



# SSU1 Checkup, a Rapid Tool for Detecting Chromosomal Rearrangements Related to the SSU1 Promoter in *Saccharomyces cerevisiae*: An Ecological and Technological Study on Wine Yeast

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Chromosomal rearrangements (CR) such as translocations, duplications and inversions play a decisive role in the adaptation of microorganisms to specific environments. In ecological *Saccharomyces cerevisiae* strains, CR involving the promoter region of the gene *SSU1* lead to a higher sulfite tolerance by enhancing the SO<sub>2</sub> efflux. To date, three different *SSU1* associated CR events have been described, including translocations XV-t-XVI and VIII-t-XVI and inversion inv-XVI. In the present study, we developed a multiplex PCR method (*SSU1* checkup) that allows a rapid characterization of these three chromosomal configurations in a single experiment. Nearly 600 *S. cerevisiae* strains collected from fermented grape juice were genotyped by microsatellite markers. We demonstrated that alleles of the *SSU1* promoter are differently distributed according to the wine environment (cellar versus vineyard) and the nature of the grape juice. Moreover, rearranged *SSU1* promoters are significantly enriched among commercial starters. In addition, the analysis of nearly isogenic strains collected in wine related environments demonstrated that the inheritance of these CR shapes the genetic diversity of clonal populations. Finally, the link between the nature of *SSU1* promoter and the tolerance to sulfite was statistically validated in natural grape juice containing various SO<sub>2</sub> concentrations. The *SSU1* checkup is therefore a convenient new tool for addressing population genetics questions and for selecting yeast strains by using molecular markers.

**Keywords:** sulfite resistance, domestication, translocation, multiplex PCR, marker assisted selection

## INTRODUCTION

Microorganisms develop various strategies for being better adapted to various environments. Among them, the yeast *Saccharomyces cerevisiae* is a noteworthy example of a microorganism whose evolution led to specialized genetic groups associated with different human-related environments (Sicard and Legras, 2011; Borneman and Pretorius, 2014; Marsit and Dequin, 2015). In a winemaking context, this species has been exposed to stressful conditions (high alcohol content, high osmotic pressure, low pH, etc.) for millennia, potentially resulting in adaptive differentiation.

In wine production, sulfite addition is widely used since the middle age as a preservative because of its antimicrobial, antioxidant, and antioxydasic activities. Produced by dissolution of sulfur dioxide (SO<sub>2</sub>), sulfite inhibits key glycolytic enzymes like *Tdh* and *Adh* proteins, binds carbonyl compounds such as pyruvate and acetaldehyde (Hinze and Holzer, 1986) affects transporter activity by binding membrane proteins (Divol et al., 2012) and down-regulates the expression of many central metabolism genes (Park and Hwang, 2008). Therefore, sulfite tolerance has been unconsciously selected by wine making practices and constitutes a desired trait in *Saccharomyces* wine yeast strains. Cellular mechanisms of sulfite tolerance have been extensively reviewed in *S. cerevisiae* (Divol et al., 2012; García-Ríos and Guillamón, 2019). They include the overproduction of acetaldehyde (Cherati et al., 2010) the regulation of sulfite reduction systems and more generally of the sulfur metabolic pathway (Divol et al., 2012). Moreover, sulfite tolerance mostly depends on the pumping of SO<sub>2</sub> through the plasma membrane. This sulfite efflux involves the sulfite pump *Ssu1p* which is encoded by the *SSU1* gene. This gene shows a high level of polymorphism (Aa et al., 2006) and deleterious mutations in its coding sequence cause SO<sub>2</sub> susceptibility (Avram and Bakalinsky, 1997; Park and Bakalinsky, 2000).

The expression level of *SSU1* has a direct consequence on sulfite tolerance and has been widely studied (Pérez-Ortín et al., 2002; Nardi et al., 2010; Engle and Fay, 2012; Zimmer et al., 2014; García-Ríos et al., 2019). Interestingly, the *SSU1* promoter sequence is involved in three Chromosomal Rearrangements (CR) (i.e., XV-t-XVI, VIII-t-XVI, and inv-XVI) that increase its expression leading to a more efficient sulfite pumping over (Pérez-Ortín et al., 2002; Zimmer et al., 2014; García-Ríos et al., 2019). These three independent CR events constitute a hallmark on parallel evolutionary routes driven by human selection. In the VIII-t-XVI translocation, the native promoter of *SSU1* is replaced by tandem repeated sequences of the *ECM34* promoter from chromosome VIII (Pérez-Ortín et al., 2002). In the XV-t-XVI translocation, the upstream region of *SSU1* is placed head to tail with the *ADH1* promoter from chromosome XV (Zimmer et al., 2014). The inversion of chromosome XVI (inv-XVI) involves the *SSU1* and *GCR1* regulatory regions, increasing the expression of *SSU1* (García-Ríos et al., 2019).

To date, the distribution of translocation (XV-t-XVI and VIII-t-XVI) and inversion (inv-XVI) events of the *SSU1* gene

have been investigated for a small number of strains (Pérez-Ortín et al., 2002; Zimmer et al., 2014; García-Ríos et al., 2019). Here, we set up a multiplex method (*SSU1* *checkup*) based on labeled primers with different fluorochromes, to identify in a single assay the three types of *SSU1* associated CR (VIII-t-XVI, XV-t-XVI, and inv-XVI) as well as the wild type forms of these chromosomes (VIII-wt, XV-wt, and XVI-wt). The *SSU1* was applied to nearly 600 yeast strains, including natural isolates and commercial starters, and provides new insights on the allele frequency of rearranged *SSU1* promoters. In addition, by using microsatellite genotyping, the genetic relationships between strains of the collection were established allowing the study of CR occurrence in nearly isogenic clones. Finally, for a subset of strains, the phenotypic impact of different CR was evaluated by measuring their parameters of growth in grape juice containing different concentrations of SO<sub>2</sub>.

## MATERIALS AND METHODS

### Origin of Samples

A total of 628 *S. cerevisiae* isolates were collected from grapes and fermented must (white, red, and sweet) originating from five different countries (France, Lebanon, Argentina, Spain, and Italy), two different *Vitis* species (mostly *V. vinifera* and to a lesser extent *V. labrusca*) and nine different varieties. Two different procedures were used for strain isolation depending on the environment considered: vineyard or cellar. For vineyard isolates, around 2 kg of healthy and mostly undamaged grapes were collected a few days before the harvest in the vineyard, crushed in sterile conditions and macerated for 2 h with 50 mg/L of SO<sub>2</sub>. The juice was fermented at 21°C in small glass-reactors (500 mL). For cellar isolates, yeast colonies were obtained from spontaneous fermentation vats containing sulfited grape juices according to local enological practices (ranging from 20 to 50 mg/L of sulfur dioxide) except for sweet wines for which no sulfur dioxide was added. For both sampling procedures, fermentations were allowed to proceed until 2/3 of the must sugars were consumed and fermented juices were plated onto YPD plates (yeast extract, 1% w/v; peptone, 1% w/v; glucose, 2% w/v; agar 2% w/v) with 100 µg/mL of chloramphenicol and 150 µg/mL of biphenyl to delay bacterial and mold growth. Around 30 colonies per sample were randomly chosen and after sub-cloning on YPD plates, each yeast colony was stored in 30% (v/v) glycerol at -80°C. Additionally, a collection of 103 industrial *S. cerevisiae* starters was constituted by streaking on YPD plates a small aliquot of Active Dry Yeast obtained from different commercial suppliers.

### Microsatellite Analysis

Strains were genotyped using fifteen polymorphic microsatellite loci (C3, C4, C5, C6, C8, C9, C11, SCAAT1, SCAAT2, SCAAT3, SCAAT5, SCAAT6, SCYOR267C, YKL172W, YPL009C) developed for estimating the genetic relationships among *S. cerevisiae* strains (Legras et al., 2007). Most of the strains were previously genotyped in our lab (Börlin et al., 2016;

Raymond et al., 2018; Peltier et al., 2018a; Borlin, 2015) and the additional 82 strains were genotyped in this work using identical experimental conditions. Briefly, two multiplex PCRs were carried out in a final volume of 12.5  $\mu\text{L}$  containing 6.25  $\mu\text{L}$  of the Qiagen Multiplex PCR master mix (Qiagen, France), 1  $\mu\text{L}$  of DNA template, and 1.94  $\mu\text{L}$  of each mix, using the conditions previously reported (Peltier et al., 2018b). Both reactions were run using an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C for 2 min, 72°C for 1 min, and a final extension step at 60°C for 30 min. The size of PCR products was determined by the MWG company (Ebersberg, Germany), using 0.2  $\mu\text{L}$  of 600 LIZ GeneScan (Applied Biosystems, France) as a standard marker, and chromatograms were analyzed with the GeneMarker (V2.4.0, Demo) program. Only strains that amplified at least 12 of 15 loci were kept. On the 735 strains collected, 586 met this criterion and were used in this study (listed on **Supplementary Table S1**). The microsatellite data set was analyzed by means of the *poppr* R package using the Bruvo's distance matrix. Strains showing a strong similarity were identified by applying a cut of value of 0.15 to the Bruvo's genetic distance matrix. In this way, 194 very closely related strains were identified and considered as "clones." This cut off value was defined in order to restore the normality of the distribution (**Supplementary Figure S1**). The assignment of clustering methods was achieved by using the *find.clusters* function (*adeget* package). The selection of the optimal groups was computed by the Ward's clustering method using the Bayesian Information Criterion (BIC) as statistical criterion (Avramova et al., 2018).

