

Article

Sexually-Driven Combinatorial Diversity in Native *Saccharomyces* Wine Yeasts

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Abstract: Natural diversity represents an inexhaustible source of yeasts for the diversification of wines and the improvement of their properties. In this study, we analysed the genetic diversity of autochthonous *Saccharomyces cerevisiae* wine yeasts in the Aljarafe of Seville, one of the warmest winemaking regions of Spain. Through multiplex-PCR analysis of five microsatellite markers and RT-PCR determination of the killer genotype, we found 94 different patterns among 150 *S. cerevisiae* yeast strains isolated from spontaneous fermentation of grape must, thereby representing a highly diverse population. Remarkably, 92% of the isolated strains exhibited high sporulation capacity. Tetrad analysis of sporulating strains rendered a microsatellite marker's combinatory that mimics patterns observed in the native population, suggesting that the high polymorphism of microsatellite markers found in these wild yeasts might result from sexual reproduction in their natural environment. The identification of unconventional M2/L-A-lus totivirus combinations conferring the killer phenotype also supports this suggestion. One idea behind this study is to determine to what extent the vineyards microbiota in areas with warm climates can provide useful natural yeasts to adapt fermentation processes to the needs imposed by global warming. Analysis of traits of oenological interest in regions potentially affected by global climate changes, such as growth tolerance to ethanol and to sugar stress in the analysed strains, indicated that this broad combinatorial diversity of natural *S. cerevisiae* yeasts provides a wide range of autochthonous strains with desirable profiles for quality winemaking in warm regions. This combinatorial diversity renders strains with diverse oenological performing abilities. Upon microvinification assays and organoleptic attests, a *S. cerevisiae* strain with interesting oenological properties has been identified. This result can be considered a successful outcome in industry–academia collaboration.



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Keywords: native yeast; spontaneous fermentation; combinatorial polymorphism; Aljarafe winemaking region; global climate change

1. Introduction

Spontaneous grape juice fermentation is a complex process involving many different microorganisms. However, even though natural *Saccharomyces cerevisiae* strains are present at a low frequency on the surface of healthy grapes, this budding yeast consistently dominates wine fermentations [1,2]. Therefore, from a biotechnological point of view, the yeast microbiota, and particularly natural *S. cerevisiae*, play a key role in the winemaking process.

Natural *S. cerevisiae* yeast strains vary from one region to another, presenting an enormous genetic and phenotypic variability depending on grape berries strain, geography, climate and many other factors of the sampling location [3]. Remarkably, the microbiota associated with grapes in one region, including yeasts, are part of the characteristics of terroir that leave footprint in the wines of each vineyard [4,5]. Thus, description and characterization of autochthonous *S. cerevisiae* strains within a vineyard represent an important step towards the conservation and exploitation of the microbial biodiversity, providing a better understanding of the microbial footprint found in the wine of the

examined vineyard. In this study, *S. cerevisiae* yeast strains were isolated and characterized from spontaneously fermenting must in the Aljarafe winemaking region in Spain. The Aljarafe has a rich heritage of native grape varieties, such as ‘Pedro Ximenez’, ‘Zalema’ and ‘Garrido fino’. To our knowledge, no previous studies have investigated yeast diversity in this region. The aim of this study was therefore to evaluate the genetic and phenotypic diversity of *S. cerevisiae* strains isolated from different vineyards and grape musts at the Aljarafe region of Andalusia, in southern Spain.

Genetic differences of *S. cerevisiae* wine strains are usually discriminated by using a variety of DNA-based methods, including mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP), inter-delta PCR [6], SNP analysis [7] and RAD-seq [8]. Microsatellite analysis, however, remains a popular method to investigate genetic diversity in vineyard- and cellar-associated *S. cerevisiae* studies [9–12]. Yeast microsatellite loci consist of short tandem-repeated DNA sequences of highly variable length [13]. Allele sizing of the microsatellite loci by PCR efficiently discriminates different strains and it is generally assumed that equal patterns correspond with the same strain or, at least, very close derivatives [12,14]. Genotypic discrimination of isolated yeast strains described here was performed by multiplex-PCR of five microsatellite regions. The killer character is also a valuable property of wine yeasts [15–17]. The production of killer toxins from M/L-A totivirus in *S. cerevisiae* allows selected yeasts to compete with other wild yeasts when conducting inoculated grape must fermentations, and this character was also studied by RT-PCR in the isolated strains [18].

Tetrad analysis of individual spores on asci is a powerful tool to study genetic traits in yeasts. The capability of wild *S. cerevisiae* yeasts to sporulate (the specialized meiotic event in yeasts) varies in populations of different origin [19]. Depending on the strain, yeasts may follow homothallic or heterothallic life cycles, the former being the most common in sporulating wine strains [20,21]. In contrast with heterothallic strains, in which haploid yeast cells of “a” and “α” mating type mate to produce diploid strains, homothallic yeasts, after meiosis, undergo a mating type switch in the daughter bud of the germinating spore, followed by conjugation between daughter and mother cells (endoduplication), thereby yielding homozygous diploid cells (except for the mating type locus) from isolated homothallic spores after sporulation [22].

