des vins. Rev. Suisse Vitic. Hort. 28:161-165.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., Adler, L. (1997). The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J., 16, 2179-2187.

Arroyo-Lopez, F. N., Perez-Torrado, R., Querol, A., Barrio, E. (2010). Modulation of the glycerol and ethanol syntheses in the yeast *Saccharomyces kudriavzevii* differs from that exhibited by *Saccharomyces cerevisiae* and their hybrid. Food Microbiol., 27, 628-637.

Arroyo-López, F. N., Pérez-Través, L., Querol, A., Barrio, E. (2011). Exclusion of *Saccharomyces kudriavzevii* from a wine model system mediated by *Saccharomyces cerevisiae*. Yeast, 28, 423-435.

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.

Bellon, J., Eglinton, J., Siebert, T., Pollnitz, A., Rose, L., de Barros Lopes, M., Chambers, P. (2011). Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. Appl. Microbiol. Biot., 91, 603-612.

Bellon, J. R., Schmid, F., Capone, D. L., Dunn, B. L., Chambers, P. J. (2013). Introducing a new breed of wine yeast: interspecific hybridisation between a commercial *Saccharomyces cerevisiae* wine yeast and *Saccharomyces mikatae*. PLoS ONE, 8, e62053.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., Rozès, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. Int. J. Food Microbiol., 121, 169-177.

Borneman, A. R., Gianoulis, T. A., Zhang, Z. D., Yu, H., Rozowsky, J., Seringhaus, M. R., Wang, L. Y., Gerstein, M., Snyder, M. (2007). Divergence of transcription factor binding sites across related yeast species. Science, 317, 815-819.

Borneman,A.R., Pretorius, I. S. (2015). Genomic insights into the *Saccharomyces sensu stricto* complex. Genetics, 199, 281-291.

Demuyter, C., Lollier, M., Legras, J. L., 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.

Drummond, A., Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol., 7, 214.

Erny, C., Raoult, P., Alais, A., Butterlin, G., Delobel, P., Matei-Radoi, F., Casaregola, S., Legras, J. L. (2012). Ecological success of a group of *Saccharomyces cerevisiae*/*Saccharomyces kudriavzevii* hybrids in the Northern European wine making environment. Appl. Environ. Microbiol., 78, 3256-3265.

Gamero, A., Tronchoni, J., Querol, A., Belloch, C. (2013). Production of aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures. J. Appl. Microbiol., 114, 1405-1414.

González, S. S., Gallo, L., Climent, M. D., Barrio, E., Querol, A. (2007). Enological characterization of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. Int. J. Food Microbiol., 116, 11-18.

Hilbe, J. (2007). STATISTICA 7: an overview. In Christensen R (Eds.), The American Statistician. London: Taylor & Francis, pp. 91-94.

Hittinger, C. T., Gonçalves, P., Sampaio, J. P., Dover, J., Johnston, M., Rokas, A. (2010). Remarkably ancient balanced polymorphisms in a multi-locus gene network. Nature, 464, 54-58.

Ishikawa, T., Noble, A. C. (1995). Temporal perception of astringency and sweetness in red wine. Food Qual. Prefer., 6, 27-33.

Izawa, S., Sato, M., Yokoigawa, K., Inoue, Y. (2004). Intracellular glycerol influences resistance to freeze stress in *Saccharomyces cerevisiae*: analysis of a quadruple

637 mutant in glycerol dehydrogenase genes and glycerol-enriched cells. Appl. Microbiol.
638 Biotechnol., 66, 108-114.

639 Jackson, R. S. (2009). Chapter 3 - Olfactory Sensations. In Jackson,R.S. (Eds.),
640 Wine Tasting (Second Edition). San Diego: Academic Press, pp. 55-128.

641 Jeffares,D.C., Rallis, C., Rieux, A., Speed, D., Prevorovsky, M., Mourier, T.,
642 Marsellach, F. X., Iqbal, Z., Lau, W., Cheng, T. M. K. et al. (2015). The genomic and
643 phenotypic diversity of Schizosaccharomyces pombe. Nat Genet, 47, 235-241.

644 Kassen, R. (2002). The experimental evolution of specialists, generalists, and the
645 maintenance of diversity. J. Evol. Biol., 15, 173-190.

646 Kück, P. and Meusemann, K. (2010). FASconCAT, Version 1.0.

647 Librado, P., Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of
648 DNA polymorphism data. Bioinformatics, 25, 1451-1452.

649 Llauradó, J. M., Rozès, N., Constantí, M., Mas, A. (2005). Study of some
650 *Saccharomyces cerevisiae* strains for winemaking after preadaptation at low
651 temperatures. J. Agric. Food Chem., 53, 1003-1011.

652 Lopandic, K., Wallner, E., Tscheik, G., Leitner, G., Querol, A., Borth, N.,
653 Tiefenbrunner, W. (2007). Genetically different wine yeasts isolated from Austrian
654 vine-growing regions influence wine aroma differently and contain putative hybrids
655 between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. FEMS Yeast
656 Res., 7, 953-965.

657 Lopes, C. A., Barrio, E., Querol, A. (2010). Natural hybrids of *S. cerevisiae* x *S.*
658 *kudriavzevii* share alleles with European wild populations of *Saccharomyces*
659 *kudriavzevii*. FEMS Yeast Res., 10, 412-421.

