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Differentiation of industrial wine yeast strains using microsatellite markers

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Aims: To differentiate nine industrial wine strains of *Saccharomyces cerevisiae* using microsatellite (simple sequence repeats, SSR) markers.

Methods and Results: Six of the strains were indigenous yeasts currently used as high-density starter monocultures by the Uruguayan wine industry. Unequivocal differentiation of these six native strains and three commercial *S. cerevisiae* wine strains was achieved by PCR amplification and polymorphism analysis of loci containing microsatellite markers.

Conclusions: We recommend the use of this reproducible and simple molecular method to routinely discriminate wine yeast strains.

Significance and Impact of the Study: Microsatellites are superior to other methods for typing yeasts because the results can be exchanged as quantitative data. Knowledge of the frequencies of the alleles for different SSR markers will eventually lead to an accurate typing method to identify industrial wine yeast strains.

INTRODUCTION

Most of the yeast strains utilized in the food industry for the production of beer, wine and bread are classified as *Saccharomyces cerevisiae* (Kurtzman and Fell 1998). However, not all *S. cerevisiae* strains are suitable for the different fermentation process, and the ability to produce a quality beverage or food differs significantly from strain to strain. Most modern wine-makers inoculate grape must with a pure culture of a selected *S. cerevisiae* strain to ensure a reliable and predictable fermentation process (Henschke 1997). As a result of these strain selection programmes, the identification and differentiation of *S. cerevisiae* strains has become a key issue to wine microbiologists. Traditional morphological and biochemical tests are of limited value in revealing the genetic diversity of industrial yeast strains of the same species. Molecular methods developed to study yeasts at both the species and subspecies level include restriction fragment length polymorphism (RFLP) of genomic and mitochondrial DNA (Degré *et al.* 1989; Querol *et al.* 1992), chromosome karyotyping (Van Der Westhuizen and Pretorius 1992; Kishimoto *et al.* 1994), randomly amplified polymorphic DNA (RAPD) (Lavalleé *et al.* 1994; de Barros Lopes *et al.* 1998) and amplified fragment length polymorphism (AFLP) (de Barros Lopes *et al.* 1999).

Microsatellites or simple sequence repeats (SSR) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp (Charlesworth *et al.* 1994). These repetitive sequences are a major component of higher organism DNAs. They are hypervariable in length (Tautz 1989) as a result of DNA-replication errors, such as slipped-strand mispairing (Strand *et al.* 1993). Thus, microsatellites show a substantial level of polymorphism between individuals of the same species and are extensively used for paternity exclusion tests (Helminen *et al.* 1988), forensic medicine (Hagelberg *et al.* 1991) and for molecular typing of different organisms including cultivars of *Vitis vinifera* (Bowers *et al.* 1999) and the pathogen yeast *Candida albicans* (Bretagne *et al.* 1997). Field and Wills (1998) have conducted computer searches for short tandem repeat patterns on several completely sequenced small genomes, including yeast. They suggest that trinucleotide repeats could be used to genotype yeast strains.

The aim of this study was to differentiate nine yeast strains of *S. cerevisiae* that are currently used in a Uruguayan winery for the production of different styles of wine. Six of them are selected native strains isolated in this

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winery and three are the more commonly used commercial strains in the Uruguayan wine industry.

MATERIALS AND METHODS

Yeast strains and media

Saccharomyces cerevisiae AB972, the strain used in the sequencing project, is a haploid, MAT alpha strain. The commercial wine *S. cerevisiae* strains used were Montrachet 522 (M522) from the University of California, AWRI796 from the Australian Wine Research Institute and CHP from Gist Brocades, Chile. The six native *S. cerevisiae* strains used in this study were isolated in Uruguay and are described in Table 1. All yeast strains were grown on YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] medium for DNA isolation. WL Nutrient (Difco) was used as a differential medium.