Global climate change is exerting an increasingly effect on grape composition, microbiology, chemistry and the sensory aspects of wine, warm regions being profoundly affected [23]. Consequently, the search of autochthonous *S. cerevisiae* strains adapted to heat-related fermentation stresses is becoming an important approach for making quality wines in these areas. The Aljarafe is at present one of the warmest winemaking regions of Spain. In this study, phenotypic characteristics of potential interest in winemaking regions influenced by climate change, such as growth tolerance to high sugar concentrations and to ethanol stress at increasing temperature, were further analysed in autochthonous yeasts from this region. Our results show an enormous *S. cerevisiae* genotypic and phenotypic diversity in this natural population, likely arisen from sexual reproduction, that may help to select autochthonous yeast strains to produce quality wines in the Spanish Aljarafe and other warm regions.

2. Results and Discussion

2.1. Genetic Diversity of Autochthonous *S. cerevisiae* Wine Yeasts at the Aljarafe Region

The study of wine yeast biodiversity represents an important step in the preservation of the local heritage. To gain deeper knowledge of the genetic diversity and population structure of native *S. cerevisiae* wine strains in winemaking at the Aljarafe region, isolated yeasts were collected from spontaneous alcoholic fermentation during the 2019 vintage in a traditional winery at this region (Bodegas Salado SL, Sevilla, Spain). To determine the predominance of strains in different grape varieties and/or vineyards, samples from adjacent grapevine cultivars of “Pedro Ximenez”, “Garrido Fino” and “Zalema” grape varieties in the “Serrana” vineyard (Huévar del Aljarafe, Sevilla), and of “Pedro Ximenez”

and “Garrido Fino” in the “Yeguas” vineyard (Carrión de los Céspedes, Sevilla) were taken. These two vineyards of “albariza” soil (about 18 Ha each) are managed by the same winery in the same manner but located at 8 km distance one to the other.

To analyse the different strains along the fermentation, time-course samples were taken from musts of the different grape varieties and vineyards as grapes were milled (T0), after one week during tumultuous fermentation (T1), and after two weeks at the end of this fermentation phase (T2). The isolated yeast colonies were preliminary identified on the basis of their cell morphology and ten *S. cerevisiae* independent colonies (randomly chosen) from each sample were profiled using multiplex PCR reactions of five *S. cerevisiae* microsatellite markers [24,25]. Overall, 150 *S. cerevisiae* yeast strains from must were analysed as follows. Samples were taken at three different times from five must fermentations. Three fermentations of “Zalema”, “Pedro Ximenez” and “Garrido Fino” grapes from the “Serrana” vineyard and another two of “Pedro Ximenez” and “Garrido Fino” grapes from the “Yeguas” vineyard. Ten different colonies were genotyped from each sample, making a total of 150 yeast strains analysed.

Allele sizing of the microsatellite loci discriminated 94 different patterns among the 150 isolated *S. cerevisiae* strains (Figure 1). The number of *Saccharomyces* strains present during spontaneous fermentation is known to be strongly variable, from less than 20 different strains [26,27] to more than 110 reported in two Spanish cellars [28]. Thus, a highly *S. cerevisiae* diverse population underlies the Aljarafe region (Figure 1).

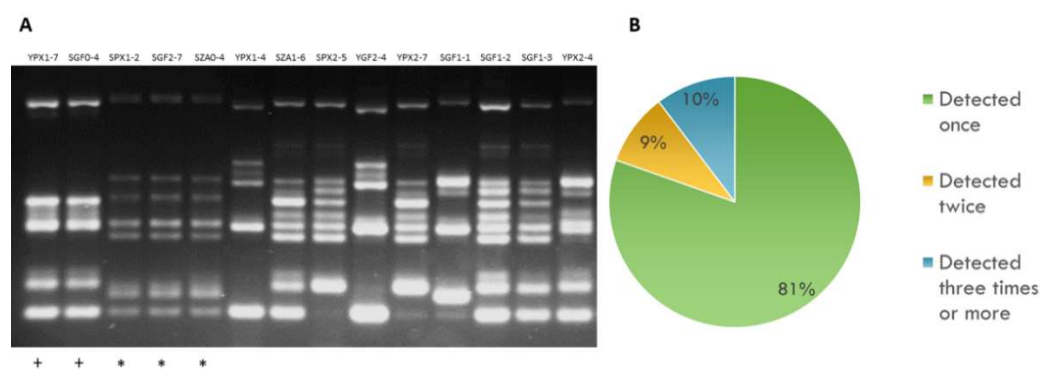


Figure 1. Broad genetic diversity of autochthonal wine yeasts in Aljarafe vineyards. (A). Representative examples of microsatellite-PCR patterns obtained in 150 *S. cerevisiae* colonies isolated from five spontaneous fermentations of must different grape varieties in two independent vineyards. Colonies were isolated from samples taken as grapes were milled and after 1 and 2 weeks of fermentation. Strains with a common pattern were identified in samples from the two studied vineyards (+) as well as in different grape varieties of the same vineyard (*). Each lane is labelled with the strain name. (B) Percentage of strains with microsatellite patterns detected once, twice and three or more times. A total of 97 unique microsatellite patterns were distinguished among the 150 isolates, most of them detected only once.