660 López-Malo, M., Querol, A., Guillamon, J. M. (2013). Metabolomic comparison of
661 *Saccharomyces cerevisiae* and the cryotolerant species *S. bayanus* var. *uvarum* and
662 *S. kudriavzevii* during wine fermentation at low temperature. PLoS ONE, 8, e60135

663 MacLean, R. C., Bell, G. (2002). Experimental adaptive radiation in *Pseudomonas*.
664 Amer. Nat., 160, 569-581.

665 Naumov, G. I., James, S. A., Naumova, E. S., Louis, E. J., Roberts, I. N. (2000).
666 Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces*

667 *cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. Int. J. Syst.
668 Evol. Microbiol., 50, 1931-1942.

669 Naumov, G. I., Naumova, E. S., Antunovics, Z., Sipiczki, M. (2002). *Saccharomyces*
670 *bayanus* var. *uvarum* in Tokaj wine-making of Slovakia and Hungary. Appl. Microbiol.
671 Biot., 59, 727-730.

672 Nevoigt, E., Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast
673 *Saccharomyces cerevisiae*. FEMS Microbiol Rev., 21, 231-241.

674 Oliveira, B. M., Barrio, E., Querol, A., Pérez-Torrado, R. (2014). Enhanced enzymatic
675 activity of Glycerol-3-Phosphate Dehydrogenase from the cryophilic *Saccharomyces*
676 *kudriavzevii*. PLoS ONE, 9, e87290.

677 Orlić, S., Arroyo-López, F. N., Huic-Babic, K., Lucilla, I., Querol, A., Barrio, E. (2010).
678 A comparative study of the wine fermentation performance of *Saccharomyces*
679 *paradoxus* under different nitrogen concentrations and glucose/fructose ratios. J.
680 Appl. Microbiol., 108, 73-80.

681 Paget, C. M., Schwartz, J. M., Delneri, D. (2014). Environmental systems biology of
682 cold-tolerant phenotype in *Saccharomyces* species adapted to grow at different
683 temperatures. Mol. Ecol., online version.

684 Pérez-Través, L., Lopes, C. A., Barrio, E., Querol, A. (2012). Evaluation of different
685 genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus
686 for industrial purpose. Int. J. Food Microbiol., 152, 102-111.

687 Pérez-Través, L., Lopes, C. A., Barrio, E., Querol, A. (2014). Study of the
688 stabilization process in *Saccharomyces* intra- and interspecific hybrids in
689 fermentation conditions. Int. Microbiol., in press.

690 Peris,D., Belloch, C., Lopandic, K., Álvarez-Pérez, J. M., Querol, A., Barrio, E.
691 (2012a). The molecular characterization of new types of *S. cerevisiae* x *S.*
692 *kudriavzevii* hybrid yeasts unveils a high genetic diversity. Yeast, 29, 81-91.

693 Peris,D., Lopes, C. A., Arias, A., Barrio, E. (2012b). Reconstruction of the
694 evolutionary history of *Saccharomyces cerevisiae* x *S. kudriavzevii* hybrids based on
695 multilocus sequence analysis. PLoS ONE, 7, e45527.

696 Peris,D., Lopes, C. A., Belloch, C., Querol, A., Barrio, E. (2012c). Comparative
697 genomics among *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* natural
698 hybrid strains isolated from wine and beer reveals different origins. BMC Genomics,
699 13, 407.

700 Peris,D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J. P., Alexander, W. G.,
701 Hittinger, C. T. (2014). Population structure and reticulate evolution of
702 *Saccharomyces eubayanus* and its lager-brewing hybrids. Mol Ecol, 23, 2031-2045.

703 Rambaut, A. and Drummond, A. (2001). Tracer v1.4. Molecular Evolution,
704 Phylogenetics and Epidemiology.

705 Rambaut, A. and Drummond, A. J. (2010). FigTree v1.3.1.

706 Redzepovic,S., Orlic, S., Sikora, S., Majdak, A., Pretorius, I. S. (2002). Identification
707 and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*
708 strains isolated from Croatian vineyards. Lett. Appl. Microbiol., 35, 305-310.

709 Remize,F., Sablayrolles, J. M., Dequin, S. (2000). Re-assessment of the influence of
710 yeast strain and environmental factors on glycerol production in wine. J. Appl.
711 Microbiol., 88, 371-378.

712 Rojas,V., Gil, J. V., Piñaga, F., Manzanares, P. (2001). Studies on acetate ester
713 production by non-Saccharomyces wine yeasts. Int. J. Food Microbiol., 70, 283-289.

714 Rossouw,D., Jacobson, D., Bauer, F. F. (2012). Transcriptional regulation and the
715 diversification of metabolism in wine yeast strains. Genetics, 190, 251-261.

716 Salvadó,Z., Arroyo-Lopez, F. N., Guillamón, J. M., Salazar, G., Querol, A., Barrio, E.
717 (2011). Temperature adaptation markedly determines evolution within the genus
718 *Saccharomyces*. Appl. Environ. Microbiol., 77, 2292-2302.

719 Sampaio,J.P., Gonçalves, P. (2008). Natural populations of *Saccharomyces*
720 *kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S.*
721 *cerevisiae* and *S. paradoxus*. Appl. Environ. Microbiol., 74, 2144-2152.

722 Starmer, W. T. and Lachance M. A. (2011). Yeast ecology. In Kurtzman,C.P., Fell, J
723 W, Boekhout, T (Eds.), The Yeasts: A Taxonomic Study. Amsterdam: Elservier, pp.
724 65-83.

725 Tamura,K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011).
726 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood,
727 evolutionary distance, and Maximum Parsimony methods. Mol. Biol. Evol., 28, 2731-
728 2739.