## The SSU1 Checkup Method

In order to experimentally detect in a single multiplex PCR test all the CR involving the gene *SSU1*, labeled primers were designed using a specific dye per chromosome position as follows: 6-FAM (Chr8: VIII-14558), ATTO550 (Chr15: XV-160994), HEX (Chr16: XVI-373707), and ATTO565 (Chr16: XVI-412453) (**Table 1**). All the primers (**Table 1** and **Supplementary Table S2**) were synthesized by Eurofins genomics (Ebersberg, Germany). A multiplex PCR was carried out in a final volume of 20  $\mu\text{L}$  using 100 nM of each primer, 1  $\mu\text{L}$  of template DNA and the Qiagen PCR multiplex PCR kit (Qiagen, France) on a T100TM Thermal cycler (Bio-Rad, France). The following PCR program allows the amplification of all the expected fragments from the rearranged and the wild type VIII, XV and XVI chromosomes: initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 90 s, 72°C for 90 s, ending with a hold at 60°C for 30 min. DNA templates for PCR were extracted in 96-well microplates using the previously described LiAc-SDS protocol (Chernova et al., 2018). Before analysis, PCR products were diluted 60 times in ddH<sub>2</sub>O and 1  $\mu\text{L}$  of this solution was mixed with 0.2  $\mu\text{L}$  of the internal size standard GenScan<sup>TM</sup> 1200 LIZ (Applied Biosystems, France) and 9.8  $\mu\text{L}$  of highly deionized Hi-DiTM formamide (Applied Biosystems, France). Samples were analyzed by Eurofins genomics (Ebersberg, Germany) on an ABI-3710 Genetic Analyzer. Each peak was identified according to the color and size and attributed to the alleles (**Figure 1**).

Each allele was also sequenced by amplifying both strands with non-labeled primers. The sequences were released on GenBank with the following accession numbers: ID MT028493-MT028507.

## SO<sub>2</sub> Tolerance Assessment

To assess SO<sub>2</sub> tolerance, a subset of 34 strains (**Supplementary Table S3**) was cultivated in white grape juice (Sauvignon blanc from the Bordeaux area, France). This must had a total SO<sub>2</sub> concentration of 14 mg/L and was spiked with 0, 25, 50, and 75 mg/L of total SO<sub>2</sub>. Cultures were achieved in 96-well plates (U flat well, Greiner, France) filled with 200  $\mu\text{L}$  of grape juice sterilized by a nitrate-cellulose membrane filtration (Millipore, France). Yeasts were pre-cultivated in YPD media (yeast extract, 1% w/v; peptone, 1% w/v; glucose, 2% w/v) for 16 h at 28°C and inoculated into the grape juice to a final concentration of  $1 \times 10^6$  cells/mL. Growth was monitored by OD<sub>600</sub> measurements for 96 h at 28°C using a microplate spectrophotometer (Synergy HT Multi-Mode Reader, BioTek Instruments, Inc., United States). Culture plates were shaken every 25 min for 30 s prior to the OD<sub>600</sub> measurements. The well position on the microplate was randomized and six replicates were done for each strain\*media condition. Data from the microplate reader were transformed with the polynomial curve  $y = -0.0018x^3 + 0.1464x^2 + 0.7757x + 0.0386$  to correct the non-linearity of the optical recording at higher cell densities as previously reported (Martí-Raga et al., 2016). Growth kinetic data were fitted using the *Richards* flexible inflection point model implemented by the *fit.growthmodel* function, R package *growthrates*. This model allows the estimation of the maximal growth rate ( $\mu_{max}$ ). A second parameter, *Lag Time*, was manually computed from raw data by considering the time necessary to reach twice the OD<sub>600</sub> of the inoculum. A linear model was applied for estimating effects of the SO<sub>2</sub> concentration and type of chromosome XVI and their possible interactions:

$$(1) \text{ Lm1: } Y_{ik} = m + \text{ChrXVI}_i + \text{SO}_2_k + (\text{ChrXVI:SO}_2)_{ik} + E_{ijk}$$

Where Y are the values of the trait ( $\mu_{max}$  and *Lag Time*), for *j* ChrXVI configurations ( $i = 1$  to 6), and *k* SO<sub>2</sub> ( $k = 1$  to 4) concentrations, *m* was the overall mean and *E<sub>ijk</sub>* the residual error. Homoscedasticity of the ANOVA was tested by *LeveneTest* function (*car* package) while the normal distribution of models' residuals was estimated by visual inspection (*qq plot*).

## RESULTS AND DISCUSSION

### Assessment of the Genetic Diversity of Starters and Natural Isolates Populations of *S. cerevisiae*

In this study, we analyzed a large dataset of 586 isolates that were genotyped using 15 microsatellite loci. This collection includes 103 industrial starters and 483 indigenous isolates from different origins (sampling mode, red or white grape must, country). Since many natural isolates were sampled in the same juice, some of them could have originated from clonal expansion and be

**TABLE 1** | Primers used for the *SSU1* checkup method and the strains used as positive controls.

Chromosome	Strain	Primer F			Primer R			Size <sup>a</sup>	Position <sup>b</sup>
		Name	Sequence	Dye	Name	Sequence	Dye		
VIII	SB	1189	ATGGCAGCTTCTAAGTTGTGG	FAM	1190	GTTTATGTTTGGTTTGGGGG	na	604	VIII-14558 to VIII-15162
	GN							667	
XV	SB	1191	AAAGAAGTTGCATGCGCCTA	ATTO550	1192	ACCTGAGTGCATTGCAACA	na	702	XV-160994 to XV-161695
	F10							702	
XVI	SB	1193	TGTCAAGTTGAGACAAACCGA	na	1194	GGGAAAGCTGTAATTTGTGT	Hex	991	XVI-372717 to XVI-373707
VIII-t-XVI	F10	1189	ATGGCAGCTTCTAAGTTGTGG	FAM	1194	GGGAAAGCTGTAATTTGTGT	Hex	555	VIII-14558 to XVI-373707
XV-t-XVI	GN	1191	AAAGAAGTTGCATGCGCCTA	ATTO550	1194	GGGAAAGCTGTAATTTGTGT	Hex	496	XV-160994 to XVI-373707
inv-XVI	P5	1196	TGCATAAGCAGGCAACTCCT	ATTO565	1194	GGGAAAGCTGTAATTTGTGT	Hex	781	XVI-373707- 412453

<sup>a</sup>PCR product lengths in base pairs; <sup>b</sup>position in the chromosome for reference strain S288c; na: not applicable.