Specific yeast strains have been found in association with different grape varieties [29]. Yet, in distant regions, clustering *S. cerevisiae* strains suggests a restricted geographic gene flow [30,31]. In our study, according to their microsatellite patterns, strains associated to a particular grapevine cultivar or vineyard were not observed. In fact, two strains with a common pattern were identified in samples from the two studied vineyards. Identical patterns were also observed in three strains isolated in different grape varieties of the same vineyard (see in Figure 1). The fact that the same strain can be found in different vineyards and on different grape varieties suggests that genetic flux may exist among yeast populations of these different grape varieties and vineyards. As previously described in other regions [32], the close location of the grapevine cultivars and vineyards studied here likely generates a common randomly distributed yeast population.

During wine fermentation, numerous studies have established that different strains are usually involved at different phases along the fermentation process [33,34]. It is also

known that only few *S. cerevisiae* strains are usually predominant, representing more than 30–50% of the total yeast isolates [35]. Accordingly, we also observed a great diversity of *S. cerevisiae* strains along the fermentation of must from each grape variety and vineyard. However, more than 90% of the strains were only detected once or twice along the whole analysis (Figure 1B). Predominance of specific strains along the different fermentation phases were not identified, neither simultaneously nor in succession in the course of the analysed fermentations (not shown).

Diversity among yeasts strains is not always evident on their chromosomal markers but could reside in episomal or mobile elements. Given their potential relevance in wine fermentation we have analysed the presence of totiviruses determining killer phenotypes. Killer yeasts may become particularly important in the predominance of specific strains during wine fermentations [36–38]. The killer phenotype in *S. cerevisiae* relies on the cytoplasmic persistence of two dsRNA totiviruses, L-A and M. M encodes the toxin, and L-A provides proteins for replication and encapsidation for both viruses [39]. The toxin produced by killer cells may provide fitness advantage increasing the ratio of killer yeasts during spontaneous or inoculated fermentations [38,40]. Nonetheless, the dominance of Killer yeasts during wine fermentation remains controversial [17]. By means of RT-multiplex PCR [18], we found killer totivirus in 39 out of the 150 strains analysed (see in Table 1).

Table 1. Fraction of killer strains over strains analysed in milled grapes (T0), during tumultuous fermentation (T1) and at the end of this phase (T2) at the indicated vineyard (Y: Yeguas and S: Serrana) and grape varieties (PX: Pedro Ximenez, GF: Garrido Fino and ZA: Zalema). As some strains appeared more than once, overall fraction of unique strains (as defined by showing different microsatellite patterns), and the fraction of killer yeasts among these different strains are also indicated.

	YPX	SPX	YGF	SGF	SZA
T0	2/10	2/10	1/10	1/10	5/10
T1	4/10	5/10	1/10	5/10	1/10
T2	4/10	4/10	1/10	1/10	3/10
Overall different strains	26/30	24/30	11/30	18/30	25/30
Killer yeasts/total different strains	9/26	7/24	2/11	5/18	9/25

In this study, the proportion of killer yeasts remained at a similar abundance, ranging 27–40% values independently of vineyards and grape varieties, with no predominant killer strains along the fermentation processes (Table 1). The fact that most of non-killer yeasts were resistant to the lethal effect of the K2 killer toxin (Figure 2), may provide some clues to explain the absence of predominant killer yeasts in the course of the analysed fermentations, as described for K1 killer yeasts [41].

We previously found that among the four known K1, K2, K28 and Klus *Saccharomyces* killer yeast types (totivirus L-A-1/M1, L-A-2/M2, L-A-28/M28 and L-A-lus/Mlus, respectively), most of the killer strains identified here were K2 (L-A-2/M2 totivirus associations) as usually found in wine yeasts [42], but asymptomatic L-A-2/M2 infected cells, as well as unexpected L-A-lus/M2 totivirus associations, were also identified [18]. Sequencing analysis confirmed these unconventional killer genotypes (not shown). Importantly, since only inherited transmission of killer totivirus takes place in yeast, a new L-A/M combination can only arise from sporulation/conjugation events, suggesting that sexual reproduction may take place often in this natural population.

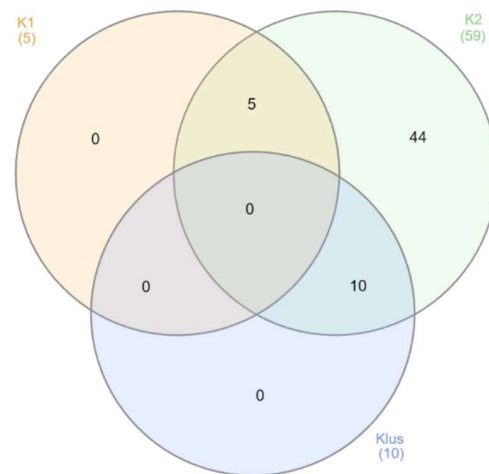


Figure 2. Number of strains resistant to K1, K2 and Klus killer toxins (Venn diagram). A total of 59 of the 62 non-killer yeast were resistant to the K2 toxin. No resistant strains to toxin K28 were found.