729 Tronchoni,J., Gamero, A., Arroyo-Lopez, F. N., Barrio, E., Querol, A. (2009).
730 Differences in the glucose and fructose consumption profiles in diverse
731 *Saccharomyces* wine species and their hybrids during grape juice fermentation. Int.
732 J. Food Microbiol., 134, 237-243.

733 Wang,Q.M., Liu, W. Q., Liti, G., Wang, S. A., Bai, F. Y. (2012). Surprisingly diverged
734 populations of *Saccharomyces cerevisiae* in natural environments remote from
735 human activity. Mol Ecol, 21, 5404-5417.

736 Zwietering, M. H., Jongenburger, I., Rombouts, F. M., van 't Riet, K. (1990). Modeling
737 of the bacterial growth curve. Appl. Environ. Microbiol., 56, 1875-1881.

738

739 *Figure legends*

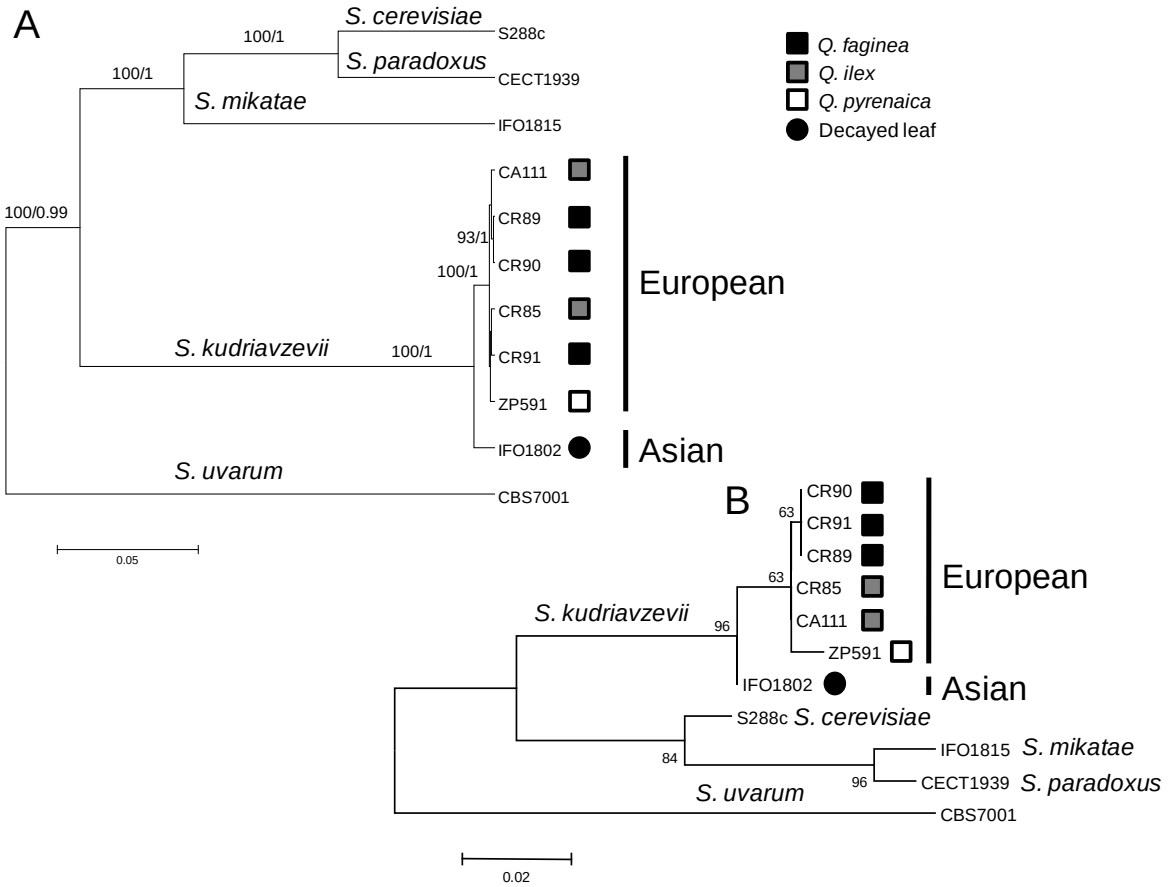
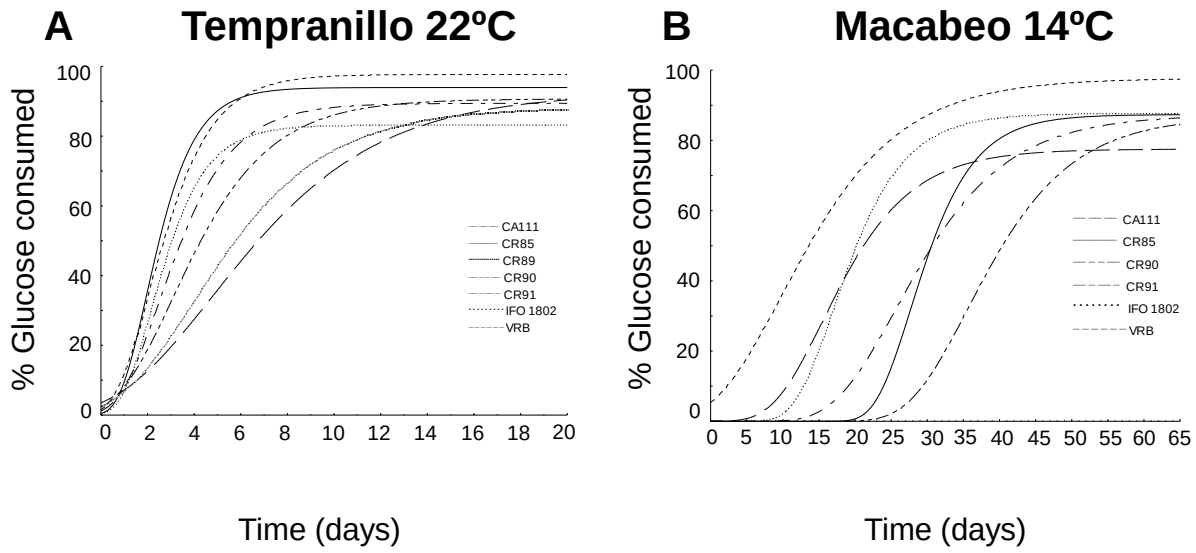
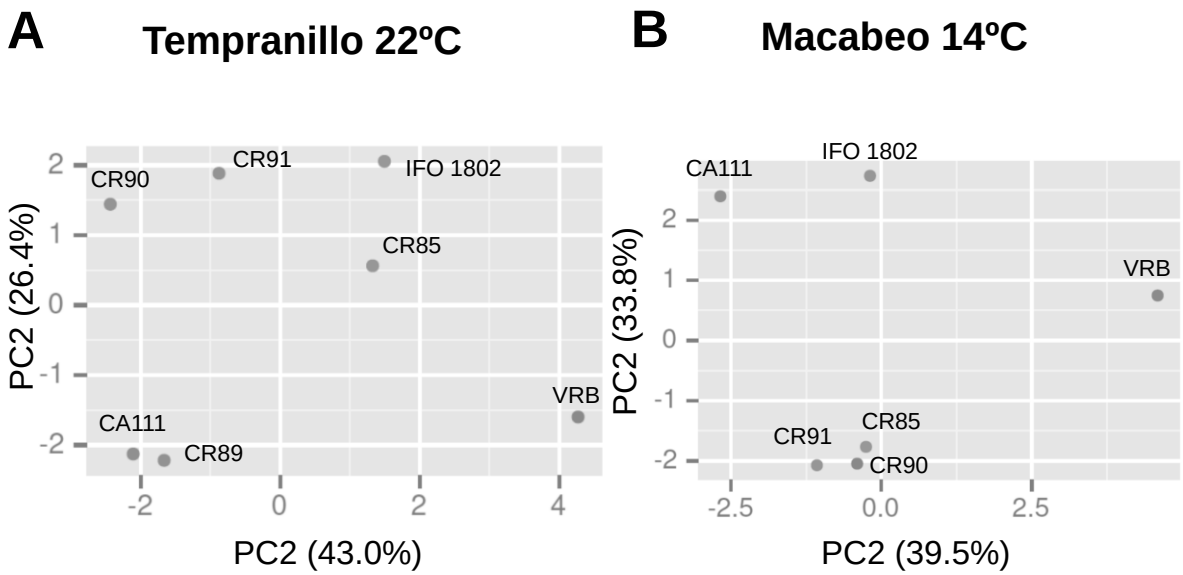