Quick preparation of DNA template for PCR

The pellet corresponding to around 10^9 cells (early stationary phase) was washed with sterile water and resuspended in 0·4 ml breaking buffer (2% Triton X-100, 1% sodium dodecylsulphate, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris pH 8 and 1 mmol l^{-1} EDTA pH 8). The cells were homogenized by vortexing for 5 min with 0·3 g glass beads (Sigma G9268) in the presence of 0·4 ml phenol, pH 8. After centrifugation at 4°C, the aqueous phase was carefully removed and 1 μl of a 1/10 dilution was used for a 20-μl PCR reaction.

High-quality DNAs, suitable as reference DNAs for long-term storage, were obtained following the method of Ausubel *et al.* (1994).

Amplification conditions

Amplification of loci containing microsatellites was performed with the following pairs of specific forward and reverse primers, respectively: 5′-GGTGACTCTAACGGCAGAGTGG-3′ and 5′-GGATCTACTTGCAGTATACGGG-3′ for locus SCYOR267C; 5′-CTGCTCAACTTGTGATGGGTTTTGG-3′ and 5′-CCTCGTTACTATCGTCTTCATCTTGC-3′ for locus SC8132X; 5′-CCCTTTTAAGGAAGAGCAAGCC-3′ and 5′-CCACTCTCAGCTTATTGGGG-3′ for locus SCPTSY7. PCR amplifications were performed in a GeneAmp PCR system 2400 (Perkin Elmer) in 20 μl reactions consisting of: 10–40 ng DNA, 200 μmol l^{-1} each dNTP, 2 μl of 10× PCR buffer minus Mg (Life Technologies), 1 unit *Taq* DNA polymerase (Life Technologies), 1.5 mmol l^{-1} $MgCl_2$ and 12·5 pmoles of forward and reverse primers for locus SCYOR267C, 4 mmol l^{-1} $MgCl_2$ and 5 pmoles of forward and reverse primers for locus SC8132X, 3 mmol l^{-1} $MgCl_2$ and 2·5 pmoles of forward and reverse primers for locus SCPTSY7. Amplification protocols were optimized for individual loci as follows: SCYOR267C: 4 min at 94°C, 40 cycles of (30 s at 94°C, 30 s at 70°C, 2 min at 72°C), and 10 min at 72°C; SC8132X: 4 min at 94°C, 35 cycles of (30 s at 94°C, 30 s at 65°C, 1 min at 72°C), and 5 min at 72°C; SCPTSY7: 4 min at 94°C, 35 cycles of (30 s at 94°C, 30 s at 55°C, 1 min at 72°C), and 5 min at 72°C.

Amplification was confirmed by running an aliquot of the PCR reaction product in 2% agarose gels. DNA concentration was then adjusted and 1/3 volume of denaturing dye solution (10 mmol l^{-1} NaOH, 95% formamide, 0·05% bromophenol blue, 0·05% xylene cyanol) was added. One to four microlitres of this mixture were denatured and electrophoresed in a 34-cm sequencing gel (5% LongRanger acrylamide plus 7 mol l^{-1} urea) and then silver stained according to the protocols and reagents provided with the Promega Silver Sequencing Kit (Promega, USA). Product sizes were determined by comparison with a standard sequencing reaction (pGEM-3zf + plasmid DNA provided with the kit) electrophoresed in adjacent lanes in the same gel. Absolute values for AB972 DNA microsatellite markers were calculated from GenBank database information and are

Table 1 Characteristics of the native and commercial wine yeast strains used in this study

