Research Article

Genetic characterization of commercial *Saccharomyces cerevisiae* isolates recovered from vineyard environments

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Abstract

One hundred isolates of the commercial *Saccharomyces cerevisiae* strain Zymaflore VL1 were recovered from spontaneous fermentations carried out with grapes collected from vineyards located close to wineries in the Vinho Verde wine region of Portugal. Isolates were differentiated based on their mitochondrial DNA restriction patterns and the evaluation of genetic polymorphisms was carried out by microsatellite analysis, interdelta sequence typing and pulsed-field gel electrophoresis (PFGE). Genetic patterns were compared to those obtained for 30 isolates of the original commercialized Zymaflore VL1 strain. Among the 100 recovered isolates we found a high percentage of chromosomal size variations, most evident for the smaller chromosomes III and VI. Complete loss of heterozygosity was observed for two isolates that had also lost chromosomal heteromorphism; their growth and fermentative capacity in a synthetic must medium was also affected. A considerably higher number of variant patterns for interdelta sequence amplifications was obtained for grape-derived strains compared to the original VL1 isolates. Our data show that the long-term presence of strain VL1 in natural grapevine environments induced genetic changes that can be detected using different fingerprinting methods. The observed genetic changes may reflect adaptive mechanisms to changed environmental conditions that yeast cells encounter during their existence in nature. Copyright © 2007 John Wiley & Sons, Ltd.

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Introduction

Wild strains of *Saccharomyces cerevisiae*, isolated from wine, cellars or vineyards are predominantly diploid, homothallic and mostly homozygous (65%), with low (Bakalinsky and Snow, 1990; Barre *et al.*, 1992; Guijo *et al.*, 1997) to high (>85%) sporulation capacity (Mortimer, 2000). Aneuploid strains, with approximately diploid DNA contents, have been described (Codon *et al.*, 1997; Nadal *et al.*, 1999; Puig *et al.*, 2000) and meiosis seems not to be a common occurrence

in their life cycle (Bakalinsky and Snow, 1990; Barre *et al.*, 1992). Such wine yeast strains present an essentially asexual life cycle and are characterized by high karyotype instability, which is believed to be a potential source of genetic variability (Bidenne *et al.*, 1992; Carro *et al.*, 2003; Longo and Vezinhet, 1993; Nadal *et al.*, 1999). Haploid laboratory strains undergo far fewer extensive changes of this kind (Longo and Vezinhet, 1993). Gross mitotic chromosomal rearrangements, such as large regions fusion between homologous and non-homologous chromosomes, occur in wine

yeasts with a frequency of ca. 10^{-5} (Puig *et al.*, 2000). Baker's yeast has a higher chromosomal variability than wine yeasts (Codon *et al.*, 1997).

In chromosome I, several membrane-associated genes are located in subtelomeric regions, and it was hypothesized that subtelomeric plasticity may allow rapid adaptive changes of the yeast strain to specific substrates (Carro *et al.*, 2003). The *SSUI-R* allele, generated by reciprocal translocation between chromosomes VIII and XVI, confers sulphite resistance to yeast cells and was described as first case of adaptive evolution, occurring probably as a consequence of the use of sulphite as a preservative in wine production (Goto-Yamamoto *et al.*, 1998; Pérez-Ortín *et al.*, 2002). Retrotransposons may also be involved in chromosomal recombinations. *S. cerevisiae* strains contain between two and 30 copies of at least five retrotransposons (Ty1–Ty5), the copy number of each being highly variable, depending on the strain examined. The internal DNA (5.3–5.7 kb) is related to retroviruses and is surrounded by ~350 bp terminal repeats (LTRs), retrotransposons Ty1 and Ty2 being flanked by delta sequences. Recombination events expel the central sequence at one LTR, leaving a single LTR behind, explaining the dispersed presence of many copies of LTRs throughout the genome. PCR amplification of segments between repeated delta sequences were successfully used for the development of PCR-based *S. cerevisiae* strain typing (Lavallée *et al.*, 1994; Legras and Karst, 2003; Ness *et al.*, 1993). Multiple Ty elements mediating reciprocal recombinations (chromosomes I/III or III/VII) was shown by fine-mapping of the junctions, demonstrating their crucial involvement in karyotype alterations in natural and industrial strains (Carro *et al.*, 2003; Rachidi *et al.*, 1999; Umezu *et al.*, 2002), together with insertions/transpositions of Y' elements (Neuvéglise *et al.*, 2000). Additionally, ribosomal DNA repeats may also contribute to chromosomal size changes (Nadal *et al.*, 1999). Small but positive fitness increments due to Ty-promoted genome rearrangements, leading to inactivation of *FAR3* and *CYR1*, were verified in laboratory populations (Blanc and Adams, 2003).