Figure 1. Nuclear and mitochondrial phylogenetic tree reconstructions. A) Bayesian and Maximum Likelihood phylogenetic tree reconstructions by a multilocus approach of previously sequenced nuclear genes (Peris et al. 2012b). B) Maximum Likelihood *COX2* phylogenetic tree reconstruction. Branch support is shown as the MEGA 5.2 bootstrap/BEAST posterior probability. Scale bars represent the number of nucleotide substitutions per site. The isolation tree source is indicated in the legend.



748
749 **Figure 2.** Kinetic curve representations calculated from the mass loss assay. A) and
750 B) represent the kinetic curves of Tempranillo and Macabeo, respectively, at different
751 temperatures, as indicated.
752



753

754 **Figure 3.** Principal Component Analysis representations. A) and B) represent the
755 PCA plots for the Tempranillo and Macabeo aromatic profiles, respectively. The
756 percentages on each axis indicate the percent of variation explained by each
757 principal component. For clarity, variable weights are shown in Figure S2.

758

Tables

Table 1. List of the *S. kudriavzevii* strains used in this study.

Strain Name	Isolation region	Source	Latitude	Longitude	Altitude (m)	Average Temperature (ºC)	Maximum Temperature (ºC)	Minimum Temperature (ºC)	Precipitation (mm)
CR85	Ciudad Real, Spain	*Quercus ilex* bark	38º58'51"	-4º51'51"	593	26	34	18	10
CR89	Ciudad Real, Spain	*Quercus faginea* bark	38º58'51"	-4º51'51"	593	26	34	18	10
CR90	Ciudad Real, Spain	*Quercus faginea* bark	38º58'51"	-4º51'51"	593	26	34	18	10
CR91	Ciudad Real, Spain	*Quercus faginea* bark	38º58'51"	-4º51'51"	593	26	34	18	10
CA111	Castellón, Spain	*Quercus ilex* bark	0º22'44"	-0º50'10"	551	24	29	18	48
ZP591	Cast.Vide, Portugal	*Quercus pyrenaica*	NA	NA	NA	NA	NA	NA	NA
IFO1802	Japan	Decayed leaf	NA	NA	NA	NA	NA	NA	NA
Uvaferm VRB	Commercial	wine fermentation	NA	NA	NA	NA	NA	NA	NA

Table 2. Genetic overview of *S. kudriavzevii* strains.

