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

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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 *Saccharomyces uvarum* and *Saccharomyces paradoxus*. Many interspecies hybrids have also been isolated from wine fermentation, such as *S. cerevisiae* × *Saccharomyces 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.

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 *Saccharomyces uvarum* in Tokaj and Alsatian wines (Naumov et al., 2002; Demuyter et al., 2004), where others, such as *Saccharomyces 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 *Saccharomyces 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. The idea that natural hybridization occurs frequently in nature, and the high quality of the resulting wines, have meant that more

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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, 2013).

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 °C and 22 °C. 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 and Gonçalves (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 °C, 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 A 600 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 (300 g L^{-1}), low pH (3.0), ethanol (EtOH) content (5%, 10%, 12% and 15%), and low and high temperatures (10 °C, 16 °C, 30 °C and 37 °C). 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

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

Strain name	Isolation region	Source	Latitude	Longitude	Altitude (m)	Average temperature (°C)	Maximum temperature (°C)	Minimum temperature (°C)	Precipitation (mm)
CR85	Ciudad Real, Spain	*Quercus ilex* bark	38°58′51″	−4°51′51″	593	26	34	18	10
CR89	Ciudad Real, Spain	*Quercus faginea* bark	38°58′51″	−4°51′51″	593	26	34	18	10
CR90	Ciudad Real, Spain	*Quercus faginea* bark	38°58′51″	−4°51′51″	593	26	34	18	10
CR91	Ciudad Real, Spain	*Quercus faginea* bark	38°58′51″	−4°51′51″	593	26	34	18	10
CA111	Castellón, Spain	*Quercus ilex* bark	0°22′44″	−0°50′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