2.2. Sexually Driven Diversity of Microsatellite Patterns

The great diversity of microsatellite patterns found here in the isolated *S. cerevisiae* yeasts is striking. Strains with only one allele per marker, thereby yielding simple patterns with five discrete microsatellite DNA bands (albeit with variable size in different strains according to the diverse alleles found in the population), or with multiple allelic forms for each microsatellite locus, yielding complex patterns with more than five DNA bands, were observed (Figure 3).

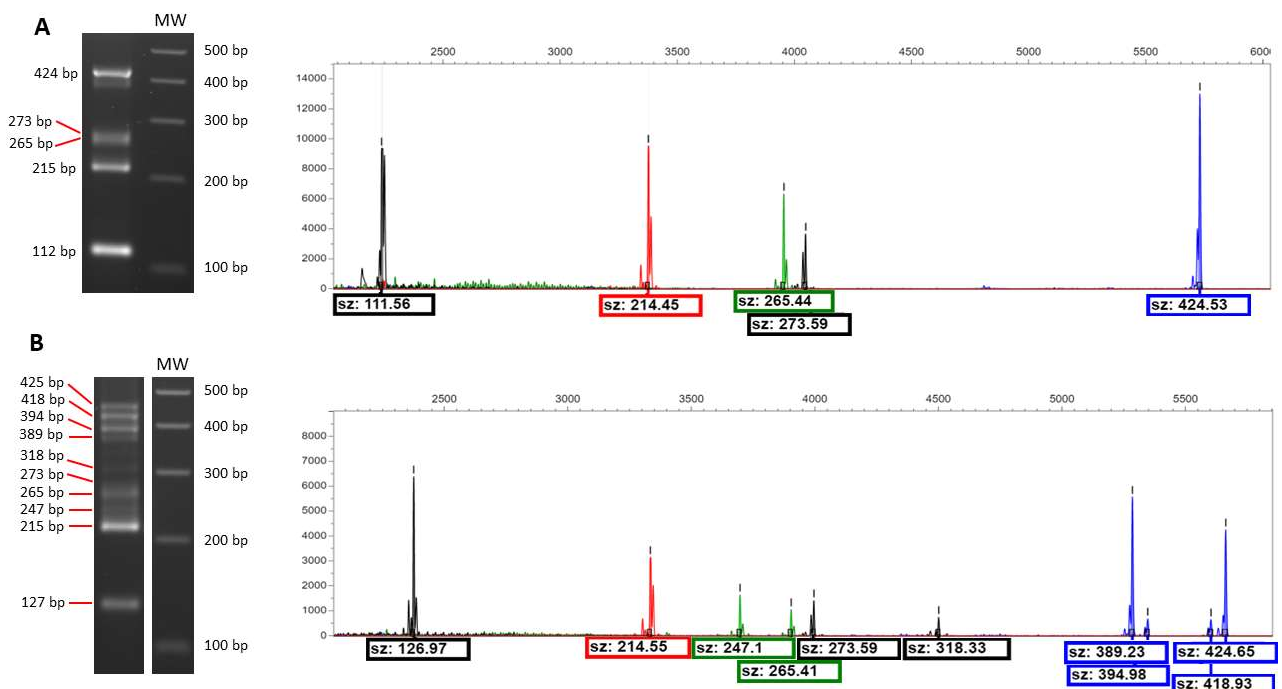


Figure 3. Representative strains harbouring simple and complex patterns of the analysed microsatellite loci. (A) Simple pattern yielding five discrete microsatellite DNA bands in gel electrophoresis (left), accurately identified by capillary electrophoresis using labelled primers to identify each of the five microsatellite bands (right). (B) Complex pattern (polymorphic strain with a complex genome ploidy) yielding more than five discrete microsatellite DNA bands in gel electrophoresis (left) and capillary electrophoresis (right) as described in A. Capillary electrophoresis peaks are colour labelled to distinguish microsatellite loci (leftmost black, C5; red, SC8132X; green, SCAAT3; rightmost black SCPTSY7 and blue, SCYOR267C). sz = size, MW = DNA size marker.

Interestingly, simple patterns were compatible with random combinations for the different alleles of each locus observed in complex patterns (Figures 1 and 3), as would be expected for a random meiotic segregation of these alleles. Thus, we hypothesize that, as previously predicted for the unconventional L-A-lus/M2 killer totivirus combination, sexual events (sporulation/conjugation) in their natural environment might be contributing to the great combinatorial diversity of microsatellite patterns observed in this *S. cerevisiae* population. Accordingly, we observed that 92% of the isolated strains efficiently sporulated in the Aljarafe population, a sporulation efficiency higher than the average reported in natural *S. cerevisiae* wine yeasts [31,43–46].

To study a possible sexually driven diversity in this population, 10 strains with complex microsatellite patterns and another 10 harbouring simple patterns were randomly chosen and subjected to further genetics analysis. As shown in Table 2, sporulation/conjugation assays indicated that 18 out of these 20 strains were able to sporulate, 13 being homothallic, 2 heterothallic and remarkably, 3 were heterozygous for this trait (homothallic/heterothallic). Two strains were non-sporulating in our assay conditions, perhaps because of possible ploidy alterations.