Group	*s*	*k*	*π*	#hap	Hd	Fs	Tajima's D
Spanish	10	5	0.00211±0.00043	4	0.9	0.490	0.29817
European*	12	5	0.00217±0.00034	5	0.933	-0.187	-0.141
European*-Japan	38	12	0.00510±0.00205	6	0.952	0.479	-1.26491

s: number of segregating sites; k: average number of differences between sequences; π: nucleotide diversity; #hap: number of haplotypes; Hd: haplotype diversity; Fs: Fu's Fs; Tajima's D (no values are statistically significant, $P < 0.05$).

* Data analyzed, including the Spanish and Portuguese strains.

Table 3. Strain growth with diverse enological stress factors.

Condition	IFO1802	CA111	CR85	CR89	CR90	CR91	Day*
300g L^{-1} Glucose	6	3	5	6	6	6	3rd
pH 3	6	6	6	6	6	6	3rd
5% EtOH	5	4	6	6	6	6	4th
10% EtOH	0	4	5	6	6	6	5th
12% EtOH	0	2	4	4	6	6	10th
15% EtOH	0	0	0	2	4	4	10th
10 ºC	6	6	6	6	6	6	6th
16 ºC	6	6	6	6	6	6	3rd
30 ºC	6	6	6	6	6	6	3rd
37 ºC	0	0	0	0	0	0	3rd

Tolerance to different fermentation stress factors is indicated by numbers from 0 (absence of growth) to 6 (colony development at the sixth dilution).

* Day of study. In the 15% EtOH and 37 ºC stress assays, maximum colony development was detected at the 4th dilution, and none of them grew, respectively.

Table 4. Growth curve parameters and the main metabolite concentrations at the end of fermentation.

A **Tempranillo must (22 ºC)**

Parameter	CA111	CR85	CR89	CR90	CR91	IFO1802	VRB
Lag phase (days)	0.74±0.01^{b,c}	0.80±0.02^{c}	0.79±0.01^{b,c}	0.72±0.01^{b}	0.80±0.01^{c}	0.90±0.03^{d}	0.63±0.03^{a}
m (g L^{-1} day^{-1})	16.44±0.45^{a}	57.89±0.38^{f}	20.05±0.19^{b}	27.74±0.18^{c}	37.27±0.41^{d}	46.42±0.16^{e}	47.32±0.7^{e}
t_{50} (days)	6.75±0.15^{f}	2.50±0.01^{a}	5.74±0.05^{e}	4.29±0.02^{d}	3.46±0.04^{c}	3.05±0.03^{b}	2.70±0.06^{a}
t_{75} (days)	11.1±0.25^{e}	3.70±0.03^{a}	9.68±0.08^{d}	6.97±0.05^{c}	5.52±0.13^{b}	5.10±0.05^{b}	4.08±0.09^{a}
Compound							
Glucose (g L^{-1})	ND	0.61±0.18^{a}	0.60±0.13^{a}	0.72±0.02^{a}	0.83±0.10^{a,b}	1.10±0.03^{b}	ND
Fructose (g L^{-1})	3.99±0.21^{a}	7.45±1.58^{a,b}	11.75±0.37^{c}	10.10±0.28^{b,c}	11.21±1.67^{b,c}	16.17±0.87^{d}	ND
Glycerol (g L^{-1})	9.32±0.09^{c}	8.04±0.43^{b}	9.11±0.18^{b}	7.82±0.02^{b}	8.15±0.08^{b}	7.89±0.18^{b}	6.03±0.10^{a}
Ethanol (%)	13.27±0.07^{b,c}	12.40±0.34^{a}	12.65±0.30^{a,b}	12.97±0.04^{a-c}	12.97±0.04^{a-c}	12.59±0.26^{a,b}	13.79±0.07^{c}

B **Macabeo must (14 ºC)**

Parameter	CA111	CR85	CR90	CR91	IFO1802	VRB
Lag phase (days)	8.63±1.12$^{a,b/b*}$	23.36±2.73$^{b,c/c*}$	29.59±5.55^{c}	19.22±7.32^{b,c}	11.68±0.83$^{a,b/b*}$	0.62±0.10$^{a/a*}$
m (g L^{-1} day^{-1})	9.48±0.82^{a}	16.49±0.29^{c}	11.17±2.00^{a,b}	11.20±0.99^{a,b}	13.61±0.29^{b,c}	8.83±0.37^{a}
t_{50} (days)	21.19±0.04^{a,b}	30.41±2.86^{b,c}	40.15±7.45^{c}	29.68±6.37^{a-c}	20.20±0.65^{a,b}	13.62±0.45^{a}
t_{75} (days)	40.20±6.14^{a,b}	36.32±3.11^{a,b}	49.00±9.20^{b}	39.54±5.13^{a,b}	27.13±0.43^{a}	22.25±0.84^{a}
Compound						
Glucose (g L^{-1})	9.14±1.20^{b}	2.93±0.01^{a}	3.20±0.92^{a}	4.73±0.44^{a}	3.65±0.07^{a}	ND
Fructose (g L^{-1})	40.49±2.36^{b}	22.51±1.39^{a,b}	23.73±1.56^{a,b}	29.25±1.79^{b}	23.40±0.22^{b}	5.26±0.68^{a}
Glycerol (g L^{-1})	10.09±0.22^{b}	9.71±0.87^{a,b}	9.65±0.66^{a,b}	10.32±0.41^{b}	9.84±0.20^{b}	7.66±0.22^{a}
Ethanol (%)	11.03±0.17^{a}	12.71±0.01^{b}	12.50±0.08^{b}	12.10±0.24^{a,b}	12.17±0.31^{a,b}	14.17±0.44^{c}

Superscript letters indicate the significant homogeneous groups obtained by one-way ANOVA analysis (Tukey test, n=2, p-value <0.05). ***** The ANOVA significant homogeneous groups when CR90 and CR91 were removed (because the high standard deviation made it difficult to draw conclusions).
m (maximum sugar consumption rate), t_{50} (time at 50% sugar consumption) and t_{75} (time at 75% sugar consumption), ND (not detected, below the limit of detection).