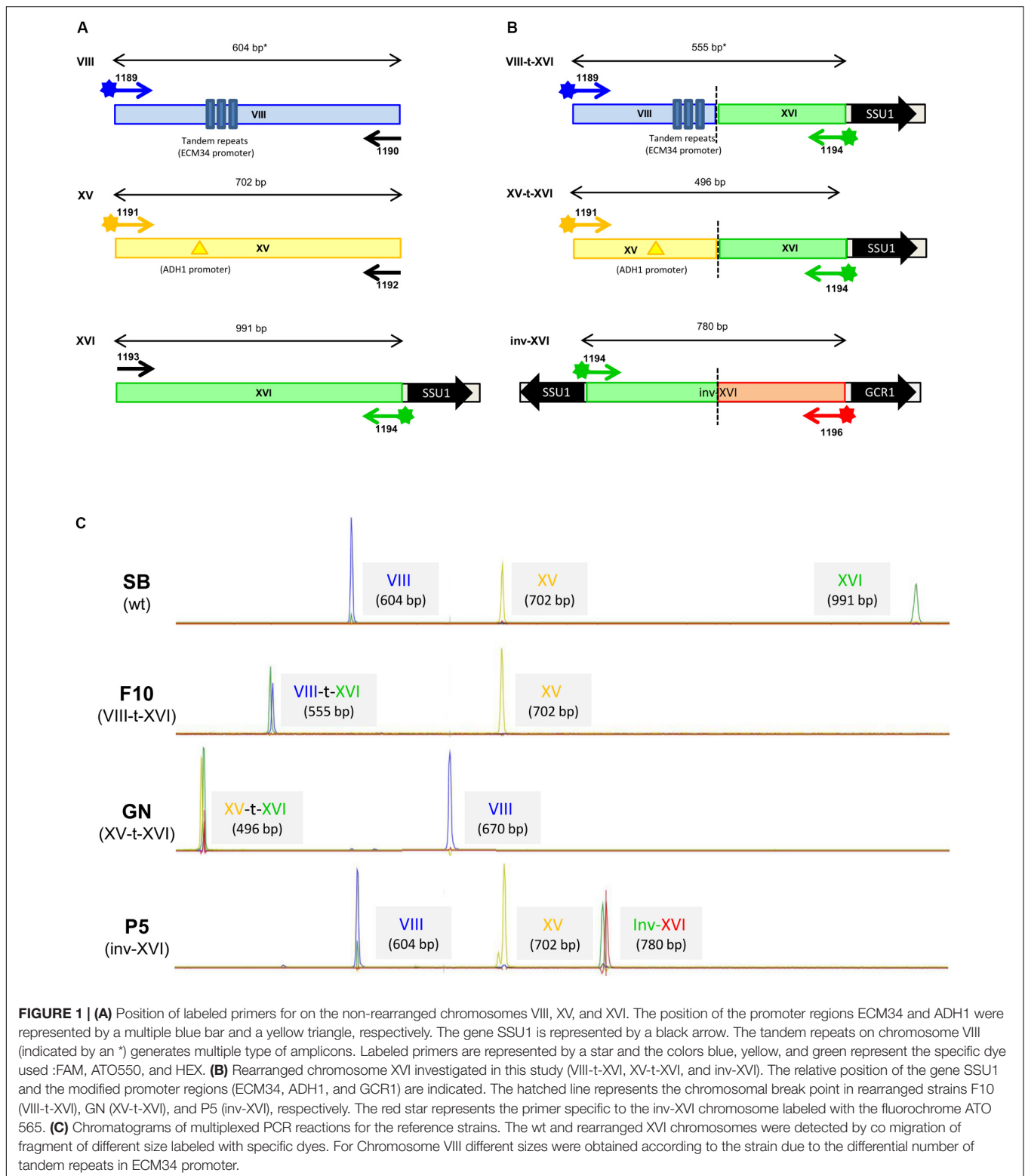
very similar from a genetic point of view. A filtering procedure was applied for keeping only one representative genotype of each clonal population by removing all but one strain having a Bruvo's genetic distance lower than 0.15 (Supplementary Figure S1). By this procedure, many natural isolates closely related to industrial starters were identified (Supplementary Figure S2) demonstrating the wide dissemination of commercial yeasts in vineyard and winery environments as previously reported (Valero et al., 2005; Borlin, 2015). In addition, 21 isogenic strains were found among commercial starters. This filtering procedure defined three subpopulations: "starters = 82," "natural isolates = 310," and "closely related clones = 194" (Supplementary Table S1). The genetic relationships for each strain within the *starters* and *natural isolates* subpopulations were then analyzed by a principal component analysis ( $k = 6$ ). The constitution of genetic groups based on microsatellites inheritance was carried out by using a k-mean based algorithm (see section "Materials and Methods").

This genetic analysis clustered the 82 commercial strains in three groups with a group C clearly separated from the other two (Supplementary Figure S3) and corresponding to "Champenoise" strains, a particular wine yeast group previously described (Legras et al., 2007; Novo et al., 2009; Borneman et al., 2016). Its detection validated our clustering analysis based on the use of k-mean clustering. The structure of the 310 *natural isolates* collected was also investigated and six subgroups were defined. Figure 2A shows the first two dimensions of the PCA; axis one clearly identified a group of isolates from Argentina, while axis 2 broadly discriminated the five other groups. The assignment of subgroups on neighbor-joining tree (unrooted) illustrates that isolates are mostly clustered according to their geographical origins (Figure 2B). Some groups are specific to sampling zones such as group 3 ( $n = 16$ ) and group 6 ( $n = 84$ ) that only contain strains sampled in Argentina and Lebanon, respectively. In contrast, group 2 ( $n = 177$ ) encompassed isolates from different geographic origins (Italy, Spain, France, and Lebanon) (Supplementary Table S4). Although not perfectly discriminating, this first analysis filtered the redundancy of our

collection and provided a clear overview of the genetic diversity of non-redundant strains.

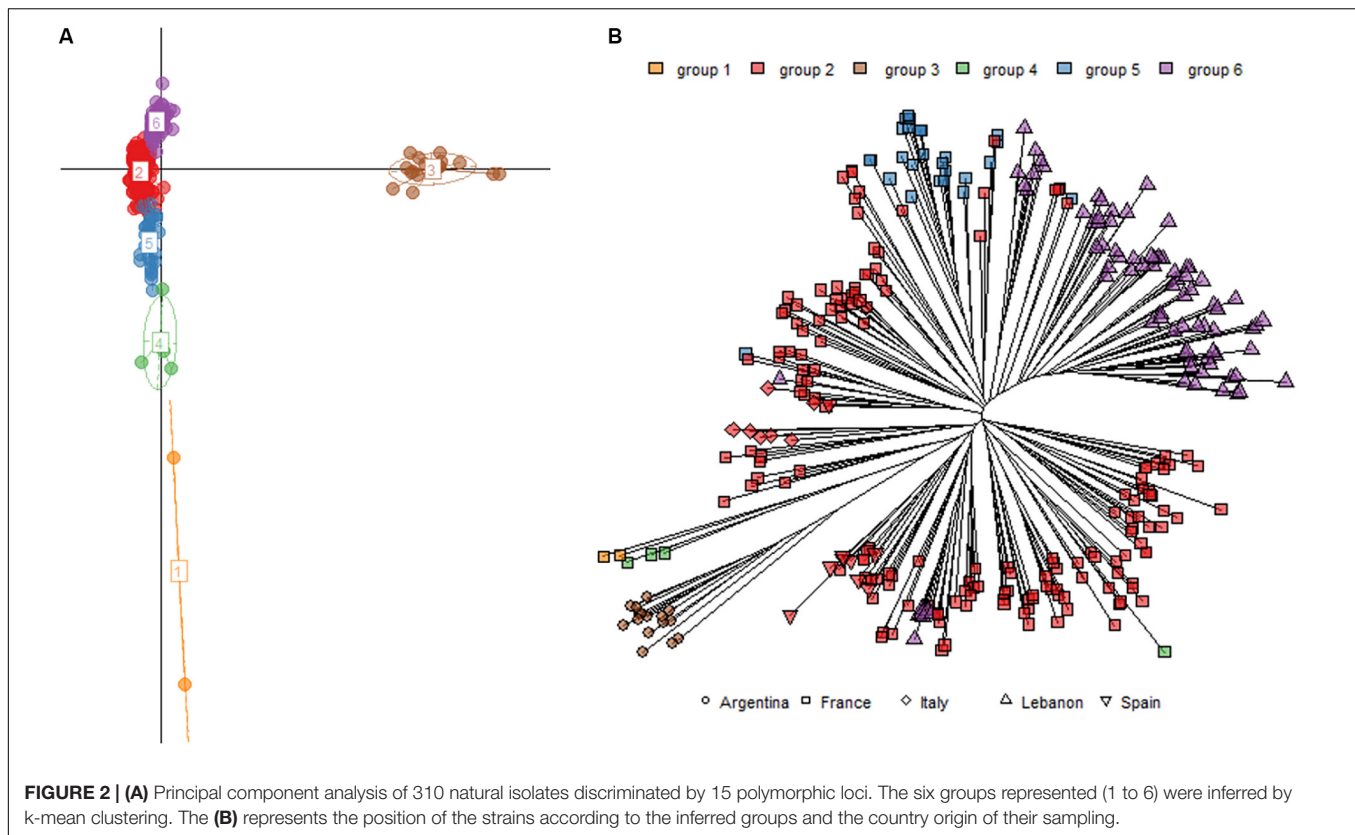
## Development of the *SSU1* Checkup, a Simple Method for Genotyping *SSU1* Chromosomal Rearrangements in *S. cerevisiae*