Table 2. Spore viability in dissected four spore's asci (number of 4:0, 3:1, 2:2, 1:3 viable: non-viable segregating tetrads. No 0:4 segregations were obtained), sexual life cycle (homothallism-heterothallism), and killer factor of the indicated strains harbouring simple and complex microsatellite patterns (+, strain with killer phenotype; -, strain without killer phenotype).

	Strain	4:0 Tetrads	3:1 Tetrads	2:2 Tetrads	1:3 Tetrads	Sexual Cycle	Killer Factor
Simple patterns	YPX2-1	8	0	0	0	Homothallic	+
	YPX2-9	0	0	4	4	Heterozygous	-
	SPX2-4	8	0	0	0	Homothallic	+
	SPX1-5	7	1	0	0	Homothallic	-
	YGF0-5	7	1	0	0	Homothallic	+
	YGF0-10	8	0	0	0	Homothallic	+
	SGF2-8	7	1	0	0	Homothallic	-
	SGF2-4	7	1	0	0	Homothallic	+
	SZA0-7	5	3	0	0	Homothallic	+
	SZA1-10	8	0	0	0	Homothallic	-
Complex patterns	YPX1-10	7	1	0	0	Homothallic	+
	YPX2-10	2	3	3	0	Heterothallic	-
	SPX2-1	7	1	0	0	Homothallic	+
	SPX2-3	7	1	0	0	Homothallic	-
	YGF1-6	8	0	0	0	Homothallic	+
	YGF2-10	0	0	0	0	Non-sporulating	-
	SGF2-2	0	0	0	0	Non-sporulating	-
	SGF2-6	7	1	0	0	Heterozygous	+
	SZA1-4	0	2	6	0	Heterozygous	+
	SZA2-4	0	0	7	1	Heterothallic	-

Tetrad analysis carried out in four spore's asci indicated that viability of the meiotic products in the sporulating strains was near 100% in homothallic strains, most of them showing simple microsatellite patterns (homozygous), while the remaining heterothallic and heterothallic/homothallic heterozygous strains presented a high degree of heterozy-

gosity (complex microsatellite patterns), low spore viability and eventually, segregation of recessive lethal mutations (see Table 2).

Wine yeast strains are primarily homothallic and diploid, aneuploid, or polyploid [47], highly heterozygous [48,49], and sporulate poorly, thus rendering few viable spores [21]. As described above, similar results were found here in non-homothallic strains. However, simple microsatellite patterns, sporulation efficiency and spore viability suggest that the homothallic yeast population is enriched in homozygous diploid (or near so) cells. No clear association was observed of the killer character with genotypic complexity, spore viability or sexual behaviour of the analysed strains (Table 2).

To determine the diversity of microsatellite patterns resulting from meiotic segregation, four spore's asci were dissected by micromanipulation and single spore progeny from asci with four viable meiotic products was examined. Tetrad analysis of the five microsatellite loci was carried out in asci from both homothallic and heterothallic strains. As expected, homothallic yeasts with simple patterns yielded homozygous strains derived from monosporic cultures, with all four spores harbouring the same five DNA fragment patterns (Figure 4A). Conversely, micromanipulated asci of homothallic strains with complex patterns generated spores with a combinatorial distribution of the microsatellite DNA fragments, according to a random meiotic segregation of these markers (Figure 4B). In heterothallic strains, random conjugation among meiotic products likely regenerate yeast cells with complex microsatellite patterns (see Table 2).