The mitochondrial genome of *S. cerevisiae* consists of 85.8 kb, has a low gene density, a very low GC content (17–18%) and extensive intergenic regions, being composed of long adenosine–thymidine (A + T) stretches and short

guanosine–cytidine (G + C) clusters that make up 62% of the genome (Foury *et al.*, 1998). Although the *S. cerevisiae* mitochondrial DNA (mtDNA) molecule is very recombinogenic, the wild-type configuration is preferentially inherited (Piskur, 1994). MtDNA restriction fragment length polymorphism (RFLP) analysis, using enzymes such as *HinfI* or *RsaI*, is associated with a high polymorphism and is a widely used genetic marker for the distinction of *S. cerevisiae* wine strains (Fernandez-Espinar *et al.*, 2000; Lopez *et al.*, 2001; Querol *et al.*, 1992a, 1992b).

Microsatellites were described as simple sequence repeats (SSR), usually less than 10 bp motif repeats, with a substantial level of polymorphism. They occur within many open reading frames, but are even more frequent in non-coding regulatory regions. In *S. cerevisiae*, microsatellites have been described as abundant and highly polymorphic in length (Field and Wills, 1998; Richard *et al.*, 1999) and used as a reproducible and portable typing method (Bradbury *et al.*, 2005; Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Legras *et al.*, 2005; Pérez *et al.*, 2001; Schuller *et al.*, 2004).

The objective of the present work was to evaluate whether the long-term presence of a commercial yeast strain (Zymaflore VL1, Laffort Oenologie) in natural grapevine environments induces genetic changes that can be detected by four genetic fingerprinting methods: interdelta sequence typing, mtDNA RFLP, chromosomal karyotyping and microsatellite analysis. We consider these studies as a first approach to gain a deeper insight in the kind of genetic changes that occur during the strains' long-term presence in nature that may give hints about microevolutionary changes occurring in the yeast's natural winemaking environments. Additionally, comparative analysis of the polymorphisms generated by each of the DNA fingerprinting methods was performed, providing a view of their discriminatory power for strain differentiation and the evaluation of 'intra-strain' variation.

Materials and methods

Fermentation and strain isolation

The yeast natural isolates were obtained from spontaneous fermentations carried out with must prepared from grapes collected in 2001, 2002 and 2003

in the Vinho Verde wine region (north-western Portugal). The vineyards (designated A, C and P in the present work) were located in close proximity (10–300 m) to wineries which used predominantly the yeast starter Zymaflore VL1. This strain is non-indigenous and was initially selected in the region of Gironde (France). Spontaneous fermentations occurred with 500 ml grape juice (obtained from about 2 kg of aseptically smashed grapes) at 20 °C with mechanical agitation (20 r.p.m.). When must weight was reduced by 70 g/l, corresponding to the consumption of about two-thirds of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on plates containing YPD medium (yeast extract 1% w/v, peptone 1% w/v, glucose 2% w/v, agar 2% w/v) and incubated for 2 days at 28 °C; 30 randomly chosen colonies were collected. The active dry yeast VL1 parental strain was kindly provided by the manufacturer. This culture was re-hydrated in a glucose solution (10% w/v) for 10 min at 30 °C, serially diluted and spread on YPD plates as mentioned above. Thirty isolates were randomly chosen and analysed by the same typing methods as isolates recovered from natural environments. All the isolates used throughout this work were kept in frozen stocks (glycerol 30% v/v) at –80 °C.

DNA isolation

Yeast cells were cultivated in 5 ml YPD medium (24 h, 28 °C, 160 r.p.m.) and DNA isolation was performed using a previously described method (Lopez *et al.*, 2001). DNA was quantified and used for interdelta sequence typing, mitochondrial RFLP and microsatellite analysis.

Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 (Pérez *et al.*, 2001) were amplified and analysed as described (Schuller *et al.*, 2004). Their characteristics are shown in Table 1.