To date, translocation events (i.e., XV-t-XVI and VIII-t-XVI) have been detected by classical PCR experiments narrowing the chromosomal break points identified by two original works (Pérez-Ortín et al., 2002; Zimmer et al., 2014). Recently, an additional chromosomal rearrangement involving the gene *SSU1* (inv-XVI) was also described (García-Ríos et al., 2019). Because these classical PCR amplifications are scarcely adapted to screen multiple genotypes in large populations, a multiplexed method (*SSU1* checkup) was set up aiming to identify, in a single PCR reaction, these three types of chromosomal rearrangements (VIII-t-XVI, XV-t-XVI, and inv-XVI) as well as the wild type alleles of the corresponding chromosomes (VIII-wt, XV-wt, and XVI-wt). Since primers are labeled with different fluorochromes they allow the identification of the different allelic combinations. Primers used for amplifying the wild type chromosomes (VIII-wt, XV-wt, and XVI-wt) were labeled with a single fluorophore (FAM, ATTO 550, Hex), providing blue, yellow, and green peaks, respectively (Figure 1A). With this set of primers, the amplifications of the rearranged *SSU1* promoter regions result to be labeled with two fluorophores, allowing the easy identification of the recombined forms (Figure 1B). The allele sizes amplified range between 388 and 991 bp and were analyzed by following the fluorescence of PCR products with an ABI sequencer (Figure 1C). In preliminary studies, we used reference strains SB (XVI-wt), GN (XV-t-XVI), F10 (VIII-t-XVI), and P5 (inv-XVI), to design and validate primers. Primers position, as well as the length of the DNA fragments amplified for the reference strains, are summarized in Table 1. The sequences of all the alleles identified were submitted to GenBank (ID 2310529). The strain P5 is a commercial starter able to sporulate (data not



shown) and would be therefore diploid. The ploidy level of strains SB, GN, and F10 has been previously determined by several genetic analyses (Albertin et al., 2009). These strains are fully homozygous diploids (2n) and were obtained by the sporulation

of the commercial starters Actiflore BO213, Zymaflore VL1 and Zymaflore F10, Laffort, France) as previously reported (Marullo et al., 2006, 2009). Their associated chromatograms showed the different types of alleles reported in this study (Figure 1C). In



order to simplify the chromatographic patterns, the detection of reciprocal translocation events (XVI-t-XV and XVI-t-VIII) was not included in the *SSU1* checkup. However, the strains GN and F10 harbor reciprocal translocations that have been verified by PCR using the primers given in **Supplementary Table S2**.

The *SSU1* checkup was used for tracking the two translocation events (VIII-t-XVI and XV-t-XVI) as well as the chromosomal inversion (inv-XVI) in a large collection of strains ( $n = 586$ ). In the VIII-t-XVI translocation, the native promoter of *SSU1* is replaced by DNA sequences of the *ECM34* promoter (located on chromosome VIII) (Pérez-Ortín et al., 2002). Alleles for this CR (i.e., VIII-t-XVI<sup>388</sup>, VIII-t-XVI<sup>478</sup>, VIII-t-XVI<sup>555</sup>, and VIII-t-XVI<sup>631</sup> bp) result from the alternative number of units of tandem repeated motifs (76 bp and/or 47 bp) localized in the promoter region of the gene *ECM34* (**Supplementary Figure S4**). In the XV-t-XVI translocation, the upstream region of *SSU1* is placed head to tail with the *ADH1* promoter (located on chromosome XV) (Zimmer et al., 2014) and a single allele has been recognized (XV-t-XV<sup>496</sup>). Finally, the chromosomal rearrangement (inv-XVI) has been recently reported by García-Ríos et al. (2019) and consist in a chromosome XVI inversion generating a new *SSU1* promoter placed head to tail with the *GCR1* promoter. This event was detected in only 19 natural isolates and showed a single allele of 781 bp (inv-XVI<sup>781</sup>). Surprisingly, twenty strains definitively failed to amplify any fragment even when performing single PCR reactions using alternative primers (**Supplementary Table S2**). This interesting result suggests that these strains could harbor another still

uncharacterized chromosomal rearrangement flanking the *SSU1* gene. Such possible new CR should be tracked by chromosome walking PCR starting from *SSU1* gene or by de novo assembly of whole genome sequences.

## Landscape of the Different *SSU1*-Promoter Alleles in Natural and Selected Populations

The *SSU1* checkup method allows the detection of four types of chromosome XVI structures in a single PCR reaction. However, this method is not quantitative, and it is not possible to know the number of copies of each haplotype. Since all the fragments amplified by the primer 1194 (Hex) are physically linked to the chromosome XVI's centromere (*CEN16*), they belong to chromosome XVI during the cell division process. Therefore, native and rearranged chromosome XVI alleles can be merged in order to have an integrated overview of the chromosome XVI inheritance. This allows following the inheritance of the different promoter versions of the *SSU1* gene.

The different alleles of chromosome XVI were counted among the 392 non-redundant *S. cerevisiae* strains analyzed. As a first approximation, we considered that all the strains analyzed are diploids. This assumption is based on the fact that 87% the *S. cerevisiae* strains are diploid and that polyploids/aneuploid strains are mostly observed in ale beer and sake strains (Peter et al., 2018). In contrast, wine yeast strains are generally euploids and diploids likely due to their homothallic character

(Mortimer et al., 1994). In our population, 65% of the population genotyped proved to be heterozygous (and therefore diploid) for at least two microsatellite loci (**Supplementary Table S1**) which is consistent with previous population genetic observations for wine *S. cerevisiae* strains (Legras et al., 2007). Assuming this hypothesis, when a strain showed a single chromosome XVI allele we assigned two identical genotypes as done for routine microsatellite analysis (Legras et al., 2007). In this way, strains showing more than two distinct alleles for chromosome XVI were considered to have an extra copy of this chromosome. For example, the strain Zymaflore VL2 inherited the alleles XVI-wt<sup>991</sup>, VIII-t-XVI<sup>555</sup>, and XV-t-XVI<sup>496</sup> (**Supplementary Table S1**) and was considered as aneuploid for the chromosome XVI (three CEN16 centromeres instead of two).

The allele frequencies computed are given in **Table 2**; the occurrence of each allele between *natural isolates* and *starters* populations was compared by a Chi<sup>2</sup> test. Among the 392 non-redundant *S. cerevisiae* strains analyzed, the most frequent alleles found were VIII-t-XVI<sup>555</sup> (0.41) and XVI-wt<sup>991</sup> (0.34). However, their allelic frequencies are not evenly distributed. Indeed, the *starters* group ( $n = 82$ ) is significantly enriched in alleles VIII-t-XVI<sup>388</sup> and XV-t-XVI<sup>496</sup> compared to the *natural isolates* group ( $n = 310$ ); in contrast, *natural isolates* mostly harbor the VIII-t-XVI<sup>555</sup> allele.

Since chromosomal rearrangements lead to more active *SSU1* genes, these alleles are supposed to be mostly dominant (Clowers et al., 2015; Peltier et al., 2018a). Therefore, for having a more accurate understanding of the functional impact of chromosome XVI forms, the percentage of homozygous strains for the different alleles is also given in **Table 2**. The homozygosity level of VIII-t-XVI alleles is much higher among *natural isolates* (49.1 vs. 13.8%) than among *starters*. Interestingly, for these two subgroups of strains, we do not find a significant discrepancy for the overall homozygosity level of the 15 microsatellite markers analyzed (23 vs. 23% for *natural isolates* and *starters*, respectively). However, the microsatellite marker C6 localized on the chromosome XVI at less than 100 kb of the *SSU1* gene, shows a similar homozygous level discrepancy than the VIII-t-XVI alleles (35 vs. 17%, for natural and industrial strains, respectively). In the same way, although allele frequencies of XVI-wt<sup>991</sup> are quite similar between the two populations, homozygous strains are more frequent in the *natural isolates* group (25.4 vs. 8.8%, corrected Chi<sup>2</sup> test,  $p = 1.10^{-4}$ ). Consequently, from a functional point of view, only seven industrial strains lacked any rearranged *SSU1* allele (*ECM34-SSU1*, *ADH1-SSU1*, or *GCR1-SSU1*). Furthermore, an overall difference of heterozygosity was not observed for the 15 microsatellite markers but was significant for the marker C6 (42.1 vs. 11.4% for natural and industrial strains, respectively). Altogether, these observations suggest that the different ratio of homozygosity observed between *starters* and *natural isolates* in the region of *SSU1* could be due to a local loss of heterozygosity that remains unexplained.