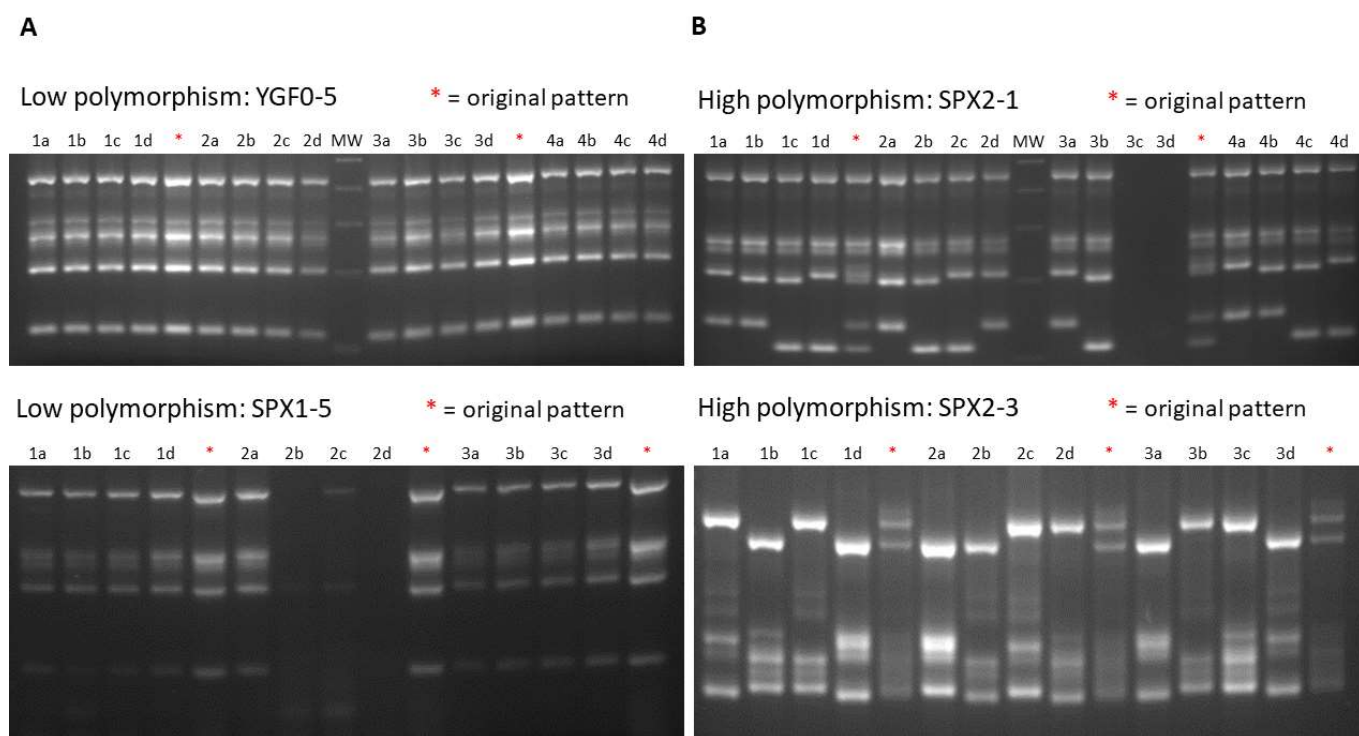


Figure 4. Patterns generated by four viable spores (a, b, c and d) per asci of strains with simple (A) and complex (B) microsatellite patterns. Microsatellite patterns of spores and their parental strains (red asterisk) of complex pattern (upper panels) and simple pattern (lower panels) strains. The microsatellite pattern of both parental complex strains segregated between the spores, resulting in the generation of simple pattern strains. Microsatellite pattern of spores from simple pattern strains remain the same comparing with the diploid, suggesting a higher genome homogeneity. MW = DNA size marker.

Overall, patterns obtained following sporulation/conjugation events in the analysed strains resembled the diversity observed in microsatellite patterns of the autochthonous yeast population in the Aljarafe region. Thus, the capability to sporulate/conjugate suggests

that sexual activity is likely driving a combinatorial genetic diversity in *S. cerevisiae* strains at this natural environment. Strains of this population hosting an unconventional L-A-lus/M2 totivirus combination also supports this idea.

The analysis of recombination rates using molecular markers provides interesting information about sexual reproduction frequency in yeasts [46,50–52]. Here, we suggest that the ratio of simple to complex microsatellite patterns in homothallic populations may also cast some light on the rate of sporulation at their natural environment. Since sporulation in homothallic strains renders homozygous monosporic cultures derived from each meiotic product (Figure 4A), we conclude that homothallic strains with simple patterns in natural populations likely result from sporulation events. Conversely, complex patterns in homothallic strains likely result from the accumulation of spontaneous mutations involving microsatellite allele size before a sporulation event occurs. In our study, we estimated a ratio of three to one simple to complex microsatellite patterns in homothallic *S. cerevisiae* strains. Thus, we conclude that sporulation may occur often in this yeast population, roughly, at a rate higher than the overall mutation rate at microsatellite loci.

2.3. Combinatorial Diversity of Phenotypic Characters of Oenological Interest

From each of the five fermentations studied, two strains with complex microsatellite pattern and two with simple ones were randomly chosen to perform further physiological characterization.

Alcoholic fermentation in the presence of oxygen is thought to provide *Saccharomyces* yeasts with a fitness advantage in high-sugar environments, such as grape juice, because ethanol is toxic to many species [53–56]. However, ethanol is a major stress factor for yeast cells during fermentation. In physiological terms, this alcohol is an inhibitor of yeast growth that slows down the maximum specific growth rate, inhibits cell division, increases cell death and enhances the frequency of “petite” mutations [49,57,58].

To determine the ethanol tolerance of this natural yeast, maximum specific growth rate ($\mu^{\max}$) at increasing ethanol concentrations (0, 5 and 10% v/v at 30 °C) was measured in the isolated strains. As shown in Figure 5, all natural wine strains showed significantly higher $\mu^{\max}$ than laboratory controls at all tested conditions. However, these strains showed a great diversity for this character, with growth rates ranging from 0.21 to 0.37 and from 0.07 to 0.12 at 5% and 10% ethanol, respectively (Figure 5A). Ethanol targets so many different molecular and cell functions [59] that almost any gene is susceptible to yield ethanol-sensitive alleles [60,61]. Thus, we conclude that the combinatorial genetic diversity observed in microsatellite markers also yields allelic combinatorial diversity of genes involved in cell growth in the presence of ethanol. Accordingly, strains with higher growth rates at 5% ethanol differed to those with higher values at 10% ethanol (Figure 5B), supporting the idea that target genes that limit growth in the presence of ethanol are different at different ethanol concentrations [49].