Interdelta sequence typing

Amplification reactions were performed on a Bio-Rad iCycler thermal cycler, using the primers $\delta 1$ (5'-CAAAATTCACCTATATCT-3') and $\delta 2$ (5'-GTGGATTTTATTCCAAC-3') (primer pair A) (Ness *et al.*, 1993) or $\delta 12$ (5'-TCAACAATGGAA-TCCAAC-3') and $\delta 2$ (primer pair B) (Legras and Karst, 2003) as described (Schuller *et al.*, 2004).

Chromosomal polymorphisms

Yeast chromosomal DNA was prepared in plugs as previously described (Bidenne *et al.*, 1992). Pulsed-field gel electrophoresis (PFGE) was performed using the TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman). The gels were run for 26 h: 6 h at 250 V with 35 s pulse time, followed by 20 h at 275 V with 55 s pulse time, at a constant temperature (14 °C).

Mitochondrial DNA restriction patterns

The reactions were performed overnight at 37 °C and prepared for a final volume of 20 μ l as previously described (Schuller *et al.*, 2004).

Table 1. Characteristics of the six microsatellite loci (ScAAT1–ScAAT6) used as genetic markers in the present study

Micro-satellite designation	Repeat	ORF or coordinates	Chromosome	Primers	Fluoro-chrome	Size (S288C)	No. of repeats (S288C)
ScAAT1	ATT	86 901–87 129	XIII	F: AAAAGCGTAAGCAATGGTGTAGAT R: AGCATGACCTTTACAATTTGATAT	6-FAM	229	35
ScAAT2	ATT	YBL084c	II	F: CAGTCTTATTGCCTTGAACGA R: GTCTCCATCCTCCAAACAGCC	HEX	393	20
ScAAT3	ATT	YDR160w	IV	F: TGGGAGGAGGGAAATGGACAG R: TTCAGTTACCCGCACAATCTA	6-FAM	268	23
ScAAT4	ATT	431 334–431 637	VII	F: TGCGGAAGACTAAGACAATCA R: AACCCCCATTTCTCAGTCGGA	TET	304	12
ScAAT5	TAA	897 028–897 259	XVI	F: GCCAAAAAATAATAAAAAA R: GGACCTGAACGAAAAGAGTAG	TET	231	13
ScAAT6	TAA	105 661–105 926	IX	F: TTACCCCTCTGAATGAAAACG R: AGGTAGTTTAGGAAGTGAGGC	HEX	266	19

Fermentation media and conditions

In order to simulate wine fermentations, batch fermentations were carried out using a previously described synthetic culture medium (MS) that partially simulates the composition of a standard grape juice (Bely *et al.*, 1990a). Pre-cultured cells of the Zymaflore VL1 parental strain, strains M2 and M4, were inoculated at a density of 1×10^6 cells/ml in fermenters with a volume of 500 ml. Batch fermentations were performed at 28 °C with continuous stirring (500 r.p.m.) and CO₂ release was determined by automatic measurement of fermenter weight loss every 20 min. Calculation of the CO₂ production rate was based on polynomial smoothing. The fermentation kinetics of pilot or industrial-scale wine fermentations are reproduced in an adequate way by this method (Bely *et al.*, 1990b).

Reproducibility

Interdelta typing and microsatellite analysis was repeated for the isolates showing different banding patterns and allelic distributions, respectively, using DNA from two independent extractions.

Results

Strain isolation

A total of 1620 yeast isolates were recovered from musts in a final fermentation stage from 54 spontaneous fermentations derived from grapes collected in the Vinho Verde wine region during 2001–2003 (Schuller *et al.*, 2005). All isolates were analysed by mtDNA RFLP (*Hinf*I), and 100 isolates revealed a banding pattern in the range 1.8–5.5 kb, identical to the commercial yeast Zymaflore VL1, as shown in Figure 1. The strain Zymaflore VL1 is a non-indigenous strain that was originally isolated from the region of Gironde, France, and was predominantly used during the last 5–10 years by wineries located in close proximity to the grape-sampling sites. As reference we used a group of 30 of the original Zymaflore VL1 strain isolates, kindly provided by the manufacturer, which revealed a unique and stable mtDNA RFLP banding pattern (Figure 1).