Table 5. Aromatic profile in must fermentation.

Compound (mg L^{-1})	Tempranillo must (22 ºC)							Macabeo must (14 ºC)					
	CA111	**CR85**	**CR89**	**CR90**	**CR91**	**IFO1802**	**VRB**	**CA111**	**CR85**	**CR90**	**CR91**	**IFO1802**	**VRB**
Higher alcohols													
Benzyl alcohol	ND	0.656±1.312^{a}	0.422±0.843^{a}	ND	ND	ND	1.425±2.015^{a}	ND	ND	ND	ND	1.824±1.364^{a}	1.632±0.935^{a}
Isobutanol	93.202±9.658^{c}	39.776±4.390^{a,b}	91.381±8.322^{c}	49.459±4.849^{b}	34.872±0.964^{a}	35.428±1.951^{a}	46.876±4.397^{a,b}	42.371±2.472^{c}	17.965±0.883^{a}	16.957±0.460^{a}	16.504±1.961^{a}	24.366±2.431^{b}	20.265±3.811^{a,b}
Isoamyl alcohol	123.362±12.975^{b}	78.334±7.730^{a}	114.690±10.914^{b}	66.780±17.645^{a}	64.688±3.060^{a}	68.031±2.208^{a}	115.126±8.704^{b}	64.592±1.622^{b,c}	56.800±1.772^{a,b}	60.276±5.475^{a,b}	58.606±0.592^{a,b}	71.151±4.259^{c}	51.776±5.913^{a}
1-hexanol	0.426±0.044^{a}	0.541±0.049^{a}	0.477±0.052^{a}	0.590±0.223^{a}	0.510±0.039^{a}	0.546±0.023^{a}	0.597±0.030^{a}	0.183±0.123^{a}	0.268±0.009^{a}	0.270±0.017^{a}	0.264±0.025^{a}	0.186±0.125^{a}	0.219±0.012^{a}
2-Phenylethanol	14.117±1.104^{a}	18.119±2.115^{a}	16.006±1.899^{a}	16.792±10.644^{a}	12.926±2.804^{a}	10.364±3.440^{a}	13.077±1.127^{a}	11.903±0.712^{a}	13.275±2.447^{a}	11.509±3.234^{a}	10.946±0.983^{a}	12.247±2.412^{a}	5.427±1.089^{a}
ΣTotal Higher alcohols	231.244	137.526	223.166	133.705	113.082	114.406	177.273	119.049	88.308	89.012	86.320	109.774	79.319
Esters													
Benzyl acetate	ND	0.026±0.053^{a}	0.025±0.049^{a}	ND	0.031±0.061^{a}	ND	ND	ND	0.030±0.060^{a}	0.040±0.079^{a}	0.100±0.068^{a}	ND	ND
Diethyl succinate	ND	0.229±0.457^{a}	0.923±1.155^{a}	ND	ND	0.184±0.368^{a}	2.969±0.876^{b}	0.532±0.644^{a}	1.904±0.991^{a}	2.272±1.107^{a}	1.413±0.460^{a}	1.701±1.515^{a}	24.723±1.028^{b}
Ethyl acetate	155.000±10.659^{b}	92.220±14.194^{a}	137.563±14.586^{b}	168.646±24.156^{b}	169.618±14.506^{b}	71.857±6.098^{a}	72.180±6.227^{a}	29.356±1.764^{a}	39.765±3.204^{a}	40.553±3.945^{a}	33.659±8.964^{a}	33.762±5.665^{a}	37.272±3.773^{a}
Ethyl lactate	ND	ND	ND	ND	ND	ND	ND	0.058±0.115^{a}	ND	ND	ND	0.061±0.123^{a}	ND
Ethyl octanoate	0.077±0.021^{a}	0.648±0.448^{b,c}	0.157±0.053^{a}	0.386±0.099^{a,b}	0.692±0.125^{b,c}	0.878±0.113^{c,d}	1.441±0.093^{d}	0.053±0.0134^{a}	0.642±0.149^{b}	0.595±0.148^{b}	0.567±0.064^{b}	0.633±0.084^{b}	0.864±0.157^{b}
Ethyl decanoate	0.059±0.046^{a}	1.065±0.917^{b}	0.222±0.179^{a,b}	0.233±0.118^{a,b}	0.552±0.212^{a,b}	0.918±0.485^{a,b}	1.276±0.152^{a,b}	0.147±0.081^{a}	1.114±0.736^{a}	1.203±0.609^{a}	1.072±0.522^{a}	1.443±1.070^{a}	0.666±0.042^{a}
Hexyl acetate	0.026±0.001^{a,b}	0.050±0.005^{d}	0.028±0.001^{a,b,c}	0.018±0.016^{a}	0.042±0.003^{b,c,d}	0.047±0.004^{d}	0.049±0.004^{c,d}	ND	0.003±0.006^{a}	ND	ND	0.013±0.002^{a}	0.016±0.002^{a}
Isoamyl acetate	0.137±0.076^{a}	0.101±0.071^{a}	0.191±0.144^{a}	0.084±0.061^{a}	0.087±0.042^{a}	0.037±0.007^{a}	0.172±0.053^{a}	0.160±0.012^{a,b}	0.221±0.032^{b}	0.178±0.015^{a,b}	0.117±0.037^{a}	0.366±0.029^{c}	0.420±0.057^{c}
Phenylethyl acetate	ND	ND	0.030±0.060^{a}	0.067±0.134^{a}	ND	ND	ND	ND	0.025±0.050^{a}	ND	0.027±0.054^{a}	0.138±0.012^{b}	ND
2-methylpropyl ethanoate	1.096±1.187^{a}	1.219±1.246^{a}	0.702±1.092^{a}	0.178±0.235^{a}	0.082±0.041^{a}	0.868±0.863^{a}	1.584±0.859^{a}	0.604±0.992^{a}	0.405±0.330^{a}	0.379±0.435^{a}	0.151±0.070^{a}	0.079±0.159^{a}	3.380±0.820^{b}
ΣTotal Esters	156.258	95.457	139.65	169.528	171.017	74.752	79.499	30.910	44.109	45.220	37.106	38.196	67.341
Yield* (10^{-3})	2.029	1.247	1.987	1.647	1.553	1.065	1.317	0.841	0.654	0.668	0.637	0.737	0.659