High sugar concentrations are among the many stressors associated with winemaking at the beginning of fermentation process. Musts usually contain 200–250 g/L of an equimolar mixture of glucose and fructose, a high osmolarity in the grape that have potentially detrimental impacts on yeast viability, growth, and fermentation performance at the beginning of winemaking [62]. In fact, many different genes involved the osmotic stress response and the high osmolarity glycerol (HOG) pathway are key elements in the growth tolerance of yeasts to high sugar concentrations [63].

On the average, the wine strains studied here were highly tolerant to high sugar concentrations as compared to laboratory control (see in Figure 5C). Nonetheless, growth tolerance varied within these different wine strains, perhaps as a consequence of a combinatorial diversity of polymorphic genes involved in osmostress tolerance, as described above for microsatellite markers and ethanol tolerance.

The genetic diversity was reflected in the oenological profile of the produced wines too. By microvinification assays in filtered must, we determined that wines obtained from each of the twenty characterized strains (Table 2 and Figure 5A) were organoleptically

different. Among them, the YGF0-10 strain produced a semi-sweet frizzante wine with excellent organoleptic properties. This strain was among the most tolerant to high ethanol and glucose concentrations (Figure 5) and was selected for future studies due to its potential winemaking interest.

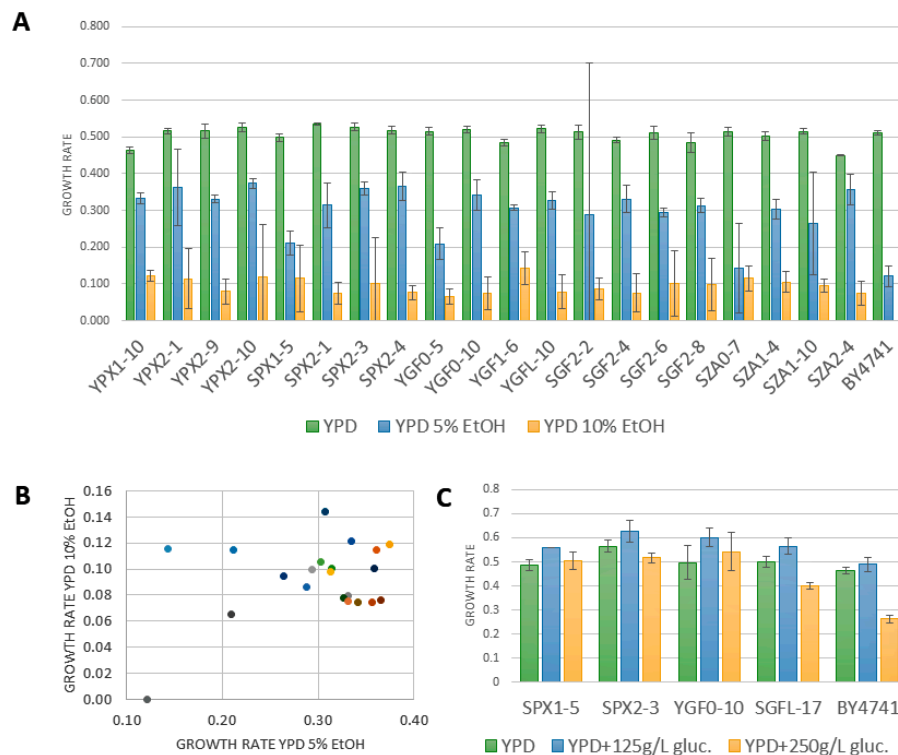


Figure 5. Phenotypic diversity of Aljarafe *S. cerevisiae* strains in growth tolerance to high ethanol and sugar concentrations. **(A)** Growth rate of the indicated strain at 0% ethanol (YPD), 5% ethanol (YPD 5% EtOH) and 10% ethanol (YPD 10% EtOH). **(B)** Relationship between growth rate at 5% versus 10% ethanol. Each dot represents these values for each strain. **(C)** Growth rate of the indicated strain at standard 20 gr/L glucose (YPD), 125 gr/L glucose (YPD + 125 g/L gluc.) and 250 gr/L glucose (YPD 250 g/L gluc.). Error bars are 95% confidence intervals.

Global climate change is progressively increasing sugar concentrations in grape, which in turn increase ethanol content in the fermenting grape must [64]. Our results suggest that at the Aljarafe winemaking region, autochthonous wine yeasts are highly resistant to the inhibitory effects of high sugar and ethanol concentrations, perhaps because the influence of this region in the microbial biota associated with both natural vineyards and traditional winemaking, as previously described in other warm regions [65–68].