Out of the 21 possible biallelic combinations of the seven chromosome XVI alleles, 18 biallelic combinations were found among the 586 strains typed using the *SSU1* checkup. The percentage of strains carrying at least one type of CR is shown

in **Figure 3**. Industrial strains are significantly enriched in translocations VIII-t-XVI and XV-t-XVI compared to natural isolates. In contrast, the inv-XVI allele was rarer and never found in industrial strains (the reference strain P5 was not included here). Interestingly, two industrial starters (3%) carry both translocated chromosomes. In addition, 11 starters (13%) have an extra copy of chromosome XVI (aneuploidy), a fraction much higher than for the *natural isolates* group (1 out of 310). It has been suggested that an extra-copy number of chromosome XVI would confer a gain of fitness during fermentation (Brion et al., 2013). As shown in **Table 2**, seven industrial strains are homozygous for the XVI-wt allele (8.5% of the population). These starters are usually recommended for red grape juice winemaking or Cognac distillation, where the SO<sub>2</sub> pressure is lower than in white wine production.

### Linking Chromosomal Rearrangement Events of Chromosome XVI and Yeast Ecology

Wine-related natural isolates allow the study of broad ecological factors influencing the chromosomal configurations of *SSU1*'s promoter. It has been shown that *SSU1* related translocations in wine yeast isolates are advantageous for growth in sulfited grape juice and contribute to a fitness gain respect to oak yeast strains (Clowers et al., 2015). Furthermore, previous studies revealed that variations in the promoter region of *SSU1* gene in wine yeasts enhance the *SSU1* gene expression during fermentation, and have a remarkable effect on the SO<sub>2</sub> resistance levels (Pérez-Ortín et al., 2002; Zimmer et al., 2014; García-Ríos and Guillamón, 2019). The subpopulation of 310 unique strains characterized in this work were split according to the sampling procedure applied: *cellar* ( $n = 205$ ) vs. *vineyard* ( $n = 105$ ) isolates (**Supplementary Table S1**). *Cellar* strains were isolated from spontaneously fermented vats in various wine estates, from sulfited grape musts according to the recommended practices of the area of origin. *Vineyard* strains were isolated from grapes manually harvested, crushed, and fermented in sterile laboratory conditions (see section "Materials and Methods").

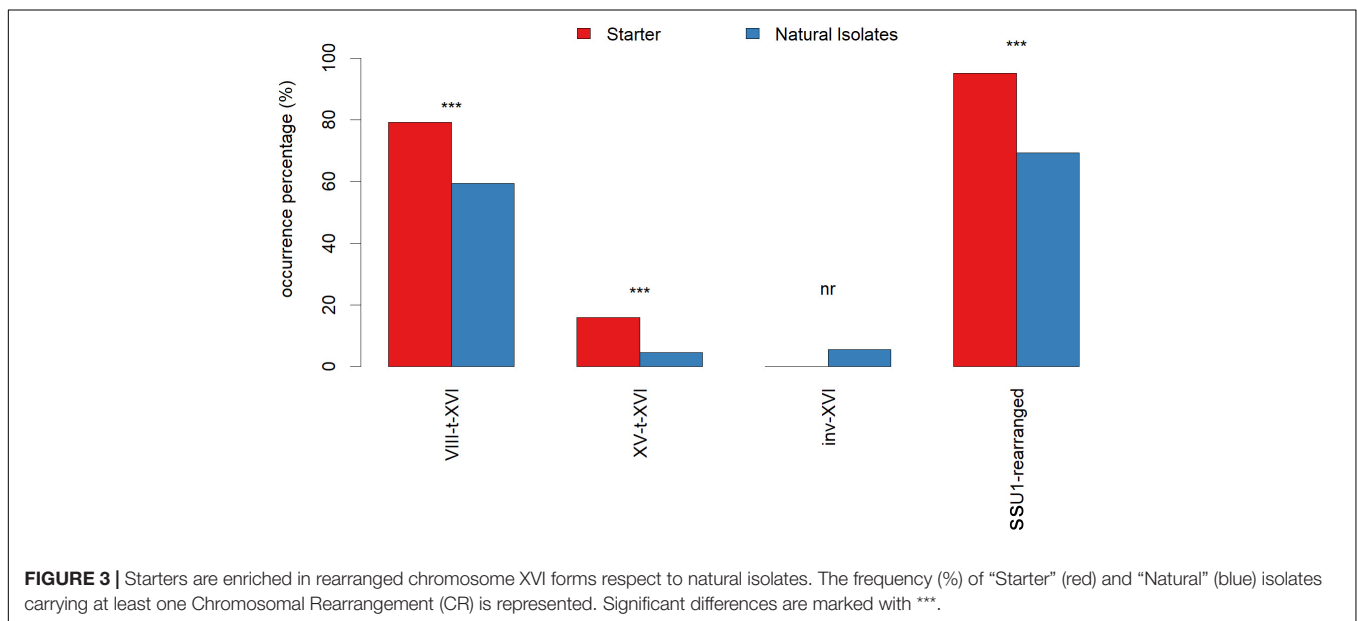
The occurrence percentage of the 18 allelic combinations found in both groups is shown in **Figure 4A**. The proportion of genotypes (555:555 and 991:555) is significantly higher in the *cellar* group while *vineyard* isolates are slightly enriched in 781:781 genotypes ( $p$ -value < 0.1, corrected Chi<sup>2</sup> test). Moreover, strains having inherited at least one rearranged chromosome XVI are significantly more frequent in the *cellar* group (**Figure 4A**). The occurrence percentage observed here could reflect that among wine-related yeast isolates, *cellar* strains undergo a stronger selective pressure than *vineyard* strains likely due to winemaking operations.

The impact of the nature of the grape juice from which strains were isolated was also tested. For this study, we focused our investigation only on *cellar* populations ( $n = 205$ ) that have been subjected to *in situ* enological treatments. Indeed, according to the enological practices and doses, grape juices are

**TABLE 2** | Allele frequency and percentage of homozygosity of different chromosome XVI forms within starters and natural isolates populations.

Chromosome XVI alleles	Allele frequencies				% of Homozygous strains			
	Total samples <i>n</i> = 392	Natural isolates <i>n</i> = 310	Starters <i>n</i> = 82	Chi <sup>2</sup> test ( <i>p</i> -value)	Total samples <i>n</i> = 392	Natural isolates <i>n</i> = 310	Starters <i>n</i> = 82	Chi <sup>2</sup> test ( <i>p</i> -value)
VIII-t-XVI <sup>388</sup>	0.093	0.021	0.369	<2.2.10 <sup>-16</sup>	3.8	2.1	10.0	1.9.10 <sup>-2</sup>
VIII-t-XVI <sup>478</sup>	0.027	0.034	0.000	nr	2.2	2.8	0.0	nr
VIII-t-XVI <sup>555</sup>	0.411	0.464	0.206	5.5.10 <sup>-8</sup>	34.6	43.2	3.8	3.1 10 <sup>-4</sup>
VIII-t-XVI <sup>631</sup>	0.008	0.010	0.000	nr	0.8	1.0	0.0	nr
VIII-t-XVI (all alleles)	0.539	0.529	0.575	0.18	41.4	49.1	13.8	6.7.10 <sup>-8</sup>
XV-t-XVI <sup>496</sup>	0.041	0.026	0.100	8.4.10 <sup>-3</sup>	1.4	0.6	3.6	nr
inv-XVI <sup>781</sup>	0.042	0.053	0.000	nr	4.4	5.6	0.0	nr
XVI-wt <sup>991</sup>	0.337	0.322	0.394	0.21	21.8	25.4	8.5	3.1 10 <sup>-4</sup>

nr: not relevant.