Figure 1. Mitochondrial DNA RFLP (*Hinf*I) of isolates recovered from natural environments (2, 22, 35, 78) in comparison to the original commercialized strain *S. cerevisiae* Zymaflore VL1 (O2)

Microsatellite analysis

As shown in Table 2, 89/100 isolates recovered from vineyards, analysed by six different microsatellite loci, shared the characteristic Zymaflore VL1 allelic distribution (pattern M1) as follows: heterozygous for loci ScAAT1, ScAAT2, ScAAT5 and ScAAT6, and homozygous for loci ScAAT3 and ScAAT4. Two natural isolates, corresponding to patterns M2 and M4, were characterized by complete loss of heterozygosity (LOH). In addition, pattern M4 showed a trinucleotide increment from 381 bp to 384 bp in locus ScAAT2. These isolates were found in different fermentations, from grapes collected in different years and in distinct sampling sites (Table 3).

Patterns M7 and M8 are characterized by the absence of alleles 219 and 256 (ScAAT5 and ScAAT6, respectively), which may be result of microsatellite expansion due to the hypothesized 'replication-slippage' model, giving rise to alleles

Table 2. Microsatellite analysis of *S. cerevisiae* Zymaflore VL1 isolates. Patterns M1–M8 were found among isolates derived from natural environments and from the original commercial strain

Loci	Pattern of microsatellite alleles (bp)						
	M1	M2	M4	M5	M6	M7	M8
ScAAT1	204/219	219	204	204/219	204/219	204/219	204/219
ScAAT2	372/381	372	384	381	372	372/381	372/381
ScAAT3	265	265	265	265	265	265	265
ScAAT4	329	329	329	329	329	329	329
ScAAT5	219/222	222	219	219/222	219/222	222	219/222
ScAAT6	256/259	256	256	256/259	256/259	256/259	259
No. of natural isolates	89	1	1	1	1	2	5
No. of original VL1 isolates	30	0	0	0	0	0	0

222 and 259. This model assumes that during DNA synthesis, the nascent strand dissociates and realigns out of register. When DNA synthesis continues, the repeat number of the microsatellite is altered at the nascent strand (Schlötterer, 2000). Pattern M8 was the most frequent variation but the isolates could be clonal, since four of them were derived from the same fermentation. The disappearance of alleles ScAAT2 372 and 381 in patterns M5 and M6 may be associated with other mechanisms. All variant patterns, M2–M8, were only detected in the isolates recovered from vineyards.

Small-scale fermentations

The two strains with patterns M2 and M4 and the original strain Zymaflore VL1 were chosen for fermentation experiments, using batch cultures with MS culture medium, whose composition is very similar to a standard grape must (Bely *et al.*, 1990a). As shown in Figure 2, Zymaflore VL1 shows a short lag phase and finishes fermentation within 110 h after inoculation, as expected for an efficient commercial yeast strain. A maximum cellular density of 21 (A_{640}) was achieved about 30 h after inoculation. The strain showing pattern M4 was severely affected in its fermentative capacities, as evidenced by a delayed fermentation initiation, a delayed maximum CO₂ production rate that was about half of the value obtained for the original strain VL1. Besides, the strain with pattern M4 had not finished fermentation after 160 h, and achieved a maximum cellular density of 16 (A_{640}) after 100 h of incubation. The strain showing pattern M2 grew very poorly (cellular density of 1, A_{640}) in synthetic MS medium.

Electrophoretic karyotyping

The same strains, with microsatellite patterns M2 and M4, were further analysed by PFGE (Figure 3). Their karyotype, patterns KD (M2) and KD (M4) showed, in comparison to the original VL1 strain (pattern K1), a chromosomal constitution similar to what would be expected for a haploid derivative, characterized by loss of structural heteromorphism and most evident for the smaller chromosomes III and VI. Flow cytometric analysis showed that the two natural strains were diploid, like strain Zymaflore VL1 (not shown).

Chromosomal polymorphisms were analysed in the isolates derived from natural environments and 30 isolates derived from the VL1 original strain (Figure 3). Major changes of chromosomal patterns were evidenced by the absence of one band in the presumable region of chromosomes VI (K2) and III (K4). Minor chromosomal changes in the same chromosomal regions were assigned to patterns K3 (chromosome VI), K5 (chromosome III) and K6 (both chromosomes III and VI), characterized by double bands closer or more distant than in pattern K1. One strain (pattern K7) is characterized by changes in chromosomal regions III and V–VIII. The 'typical' Zymaflore VL1 pattern K1 was the most frequent among both groups of isolates (Table 3).