Natural sexual reproduction usually occurs at a low frequency in wine yeasts populations [69]. However, the combinatorial diversity of genotypic and phenotypic characters observed in autochthonous Aljarafe yeasts analysed here suggest that sexual reproduction is frequent in this population, likely favouring a rapid adaptation to global environmental changes. The increase in temperature increases grape sugar and, due to its synergistic effect with alcohol, also increases the sensitivity to ethanol of the yeasts that ferment the must. The combinatorial diversity of this characteristic is of oenological interest, as the high tolerance to sugar and ethanol found in this population might constitute a great source of *S. cerevisiae* strains to improve winemaking under climate changing conditions, especially in hot areas, especially vulnerable to climate change.

3. Materials and Methods

3.1. Strains and Culture Medium

The yeast strains used in this study are summarized in Supplementary Table S1. Strain BY4741 [70] was used as a laboratory standard during the determination of growth rates. Wine yeast were isolated from a sample of spontaneously fermenting must provided by Bodegas Salado SL (Umbrete, Seville, Spain). Natural *S.cerevisiae* strains were named as follows. The first character denotes the vineyard of origin (Y: Yeguas and S: Serrana), the next two characters, the grape varieties (PX: Pedro Ximenez, GF: Garrido Fino and ZA: Zalema), the first number indicates the step of fermentation they were isolated (milled grapes, 0; early tumultuous fermentation, 1 and at the end of this phase, 2) and the last number after a hyphen is the isolate number. Yeast cells were grown in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose). For plates with solid medium, 2% agar was added. MB (methylene blue) medium was YPD buffered at pH 4 with sodium citrate and containing 0.003% methylene blue. To induce sporulation, cells were plated in SPA media (potassium acetate, 1%; yeast extract, 1%; glucose, 0.05%).

3.2. Determination of Yeast Killer Genotype and Killer Activity

Colonies to be tested for killer activity were plated onto MB medium previously seeded with a lawn of a sensitive strain. Additionally, to determine the type of toxin exported by killer strains, colonies were replicated to four different MB medium plates previously seeded with a lawn of each of the four known killer strains, where each was only immune to their own toxin. MB plates were incubated at 25 °C for 2 to 4 days. A clear zone around the colonies indicated killer toxin production. In the case of determination of killer genotype, we performed a Multiplex RT-PCR as described in [18].

3.3. Microsatellites and Capillary Electrophoresis

Five microsatellite loci were amplified for strain determination: SCYOR267C, SCPTSY7, SC8132X, ScAAT3 and C5 [24,25]. Primers used for microsatellite amplification are listed in Table 3. PCR amplification was performed using NZYTAQ II DNA polymerase (Nzytech, MB354) and the following programme: 94 °C 5 min, (94 °C 30 s, 54 °C 45 s, 72 °C 30 s) × 30 cycles, 72 °C 5 min. Following PCR, the products were loaded into an agarose gel (2.5% wt/vol) and electrophoresis was performed.

Table 3. Microsatellite PCR primers.

Name	Sequence	Locus	Fluorochrome
SCY F	GGTGACTCTAACGGCAGAGTGG	SCYOR267C	6-FAM
SCY R	GGATCTACTTGCAGTATACGGG		
SCP F	CCCTTTTAAGGAAGAGCAAGCC	SCPTSY7	NED
SCP R	CCACTCTCAGCTTATTGGGG		
SC8 F	CTGCTCAACTTGTGATGGGTTTGG	SC8132X	VIC
SC8 R	CCTCGTTACTATCGTCTTCATCTTGC		
SCAAT F	TGGGAGGAGGGAAATGGACAG	ScAAT3	PET
SCAAT R	TTCAGTTACCCGCACAATCTA		
C5 F	TGACACAATAGCAATGGCCTTCA	C5	NED
C5 R	GCAAGCGACTAGAACAAACATCACA		

For capillary electrophoresis, the microsatellite amplification was performed with fluorescently tagged primers as shown in Table 3. After PCR, 0.25 µL of PCR product were mixed with 9.25 µL of formamide and 0.5 µL of GeneScan 600 LIZ (4408399, ThermoFisher, Madrid, Spain) and injected in SeqStudio™ Genetic Analyzer (ThermoFisher) according

to manufacturer's instructions. After capillary electrophoresis, the results were analysed using the Microsatellite Analysis software provided by Thermofisher Cloud.

3.4. Sporulation and Tetrad Dissection

To test sporulation capability strains were incubated at 30 °C for 5 days in SPA media and inspected with a microscope. Tetrad dissections were performed according to [71].

3.5. Determination of Growth Rate

Exponential yeast cultures were diluted at an optical density of 0.05 in triplicated cultures. For each culture, optical density was measured each 15 min for 24–48 h. After plotting the growth curve, the growth rate was calculated using the slope of the curve according to [72]. The growth rate was calculated as the slope of the log-transformed growth data during maximal exponential growth. The average of the three values obtained are represented, and, as error bars, the 95% confidence interval was calculated and plotted. These confidence intervals allow the visual comparison of the statistical significance of the differences in growth rate found between any pair of strains and conditions [73].

3.6. Microvinifications

50 mL of a saturated yeast culture was inoculated in 950 mL of filtered grape must and incubated at 25 °C for 1 month. After fermentation, wine was stored for another month at 4 °C, decanted and tasted.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation8100569/s1>, Table S1: Strains used in this study.

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