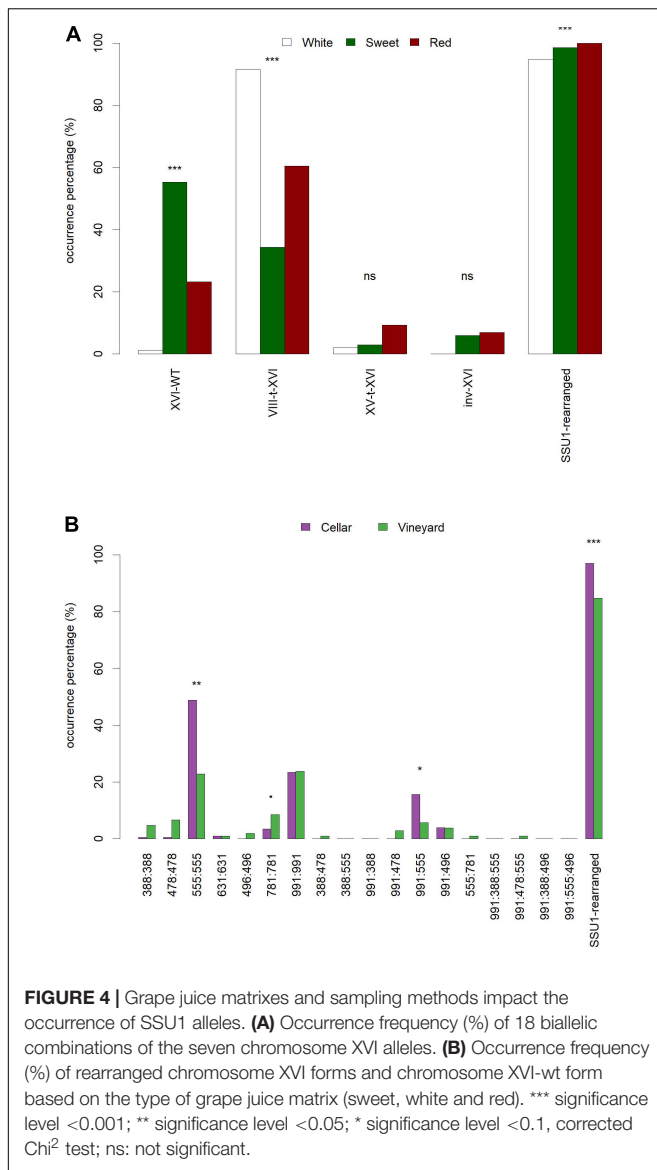
not equally sulfited. This is consistent with the recommendations of International Organization of the Vine and Wine (OIV) that regulates the limits for total SO<sub>2</sub> in wines (150 mg/L for red wines and 200 mg/L for white wines and rosés) (OIV, 2019). Strains were split in three groups depending on the type of grape juice matrix: *sweet* (*n* = 67), *white* (*n* = 95) and *red* (*n* = 43). As shown in **Figure 4B**, strains isolated from *white juice* are enriched in the VIII-t-XVI rearrangements and a few of them are homozygous for the XVI-wt<sup>991</sup> allele. In contrast, strains isolated from *sweet* grape juices are strongly enriched in the native chromosome form. For this group, the occurrence percentage of XVI-wt is 0.55, which is twice as much as the overall percentage of cellar population (0.26). These results are consistent with the traditional enological practices used in the Bordeaux area, where the addition of sulfite in the musts is routinely used for dry white wine fermentation, but mostly avoided in the beginning of sweet wine fermentation to limit SO<sub>2</sub> binding phenomena. This suggests that the selection

of CR is strongly influenced by the winemaking practices used in cellars.

### Analysis of *SSU1* Allelic Variability in Closely Related Populations

The impact of translocations in the phenotypic adaptation of yeast has been widely investigated by using genetically engineered strains (Tosato and Bruschi, 2015; Fleiss et al., 2019). However, the survey of chromosomal rearrangements in clonal populations is much less described. The *SSU1* checkup method provides an indirect opportunity to analyze this CR variability. In this section, the pool of 194 closely related clones was used in order to identify nearly isogenic groups of strains. In order to minimize the genetic distance inside a group, only strains showing less than two VNTR (Variable Number of Tandem Repeat) or LOH (Loss of Heterozygosity) were grouped together. By this way, 16 nearly genetic groups were identified encompassing 125 strains



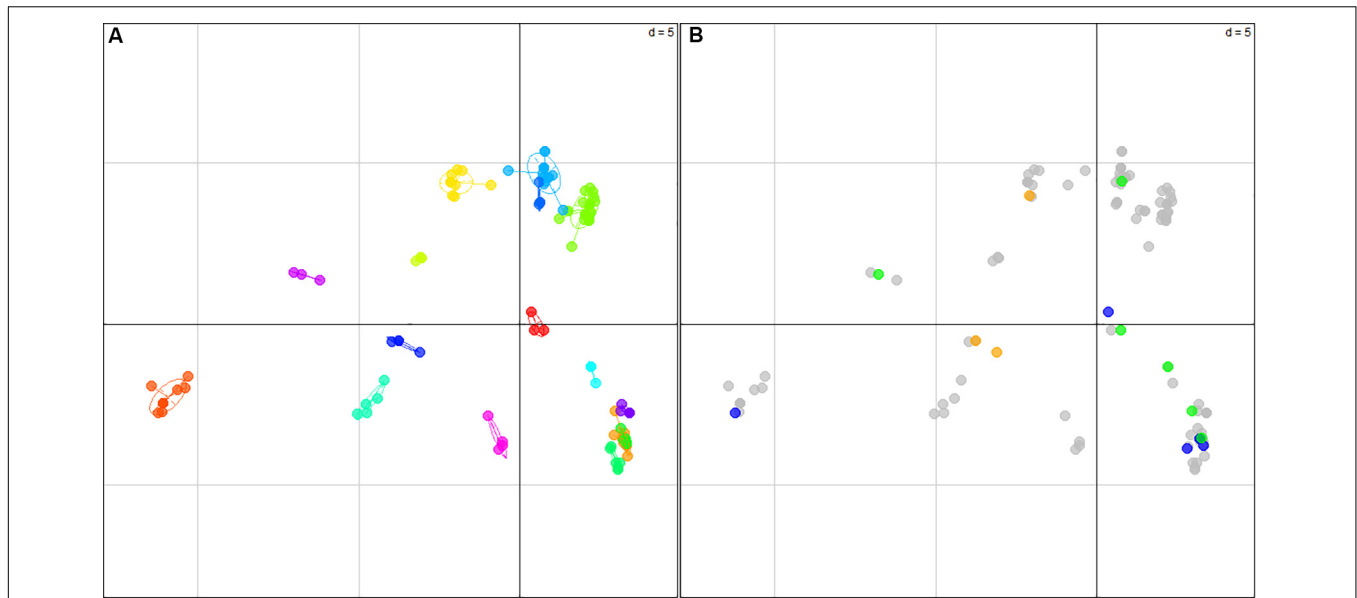


(Supplementary Table S5). Group sizes ranged between 3 and 22 individuals, with a Bruvo's genetic distance between the strains of each group always lower than 0.106. In most of the cases, strains belonging to the same group were isolated from the same vat/cellar/area samples; however, in groups 6, 11, and 15 strong similarities were found between strains from white and red samples. This is consistent with the fact that isogenic strains can be isolated from different grape juices/cellar as previously demonstrated (Borlin, 2015; Franco-Duarte et al., 2015). The relative distance between each group was illustrated by a Principal Component Analysis (Figure 5A).

Considering that each isogenic group is derived from a common clonal population, we analyzed the heterogeneity fraction in each group for three types of loci: microsatellites, chromosome VIII and chromosome XVI. For each group, the most frequent genotype was used as a reference (Supplementary Table S5). For microsatellite loci, few VNTR and LOH variations

were detected among individuals. Within the 15 microsatellite markers, the average heterogeneity fraction was 2.4 and 2.1%, for VNTR and LOH, respectively (Table 3). In the same way, the heterogeneity fraction for chromosomes VIII and XVI were computed. Interestingly, a noteworthy variability was found for 10 out the 16 groups. For chromosome VIII, LOH events were observed only in groups 4 and 14 while tandem repeat shifts of the *ECM34* promoter (VNTR) impacted in four groups. The overall allelic variability for the chromosome VIII locus was 4.5%, which is slightly higher than the average variability observed for neutral markers (microsatellites) (Table 3).

The inheritance of chromosome XVI is more complex to analyze due to the combined influences of VNTR, LOH and the different CR (Figure 5B). These changes were observed in 7 out the 16 groups, being their overall frequency 2.9, 1.3, and 7.7%, respectively. These allelic changes were mostly found within strains isolated at the same place and showing the same microsatellite pattern. For VNTR, four isogenic groups showed allelic variations in the VIII-t-XVI translocation. These variations were due to a different number of tandem repeats on the *ECM34* promoter; their frequencies were similar to those observed for chromosome VIII (2.9 vs. 3.7%). LOH variations were observed for two groups (i.e., 3 and 11). For instance, the main genotype observed in the group 11 was 555:555 (12 out 16 strains) but two strains (*3bibi6\_26* and *3bebi3\_13*) have the genotype 991:555. This difference suggested that the strains that have inherited the XVI-wt<sup>991</sup> form might have resulted from a hybridization event; alternatively the strain group homozygous for the translocation VIII-t-XVI<sup>555</sup> could have been the result of meiotic segregation (Mortimer et al., 1994). Interestingly, the C6 microsatellite (localized on chromosome XVI) did not have the same inheritance in the strains *3bibi6\_26* and *3bebi3\_13*. Also, in the same group 11, the strains CLA2016 1 and 2 isolated from Chardonnay (white grape juice) showed a longer VIII-t-XVI allele (617:617) but shared exactly the same microsatellite pattern than the 12 strains isolated from Merlot vats. This supports the idea that alternative numbers of tandem repetitions in the *ECM34* promoter can be found in clonal populations. More surprisingly, different CR were also observed within isogenic groups. This is the case of strains 13AQGUICUV1 (555:555) and 14AQGUICUV1 (991:496) isolated from the same area in sweet wines. The first strain is homozygous with a VIII-t-XVI form while the second strain is heterozygous with both XVI-wt and XV-t-XVI forms. These two clones could have resulted from the meiotic segregation of an aneuploid strain carrying the three chromosome XVI alleles (VIII-t-XVI<sup>555</sup>, XVI-wt<sup>991</sup> and XV-t-XVI<sup>496</sup>). One more time, the microsatellite C6 has not the same inheritance between these two strains supporting this hypothesis. A similar case of heterogeneity was also observed for strains 2duSPO9 (555:555) and 3mabi1\_10 (991:496) that belong to group 6. Interestingly, the strain 2duSPO9 (555:555) was isolated from a Sauvignon blanc juice while all the other strains of this group were isolated from red grape juice (Supplementary Table S5). This is consistent with the fact that the translocation VIII-t-XVI<sup>555</sup> was more frequently found in samples isolated from white grape juice (Figure 4B). All these findings were verified by simple PCR reactions,