Interdelta analysis

As shown in Figure 4 and Table 3, interdelta sequence amplification patterns with primer pair B generated a more polymorphic banding pattern than primer pair A, which is in accordance with previous findings for commercial *S. cerevisiae* strains

Table 3. Summary of the predominant and variant genetic profiles for all molecular typing methods used throughout this work (microsatellite analysis, electrophoretic karyotyping, interdelta sequence analysis), among 100 strains obtained from 12 spontaneous fermentations in different vineyards and years, in comparison to the profiles found among 30 isolates that derived from the original commercialized strain Zymaflore VLI. The frequency of predominant genetic patterns is indicated (%)

Sampling Year	Vineyard	Fermentation No.	VLI isolates (n)	Isolates with microsatellite pattern (n)			Isolates with karyotype pattern (n)			Isolates with interdelta sequence amplification pattern (n)																								
				M1	M2	M4	M5	M6	M7	M8	KD	K1	K2	K3	K4	K5	K6	K7	DA1	DB1	DB2	DB3	DB4	DB5	DB6	DB7	DB8	DB9	DB10	DB11	DB12	DB13	DB14	DB15
2001	C	1	1									1							1															
	C	2	28	25				2	1			11	1	3	10	2	1			28	24	1	1	1										
	C	3	1	1								1							1															
2002	P	4	22	20				1				2		1	2				22	19				1	1	1								
	P	5	5	5										1	1				5	4							1							
	P	6	11	11								4		4					11	10	1													
2003	P	7	2	1							1								2	2														
	A	8	1	1															1														1	
	A	9	1	1															1															
	C	10	16	12						4									16	13						1							1	
	P	11	6	6															6	5								1						
	P	12	6	5							1								6	5	1						1						1	
Isolates recovered from grapes (n)				100	89	1	1	1	2	5	2	15	1	9	2	13	2	1	100	85	3	1	1	1	1	1	1	1	1	1	1	1	1	
Isolates derived from the original VLI strain (n)				89	11							33	67						100	85	15													1
Isolates derived from the original VLI strain (%)				30	30							28							2	30	29													
Isolates recovered from grapes (%)				100	0							93	7						100	97	3

Figure 2. Growth curves (A) and fermentative profiles (B) of two natural strains showing loss of heterozygosity by microsatellite analysis [as shown in Table 2, patterns MS2 (▲) and MS4 (◆)] and their chromosomal constitution, characterized by loss of structural heteromorphism, in comparison to the commercialized strain Zymaflore VL1 (●)

(Legras and Karst, 2003; Schuller *et al.*, 2004). While patterns DA1 and DB1 are characteristic for the original parental strain Zymaflore VL1, natural isolates showed other patterns (DB2–DB15) when using primer pair B, characterized by one (DB3, DB7, DB8, DB9, DB10, DB11, DB15) or two (DB4, DB6, DB13) additional bands, one more amplified band (DB5, DB12) or one additional band and one more amplified band (DB14). All these variants were characteristic for a single fermentation, except DB2, a pattern found in isolates derived from fermentations carried out with grapes collected in different vineyards and years (Table 3).

In global terms, the VL1 yeast population, recovered from vineyards in close proximity to wineries

where this yeast has been used during the last 5–10 years, shows a higher genetic variability than a group of isolates derived from the original VL1 parental strain. Variant genetic profiles were found in different percentages for distinct typing methods and were much more frequent for the natural isolates, independent of the method used (11% and 0% for microsatellite analysis, 67% and 7% for electrophoretic karyotyping, 15% and 3% for interdelta sequence typing using primer pair B, among recovered and original VL1 isolates, respectively) and electrophoretic karyotyping detected the highest polymorphism among the strains tested. Correlations between karyotype patterns and the other molecular markers were only possible to establish

Figure 3. Electrophoretic karyotyping patterns KD and K1–K7. The assignment of different bands to chromosomes is merely indicative and was estimated by similarity between banding patterns of the present strains and laboratory strain S288C

Figure 4. Interdelta sequence analysis with primer pair A and B, showing the most frequent and characteristic Zymaflore VL1 pattern (DA1 and DB1, respectively) and variant patterns DB2–DB15 that are characterized by additional (●) or more amplified bands (○)

for patterns KD (M2) and KD (M4), diploid strains characterized by loss of heterozygosity and loss of structural heteromorphism. No other general correspondences could be established for the remaining patterns that belong to distinct typing methods.