Superscript letters indicate the significant homogeneous groups obtained by one-way ANOVA analysis (Tukey test, n=2, p-value <0.05).
ND (not detected, below the limit of detection).
Underlined and double underlined values indicate those compounds above their odor thresholds and above the off-odor thresholds, respectively (Table S2).
* Yield aroma production, calculated as g of aroma (total higher alcohols + total esters) produced / g of sugar (glucose + fructose) consumed.

Description of supporting information

Table S1. Gene accession numbers.

Table S1. Gene accession numbers												
Strain	**BRE5**	**CAT8**	**COX2**	**CYC3**	**CYR1**	**EGT2**						
CA111	JN709166	JN709225	JN676816	JN709276	JN709319	JN709358						
CR85	JN709167	JN709204	JN676817	JN709272	FN547371	JN709359						
CR89	JN709168	JN709222	JN676818	JN709273	JN709320	JN709360						
CR90	JN709169	JN709223	JN676819	JN709274	JN709321	JN709361						
CR91	JN709170	JN709224	JN676820	JN709275	JN709322	JN709362						
ZP591	Saccharomyce	Saccharomyces	HQ414037	Saccharomyces	Saccharomyces	Saccharomyces sensu stricto (http://saccharomycessensustricto.org/cgi-bin/s3)						

Table S2. Aromatic compound thresholds.

Compound	Odor threshold (mg L^{-1})	Sensory description	Off-odor threshold (mg L^{-1})	Reference
Higher Alcohols			>300	Jackson, 2009
Benzyl alcohol	200	sweet, fruity		Gámez-Míguez et al., 2007
Isobutanol	0.5-40	wine, solvent, bitter		Guth, 1997; Lambrechts and Pretorius, I S, 2000
Isoamyl alcohol	30	whiskey, malt, burnt		Guth, 1997
1-hexanol	8	resin, flower, green		Guth, 1997
2-Phenylethanol	10-14	honey, spice, rose, lilac		Ferreira et al., 2000; Guth, 1997
Esters			>150	Jackson, 2009
Benzyl acetate		apple-like		Jackson2008
Diethyl succinate	200	vinous		Etiévant1991
Ethyl acetate	12.26	pineapple	>100-150	Etiévant1991
Ethyl lactate	154.64	acid, medicine		Ferreira et al., 2000
Ethyl octanoate (caprylate)	0.002-0.005	fruit, fat		Ferreira et al., 2000; Guth, 1997
Ethyl decanoate (caprate)	0.2	grape		Ferreira et al., 2000; Guth, 1997
Hexyl acetate	0.115	pear, fruity		Takeoka et al., 1996
Isoamyl acetate	0.03	banana		González et al., 2006
Phenylethyl acetate	0.25	rose, honey, tobacco		Takeoka et al., 1996
2-methylpropyl ethanoate	1.6	floral		González et al., 2006
Hexanoic acid	0.42	sweat, acid, rancid		Carrau et al., 2008