**FIGURE 5 |** Alleles of chromosome XVI show a rapid evolution in isogenic populations. **(A)** Principal Component Analysis of 125 closely related clones (nearly isogenic) discriminated by 15 polymorphic loci. The sixteen groups were inferred manually by considering as isogenic the strains having an identical genotype for at least 13 microsatellite loci. The projection of each strain according to the 16 groups represents 14.3% of the total inertia. The Bruvo's genetic distance within each group is always lower than 0.105. **(B)** represents the same projection, but strains were colored according to the type of change occurring on chromosome XVI. Gray dots represent the major allelic form found in each subgroup while orange, blue, and green dots represent LOH, VNTR, and CR, respectively.

**TABLE 3 |** Heterogeneity fraction of VNTR, LOH and CR in isogenic populations.

Group	Number of strains	Average Bruvo's distance <sup>a</sup>	Microsatellites		Chromosome VIII		Chromosome XVI		
			VNTR <sup>b</sup>	LOH <sup>b</sup>	VNTR <sup>b</sup>	LOH <sup>b</sup>	VNTR <sup>b</sup>	LOH <sup>b</sup>	CR <sup>b</sup>
1	5	0.09	2.7	6.7	0	0	0	0	0
2	3	0.04	0	0	0	0	0	0	0
3	12	0.03	1.7	1.7	0	0	0	8.3	0
4	6	0.006	2.2	0	16.7	50	0	0	0
5	6	0.105	7.8	0	0	0	16.7	0	0
6	15	0.05	3.1	2.2	6.7	0	0	0	6.7
7	4	0.07	1.7	6.7	0	0	0	0	0
8	3	0.05	0	2.2	0	0	0	0	0
9	3	0.02	0	2.2	0	0	0	0	33.3
10	22	0.06	1.5	5.5	0	0	0	0	0
11	16	0.014	2.5	0	0	0	12.5	12.5	0
12	10	0.037	2.7	0	0	0	0	0	0
13	4	0.044	5	0	25	0	0	0	50
14	3	0.023	0	2.2	0	33.3	33.3	0	33.3
15	4	0.055	6.7	1.7	0	0	0	0	0
16	9	0.07	1.5	3	11.1	0	11.1	0	0
TOTAL	125	0.05	2.4	2.1	3.7	5.2	2.9	1.3	7.7

<sup>a</sup>Average genetic distance computed from the genetic distance matrix between all the strains of the group. <sup>b</sup>The average heterogeneity fraction was expressed in percentage of change per locus (15 loci for microsatellites and one locus for chromosomes VIII and XVI). VNTR: Variable Number of Tandem Repeat, LOH: Loss Of Heterozygosity, CR: Chromosomal Rearrangement.

after additional DNA extractions. Although the number of events observed is not sufficient for providing robust data, our results illustrate an important heterogeneous fraction among *SSU1* alleles in clonal populations that would likely be due to meiotic recombination events. These microevolutionary changes

between an industrial strain and its descendants selected after persistence in nature were previously reported using inter-*delta* markers (Franco-Duarte et al., 2015). In the case of the *SSU1* promoter, these allelic changes would play a significant role in the adaptative responses to different environments

especially due to the use or not of SO<sub>2</sub> in the early stages of vinification.

## Impact of Chromosomal Rearrangements on Yeast Fitness Parameters in Sulfited Grape Juice

Finally, we compared groups of unrelated strains harboring six different promoter regions of the gene *SSU1*. Five representative yeast strains of each group were selected by choosing strains with contrasted microsatellite inheritance and sampling origins. Indeed, strains belonging to each group showed an average Bruvo's genetic distance higher than 0.50. These genetic distances were similar to those observed for the total population (Supplementary Figure S5). Therefore, the strains selected could be considered as genetically unrelated. To simplify the interpretation of the data, the strains selected are homozygous for the six promoter regions. Growth kinetics of these strains in filtered Sauvignon blanc grape juice spiked with different SO<sub>2</sub> concentrations were analyzed by OD<sub>600</sub> measurements. Since some strains did not reach an OD<sub>600</sub> plateau after 96 h, only  $\mu_{max}$  (maximal growth rate) and *Lag Time* (Lag phase

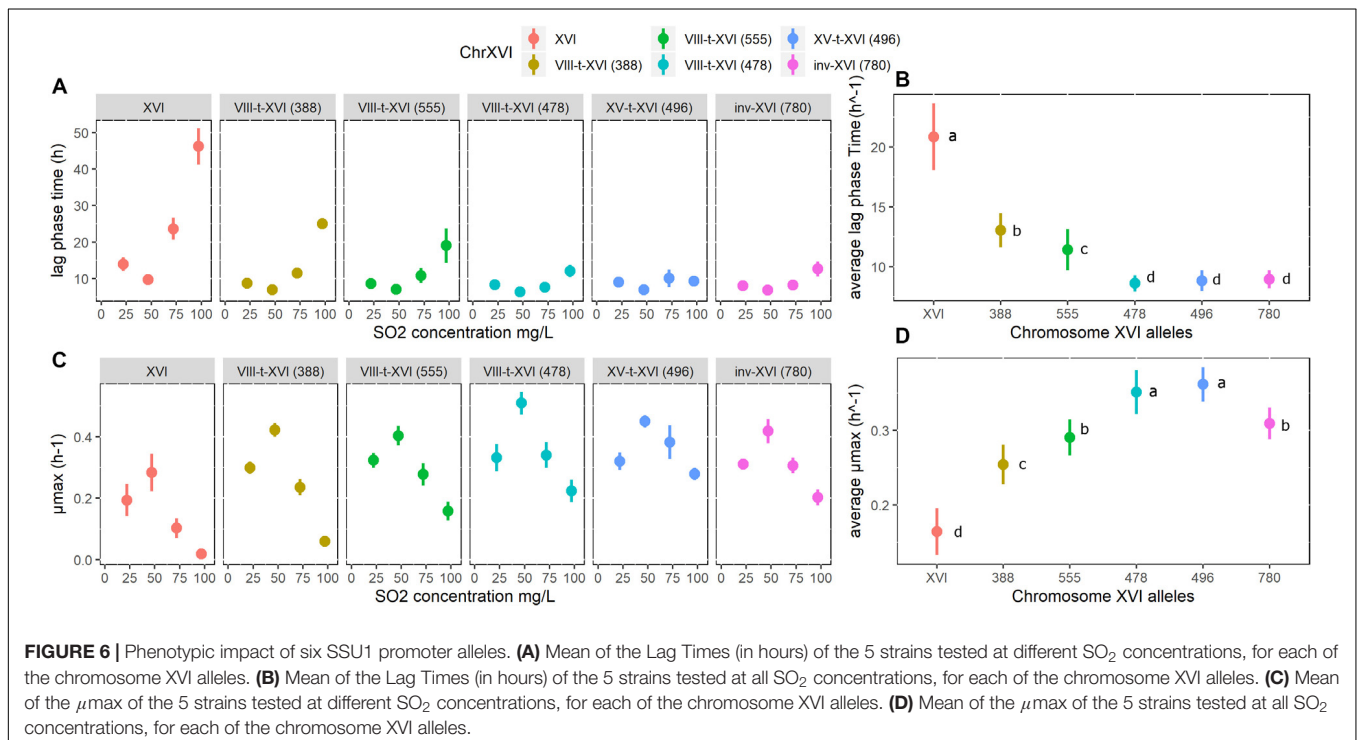
time) parameters were analyzed. An overview of the kinetics for the reference strains GN, SB, P5 and Fx10 is given in Supplementary Figure S6. The effect of SO<sub>2</sub> concentration and type of chromosome XVI were estimated by a two-way ANOVA (model Lm1, see section "Materials and Methods"). The variance explained by factors is given in Table 4. As expected, SO<sub>2</sub> addition to the grape juice significantly impacted the  $\mu_{max}$  and the *Lag Time* parameters explaining 14.8 and 18.4% of total variance, respectively. This confirms the selective pressure imposed by increasing SO<sub>2</sub> concentrations, which delayed the beginning of exponential growth and reduced the maximum growth rates of *S. cerevisiae* strains. In addition, the type of chromosome XVI significantly impacted these two parameters (Table 4) contributing in higher proportion to the total variance observed (23.5% for *Lag Time* and 17.0% for  $\mu_{max}$ ). Finally, a significant interaction was detected between SO<sub>2</sub> concentration and the type of chromosome XVI type for the *Lag Time* parameter ( $p < 1.10 \cdot 10^{-6}$ ).