Discussion

In the present study, 100 isolates of the commercial *S. cerevisiae* strain Zymaflore VL1 were recovered from spontaneous fermentations carried out with must prepared from grapes collected close to wineries where this commercial yeast has been used

consecutively for the last 5–10 years. These isolates were identified based on their mtDNA RFLP and evaluation of genetic polymorphisms was done using other methods (PFGE, interdelta sequence typing and microsatellite analysis). The patterns obtained were compared to those obtained from 30 isolates derived from the original Zymaflore VL1 strain, which is used to produce active dry yeast.

Microsatellite allelic polymorphisms were found among the 100 isolates recovered from nature. Some of the isolates may be clonal, since they derived from the same fermentation sample (four of five isolates with pattern M8 and two isolates with

pattern M7). Microsatellite patterns M2 and M4 are characterized by loss of heterozygosity for loci ScAAT1, ScAAT2, ScAAT5 and ScAAT6, localized on chromosomes XIII, II, XVI and IX, respectively. The DNA content of these strains is identical to the parental strain VL1, as determined by flow cytometric analysis (not shown). Considering that these strains had an mtDNA RFLP profile identical to strain VL1, the occurrence of sporulation and subsequent 'self-diploidization', such as the previously described 'genome renewal' (Mortimer, 2000; Mortimer *et al.*, 1994), could be an explanation of our findings. Sporulation of strain Zymaflore VL1 occurs with rather low efficiency (5–10%, our unpublished results) despite the apparent chromosomal heteromorphism. Since it has been hypothesized that sporulation does not occur in must fermentation (Puig *et al.*, 2000), and considering that the strains were derived from two different samples, it seems possible that these changes are associated with the yeast's long-term presence in natural environments. It has been reported that loci ScAAT1 and ScAAT3 were the most polymorphic (Pérez *et al.*, 2001; Schuller *et al.*, 2004), but no variant alleles were found in the present study. Microevolutionary trinucleotide expansions were found for loci ScAAT5 and ScAAT6. Recent reports point towards an evolutionary role for microsatellites as important sources of adaptive genetic variation and indicate that expansion and contraction of repeats result in gradual, quantitative and fully reversible functional changes, enabling rapid evolutionary adaptation to particular environments (Verstrepen *et al.*, 2005).

Transposable elements such as delta elements, either associated with Ty1 and Ty2 retrotransposons or distributed in a random manner throughout the genome of *S. cerevisiae*, indicative of past Ty insertions, are contributing to the variability found within isolates of strain VL1. Although karyotype variability may be Ty-mediated, no correlation between the patterns obtained from the two markers was apparent. Variation in delta sequence chromosomal positions may not be associated with gross chromosomal rearrangements, which in turn can be mediated by other repetitive DNA sequences, as mentioned above.

Our data confirmed that natural isolates of strain Zymaflore VL1 show considerable chromosomal DNA polymorphisms (Bidenne *et al.*, 1992; Longo and Vezinhet, 1993), most evident for the smaller

chromosomes III and VI. Such rearrangements, abundantly described in *S. cerevisiae*, are considered to be involved in adaptive evolution (Dunham *et al.*, 2002; Infante *et al.*, 2003; Pérez-Ortín *et al.*, 2002). Minor chromosome size polymorphisms were also observed in *Cryptococcus neoformans* and described as rapid microevolutionary changes as result of adaptation to laboratory conditions (Franzot *et al.*, 1998).

Yeast cells undergo about 70 generations for both dry yeast production and cellular multiplication (Longo and Vezinhet, 1993), and explain the genetic variability found among original commercial isolates. During must fermentation, yeast cells undergo five to seven divisions. Our (unpublished) results show that the amount of genetic variability of the original VL1 strain, measured by the molecular markers used throughout this work, does not change when cells are inoculated in musts and undergo a fermentative process.