Strain	Origin	Colony colour in WLN	Enological application	Aroma description
CP 863	1986, Trebbiano white juice	Soft green	Low temp., high killer action, low foam	Fresh fruit, no H_2S
CP KU1	1987, semillon white juice	White	Low temp., high killer action	Fresh fruit, no H_2S
CP 873	1987, semillon white juice	Dark green	Low temp., killer action	Fresh fruit, no H_2S
CP 874	1987, semillon white juice	Soft green	Low temp., higher foam, killer action	Fruity, no H_2S
CP 881	1988, Chardonnay white wine	White	Alcohol resistant, for sparkling wine, killer resistant	Fruity, no H_2S
CP 882	1988, Chardonnay white wine	Soft green	Very flocculant, for sparkling wine, alcohol and killer resistant	Fruity, no H_2S
M 522	University of California	Dark green	Alcohol resistant, traditional wine producer, sensitive to killer	Soapy, rubbery
CHP	CIVC 8130	White	Low temp., alcohol resistant, for sparkling wine, killer action	Soapy
AWRI 796	Australian Wine Research Institute	White	Alcohol resistant, killer action	Soapy, rubbery

the following: 409 bp for locus SCYOR267C, 207 bp for locus SC8132X and 332 bp for locus SCPTSY7. The SSRs present in the DNA of the reference strain AB972 correspond to $(gtt)_{20}$ for locus SCYOR267C, $(ctt)_{15}$ for locus SC8132X and $(aat)_{33}$ for locus SCPTSY7.

RESULTS

The indigenous strains were isolated from grape must during the late 1980s, when no ammonium salts were utilized in the winery industry. They were selected on the basis of their low nitrogen demand, killer or neutral phenotype and sensory analysis. The origin and phenotypic characteristics of these strains are described in Table 1. Comparative fermentation profiles were performed for six native strains and three commercial wine yeasts (results not shown) in a low nitrogen grape must (less than 150 mg l^{-1} of free amino nitrogen). Dry white wines were obtained in all cases after nine days. However, native strains resulted in fruity and very fruity wines compared with the three commercial strains that, under these conditions of low nitrogen musts, resulted in soapy and rubbery descriptors (see Table 1). Strain differentiation by plating and colony colour differentiation in WL Nutrient medium was not suitable for most strains (see Table 1) and, therefore, a rapid and reliable identification method was developed.

The native wine yeast strains and commercial strains M522, AWRI796 and CHP are normally used in the same winery and, therefore, accurate differentiation among them is needed. For this purpose, microsatellite markers located in different yeast chromosomes and showing high allelic polymorphism and heterozygosity were used (Field and Wills 1998). Amplified DNA from *S. cerevisiae* AB972 was used as an absolute size reference for each locus.

Amplification products for microsatellite locus SC8132X (see Fig. 1) showed a high level of polymorphism: seven different allele lengths and five heterozygotes (CHP, CP 881, CP KU1, M522 and CP 882). Considering the results for this marker, M522 could not be distinguished from CP 882. The rest of the DNAs analysed could be differentiated with this marker. Absolute sizes of microsatellite markers for the sequenced reference strain are known and, therefore, the allele sizes for the other DNAs could be scored, counting the nucleotide differences according to the sequencing ladder (see Table 2). PCR amplification of microsatellite locus SCYOR267C with the DNAs of all yeast strains (see Table 2) resulted in five different allele lengths and two heterozygotes (CHP and CP 881). This microsatellite marker did not show any polymorphism between strains CP KU1 and CP 863. DNAs from strains CP 873, CP 874 and CP 882 shared another allele for this marker. However, this marker allowed us to differentiate the other four strains. Amplification products for microsatellite locus SCPTSY7 were also analysed (see Table 2). In this case, five different alleles and one heterozygote were obtained (CP 882). DNAs from strains CP KU1 and CP 863 shared the same allele length for this marker. Strains CP 873, CP 874 and CP 881 also shared another allele size. This marker did not provide a higher discriminatory power than the previous two loci. Therefore, we concluded that only two markers (SCYOR267C and SC8132X) were enough to unequivocally discriminate among these nine wine yeast strains.