The specific impact of the form of the *SSU1* promoter is illustrated in Figure 6. Since five unrelated strains were tested in each group, the impact of the different *SSU1* promoters is partially decoupled to the strain effect. As expected, "non-rearranged

**TABLE 4** | Analysis of variance of SO<sub>2</sub> and *SSU1* promoter forms for lag time and  $\mu_{max}$ .

Trait	SO <sub>2</sub>		Chr XVI		SO <sub>2</sub> :Chr XVI interactions		Residual	Levene test ( $p$ -value)
	Effect	$p$ -value <sup>a</sup>	Effect	$p$ -value <sup>a</sup>	Effect	$p$ -value		
Lag time	18.4	$<2.2 \cdot 10^{-16}$	23.5	$<2.2 \cdot 10^{-16}$	7.0	$1.1 \cdot 10^{-5}$	50.7	0.06
$\mu_{max}$	14.8	$1.8 \cdot 10^{-11}$	17.0	$6.9 \cdot 10^{-10}$	2.5	0.12	65.6	0.002

<sup>a</sup>ANOVA  $p$ -value.



**FIGURE 6** | Phenotypic impact of six *SSU1* promoter alleles. (A) Mean of the Lag Times (in hours) of the 5 strains tested at different SO<sub>2</sub> concentrations, for each of the chromosome XVI alleles. (B) Mean of the Lag Times (in hours) of the 5 strains tested at all SO<sub>2</sub> concentrations, for each of the chromosome XVI alleles. (C) Mean of the  $\mu_{max}$  of the 5 strains tested at different SO<sub>2</sub> concentrations, for each of the chromosome XVI alleles. (D) Mean of the  $\mu_{max}$  of the 5 strains tested at all SO<sub>2</sub> concentrations, for each of the chromosome XVI alleles.

strains" (i.e., XVI-wt/XVI-wt) present the highest *Lag Time* and the lowest  $\mu_{max}$  at high SO<sub>2</sub> concentrations. The translocation XV-t-XVI and the inversion (inv-XVI) appear to be very efficient to cope with higher SO<sub>2</sub> concentrations. Indeed, strains carrying these alleles were poorly affected by the addition of 75 mg/L of SO<sub>2</sub>. These findings are consistent with the elevated *SSU1* expression levels reported for these two chromosomal rearrangements (Zimmer et al., 2014; García-Ríos and Guillaumon, 2019). For the VIII-t-XVI translocations, only three allelic forms (VIII-t-XVI<sup>388</sup>, VIII-t-XVI<sup>478</sup>, VIII-t-XVI<sup>555</sup>) were tested. The fourth allele (VIII-t-XVI<sup>631</sup>) was found in only three strains, two of them being clearly isogenic. The allele VIII-t-XVI<sup>478</sup> was the most efficient in reducing the *Lag Time* and preserving the  $\mu_{max}$ . The other two alleles (VIII-t-XVI<sup>388</sup> and VIII-t-XVI<sup>555</sup>) resulted in the least favorable rearrangements for sulfite tolerance (Figure 6).

Differences in the length of the VIII-t-XVI translocation alleles mainly result from the variable number of 76 bp tandem repeat units (Goto-Yamamoto et al., 1998). The number of tandem repeats (76 and 47 bp) for the VIII-t-XVI translocation was verified by DNA sequencing and results are summarized in **Supplementary Figure S4**. Alleles VIII-t-XVI<sup>388</sup>, VIII-t-XVI<sup>478</sup>, and VIII-t-XVI<sup>555</sup> show two, three, and four 76 bp tandem repeats plus one additional 47 bp tandem repeat, respectively. Since the allele VIII-t-XVI<sup>478</sup> out competed the other two forms (Figure 6), we can conclude that the number of tandem repeats and the SO<sub>2</sub> tolerance are not fully correlated, as previously reported (Yuasa et al., 2004). Therefore, undetermined natural allelic variations in the *SSU1* coding sequence, in flanking regions, or in other genomic loci are possibly also involved in this trait.

By comparing for the first time the effect of six configurations of the *SSU1* promoter on yeast fitness we pave the way for screening sulfite tolerance of strains in a simple genetic test. The alleles XV-t-XVI, inv-XVI and VIII-t-XVI<sup>478</sup> confer an efficient adaptation for growing in grape juices with high concentrations of SO<sub>2</sub>. In addition, we illustrate that for the translocation VIII-t-XVI, the sulfite resistance (maximal growth rate and lag phase) is not perfectly related to the number of 76 bp tandem repeats. Indeed, from a technological point of view, the allele VIII-t-XVI<sup>478</sup> would be the most efficient form to tolerate high sulfite concentrations in natural grape juice.

## CONCLUSION

In yeast, CR are thought to play a role in adaptation and species evolution and might have physiological consequences (Tosato and Bruschi, 2015). The setup of a *SSU1* checkup provides a rapid molecular tool for obtaining a complete overview of three CR involving the promoter region of *SSU1*. This molecular diagnostic was helpful to address some questions related to the impact of domestication of wine yeast and to progress in the identification of physio-ecological parameters that reshape the genome organization in natural isolates. From an ecological point of view, the *SSU1* checkup could be in the future a key molecular tool for addressing different questions in relation to the use of sulfites in wine. Indeed, in the recent years, the consumer-driven

push for decreasing the levels of SO<sub>2</sub> in the wine industry and the low-sulfite wine market are increasing and could modify the allelic frequency of *SSU1* promoter alleles. From a more applied point of view, we established a link between quantitative phenotypes and the inheritance of CR in genetically unrelated groups paving the way for yeast selection programs mediated by molecular markers.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank (ID 2310529).

## AUTHOR CONTRIBUTIONS

PM, OC, and IM-P designed the experiment. MR, OC, MBö, NF, MBe, and AR did the experiment. PM, OC, and MR wrote the manuscript. MBö, WA, J-LL, PM, OC, and MR analyzed the data. PM, AR, and IM-P got the grants. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01331/full#supplementary-material>

**FIGURE S1** | Bruvo's distance distribution and cut off threshold used for removing very similar strains.

**FIGURE S2** | Natural isolates closely related to industrial starters.

**FIGURE S3** | **(A)** Principal Component analysis of 82 commercial starters discriminated by 15 polymorphic loci. The three groups represented (A to C) were inferred by k-mean clustering. The **(B)** represents the position of the strains according to the inferred groups.

**FIGURE S4** | Schematic representation of a VIII allele of *S. cerevisiae*. The figure shows the location of the 76 bp Tandem Repeats and the 47 bp Tandem Repeats, as well as the translocation point (TP) and the upstream (5' region; from the end of the forward primer to the beginning of the 76 bp tandem repeats) and downstream (3' region; from the end of the 47 bp tandem repeats to the translocation point) flanking regions. The hybridization position for the primers forward (F; p1189) and reverse (R; either p1190 or p1194), are indicated.

**FIGURE S5** | Genetic distance between individuals sharing six SSU1 promoter types, only the strains homozygous for these alleles were considered. The color blue and red indicate the distribution of pair wise Bruvo's genetic distance for the total set of strains and the subset of five strains selected.

**FIGURE S6** | Growth curves of reference strains in different grape juice containing different SO<sub>2</sub> concentrations expressed in mg/L. The data presented are the average of two independent replicates for the strain Fx10 (red), GN (green), P5 (cyan), and SB (purple). Standard error was figured out by the shaded area.

**TABLE S1** | List of the 586 strains analyzed in this work.

**TABLE S2** | Additional primers used.

**TABLE S3** | List of the 30 strains phenotyped.

**TABLE S4** | Contingency table of natural isolates origin according to the microsatellite groups.

**TABLE S5** | List of the 16 isogenic subgroups identified encompassing 125 strains.

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**Conflict of Interest:** MB and PM are employed by the Biolaffort company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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