From our present data we cannot draw conclusions about the relative proportion of VL1 variants, M2 and M4 strains, at the beginning of fermentation. However, their contribution to the global yeast flora collected at the final stage of both of the spontaneous fermentations from which they were derived was 3% (1/30 isolates). Additional fermentations were performed with grape musts inoculated with strains M2, M4 and the parental VL1 strain. Strains M2 and M4 showed the same fermentative patterns as the parental VL1 strain (our unpublished results), showing that the genetic changes in strains M2 and M4 affect growth and fermentative behaviour in the MS medium, but not in grape must under fermentative, anaerobic conditions. Under aerobic conditions, the three strains had very similar specific growth rates in YPD medium, MS medium or grape must ($0.182 \pm 0.1/h$, $0.192 \pm 0.15/h$ and $0.251 \pm 0.1/h$, respectively).

Molecular markers developed for *S. cerevisiae* typing during the last few years have revealed important aspects of the genetics of wine yeast and their population dynamics during wine fermentation. In a previous work, a survey of the polymorphisms generated by each of the methods used in the present study was carried out. The results showed that microsatellite typing (loci ScAAT1–ScAAT6), interdelta analysis with primer pair B, and mtDNA RFLP (*HinfI*) had the same discriminatory power, distinguishing among 21 profiles in a group of 23 commercial wine yeast

strains (Schuller *et al.*, 2004). The present study allows the evaluation of each typing method in respect to 'intra-strain' variability. Within a previous large-scale biogeographical study we assembled a collection of about 300 *S. cerevisiae* strains that were genetically characterized regarding their microsatellite allelic combinations, interdelta amplification patterns and mtDNA RFLP. We found about 300 unique mtDNA RFLP patterns that correspond, with a few exceptions, to unique microsatellite allelic combinations and interdelta amplification patterns, respectively. Several isolates showed similar mtDNA RFLP patterns to that found for strain Zymaflore VL1. However, when analysed by microsatellite markers ScAAT1–ScAAT6, they showed completely different allelic combinations. Furthermore, a group of 50 isolates was randomly selected among the whole collection, comprising 1620 strains, and microsatellite analysis was performed. Isolates with the same/different microsatellite amplification profiles always showed the corresponding same/different mtDNA RFLP patterns (Schuller and Casal, 2007). We consider mtDNA RFLP to be a portable genetic marker, but without the capacity to reveal intra-strain genetic variability. While electrophoretic chromosome karyotyping is still considered the method of choice for the evaluation of chromosomal rearrangements, the usefulness of delta sequence typing for strain delimitation using primer pair A or B should be carefully evaluated. The use of primer pair A is associated with a low resolution among strains when compared to primer pair B (Legras and Karst, 2003; Schuller *et al.*, 2004). In the present study it was shown that higher pattern stability among isolates belonging to the same strain is obtained for the first primer pair, while the opposite is verified for primer pair B, which may lead to misidentifications. Microsatellite markers are suitable for the detection of microevolutionary changes, such as microsatellite expansions or contractions and loss of heterozygosity.

Although the yeast species associated with wine grapes have been studied over many years, knowledge about their ecology is considered uncertain and controversial, especially with respect to the principal wine yeast, *S. cerevisiae*. However, the predominating view is that *S. cerevisiae* is a domesticated species that has continuously evolved in

association with the production of alcoholic beverages (Mortimer, 2000). Under this model, the occasional strains of *S. cerevisiae* found in nature are thought to be migrants from human-associated fermentations. In a previous systematic biogeographical survey, we demonstrated the high biodiversity of *S. cerevisiae* strains in vineyards belonging to the Vinho Verde region (Schuller *et al.*, 2005). The presence and distribution of commercial yeast strains used by the wineries was also evaluated, including data from an identical study that was carried out in the Languedoc region (Valero *et al.*, 2005). The permanent implantation of commercial strains in the vineyard did not occur; these strains were subject to natural fluctuations of periodic appearance/disappearance, like autochthonous strains. After completing wine fermentation, *S. cerevisiae* cells, together with grape skins and water run-off, are released to the environment surrounding the winery, where they encounter adverse growth and survival conditions, completely different from wine fermentations. We hypothesize that the genetic changes that we observed among the 100 yeast isolates recovered from natural environments reflect adaptive mechanisms to changed environmental conditions and are consequence of the high genomic plasticity described for *S. cerevisiae*. These strains will be used to gain a deeper understanding of adaptive mechanisms that yeast strains may undergo in natural environments.

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