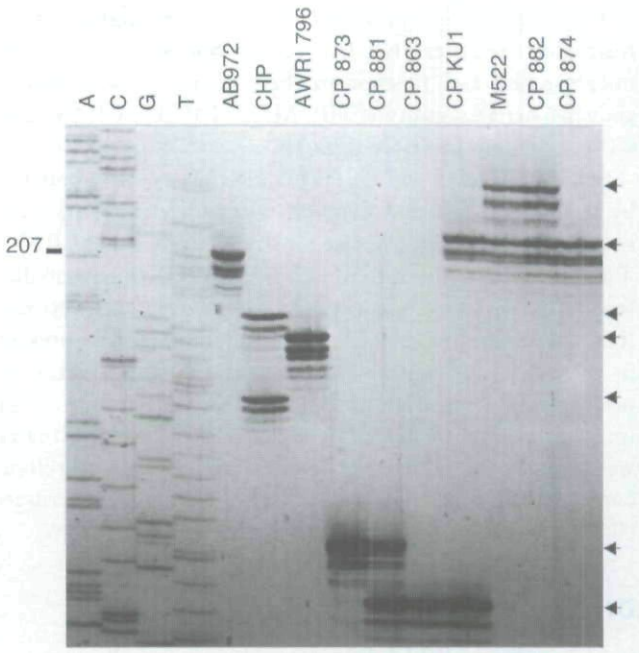

Fig. 1 Allelic diversity at microsatellite locus SC8132X. Amplification products were detected by silver staining. Lanes A, C, G and T correspond to the sequencing reaction used as a size standard, DNA from strain AB972 was the absolute size reference standard with an allele size of 207 bp. Allele lengths were determined from the bands indicated by arrows

Table 2 Allele sizes (bp) at three microsatellite loci

	Microsatellite loci		
Yeast strain	SCYOR267C	SC8132X	SCPTSY7
AWRI796	427	195	311
CHP	421–427	186–197	N.D.*
M522	458	210–218	300
CP 863	392	156	352
CP 873	433	165	303
CP 874	433	210	303
CP 881	433–458	156–165	303
CP 882	433	210–218	303–350
CP KU1	392	156–210	352

*N.D., not determined.

The amplification products obtained were digested with restriction enzymes that had recognition sites within the microsatellite loci to confirm their identity. Yeast strains showing heterozygosity (CHP, M522, CP 881, CP 882 and CP KU1) were probably diploids.

Strains CP KU1 and CP 863 could only be differentiated by locus SC8132X and their phenotypic characteristics are similar. This was also the case for strains CP 873 and CP 874. To confirm that the differences in SC8132X microsatellite lengths observed were reflecting a genetic difference between these strains and were not due to a mutation already present in the cell that originated the colony randomly picked to perform DNA isolation, independent experiments of DNA isolation were performed. The allele sizes obtained in all cases were the same as those previously obtained and, therefore, confirmed that a different allele size for a unique microsatellite marker is enough to differentiate two yeast strains.

DISCUSSION

In this work we could differentiate unequivocally between nine wine industrial *S. cerevisiae* strains using only two microsatellite markers. The level of polymorphism and heterozygosity observed is in agreement with data previously reported by Field and Wills (1998) for laboratory strains.

Microsatellite polymorphism analysis is a very reproducible method because specific primers and, therefore, high annealing temperatures are used for the amplification of short DNA fragments. Moreover, SSR results can be expressed objectively as allele lengths and can thus be communicated and exchanged as quantitative data rather than as images of band patterns (as is the case for RAPDs and AFLP). The method can also be automated for the analysis of multiple samples and multiplexed for the analysis of multiple microsatellite loci. When different laboratories obtain slightly different allele sizes for a single microsatellite marker because of differences in methodology (fluorescein labelled primers and automatic sequencing *vs.* silver staining of sequencing gels), such differences can easily be reconciled by the inclusion of common DNAs as standard references for comparison. At present and using the quick method described to isolate DNA, we can perform this microsatellite polymorphism analysis in 14 h. We recommend the use of microsatellite markers to routinely discriminate wine yeast strains. Furthermore, the exchange of information on allele sizes for multiple microsatellite loci, obtained by different laboratories, might allow the construction of a database to accurately genotype industrial wine yeast strains in the future.

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