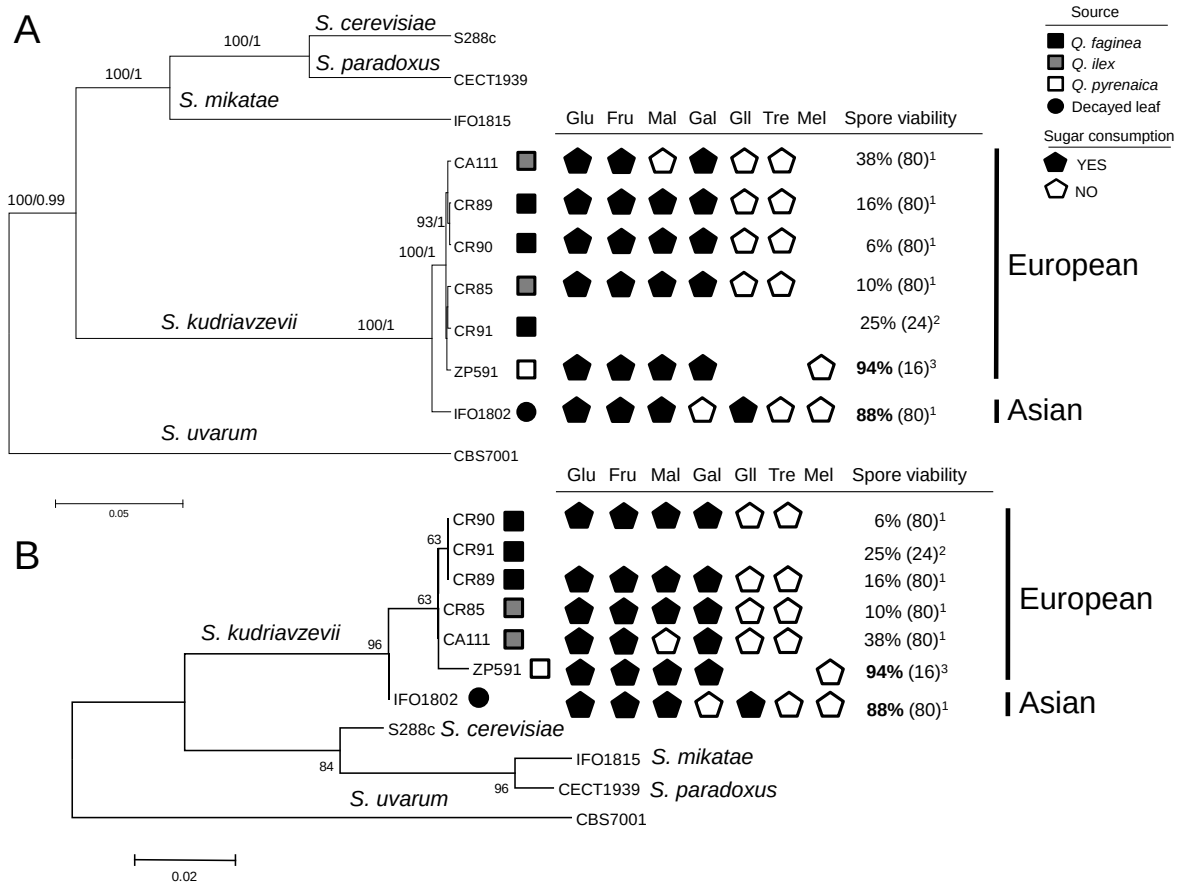
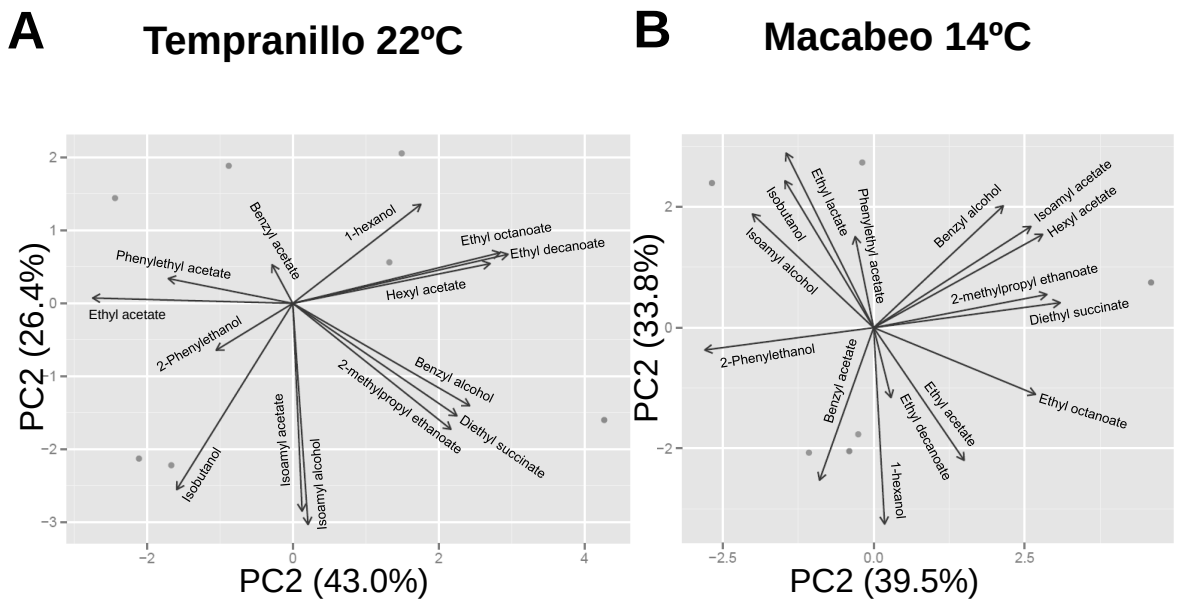


Figure S1. Mulitolocus and *COX2* phylogenetics, sugar uptake and spore viability. A) Nuclear and B) mitochondrial phylogenetic trees represented together with sugar uptake and spore viability phenotypes. Spore viability shows the percent of viable spores with the numbers of spores tested in parentheses. Spore viability higher than 75% is highlighted in bold. Superscripts 1, 2 and 3 for Lopes *et al.* 2010, this study and the Hittinger *et al.* 2010 data, respectively. Abbreviations: Glucose (Glu), Fructose (Fru), Maltose (Mal), Galactose (Gal), Galactitol (Gll), Trehalose (Tre), Melobiose (Mel).



Figures S2. Principal Component Analysis representations. A) and B) represent the PCA plots for the Tempranillo and the Macabeo aromatic profiles, respectively. Percentages on each axis indicate the percent of variation explained by each principal component. Variable weights are shown.