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Use of RAPD Analysis for Differentiation among Six Enological *Saccharomyces* spp. Strains

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Summary

Two molecular typing techniques, amplified ribosomal DNA restriction analysis and random amplified polymorphic DNA assay, were evaluated for discrimination among six strains previously identified as synonymous of *Saccharomyces cerevisiae* by classical physiological characteristics and enological criteria. The first method that used restriction patterns originated by the amplification of internal transcribed spacers (ITS1 and ITS2) and the ribosomal gene 5.8S did not allow the differentiation among the six strains. On the contrary, reproducible amplicon fingerprints for the *Saccharomyces* spp. strains were obtained by random amplified polymorphic DNA assay using twenty one different primers. Among them, only four primers allowed discrimination among the six *Saccharomyces* spp. tested. The percentage of similarity between all species tested ranged from 40 to 80 %. The potential of the random amplified polymorphic DNA assay analysing the diversity of wine yeast species and developing polymerase chain reaction primers for wine yeast characterisation is discussed.

Key words: DNA fingerprinting, RAPD, ARDRA, wine yeast identification, *Saccharomyces cerevisiae*

Introduction

Traditionally, wine has been manufactured by spontaneous fermentation of grape must with indigenous yeasts from grapes and winery equipment. On the contrary, most modern wine-makers inoculate the musts with selected yeast strains either in pure or in mixed yeast form. This allows a better vinification control and assures a faster fermentation free of contaminating yeasts (*1,2*).

The number of wine yeast strains available in the world market has increased as the result of studies that show the strong influence of indigenous yeasts for the wine quality of a given world region. In Portugal, the active dry enological yeasts available in the market are not yet representative of the indigenous yeasts of most of our wine regions (*3*). Therefore, enological, physiological and molecular characterisation of our native wine yeasts is of great importance to assure Portuguese wine quality. As most of the wine yeast strains belong to the genus *Saccharomyces*, they are not easily differentiated and/or identified on the basis of classical methods when more than one species is present in a yeast starter used for wine-making process (*4*). This represents a challenge for wine microbiologists that have to assure the correct propagation of the yeast(s) with a predictable influence on the vinification process to obtain the desired product quality (*5*). The traditional methods for identifying yeasts rely on morphological, physiological and biochemical criteria (*6*). These techniques are gener-

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ally laborious and time consuming, hence they are not appropriate for routine identification (*7*). In the last decade, methods based on molecular biology have been developed and are now being applied to the field of yeast taxonomy. One such method is a variant of the polymerase chain reaction (PCR) technique based on the amplification of random fragments of DNA (RAPD). This technique uses short (5–15mer) oligonucleotide primers of arbitrary sequence that at low annealing temperature hybridise *at loci* distributed at random throughout the genome, allowing the amplification of polymorphic DNA fragments (*8*). The products obtained have been shown useful in differentiation of species and strains of many organisms. Couto *et al.* (*9*) were able to differentiate several isolates of spoilage yeasts demonstrating that this technique is a useful tool for discriminating between *Zygosaccharomyces bailli*, *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* species, using selected decameric oligonucleotides primers.

PCR amplification of specific sequences for the identification of organisms has also become common because of the relative ease of manipulation and high reproducibility. Restriction patterns of the intergenic rDNA spacer between the 18S and 28S rRNA genes (amplified ribosomal DNA restriction analysis – ARDRA) have been also used to differentiate among species of *Saccharomyces* spp. (*10*,*11*). Guillamón *et al.* (*10*), were able to differentiate between type strains of *Sacch. cerevisiae* and *Saccharomyces bayanus* with the restriction patterns originated by endonuclease H*ae*III.

In our previous attempts, six *Saccharomyces* spp. isolates were selected from Alentejo white wine musts. Five out of six were classically identified as *Sacch. cerevisiae* strains according to Kreger-van Rij (*6*). One species was identified as *Sacch. montuliensis* according to enological criteria. However, Vaughan-Martini and Martini (*12*) based on DNA-reassociation analysis suggested that strains of *Saccharomyces* which have recently been classified as synonymous of *Sacch. cerevisiae* might be included into three or even more separate species. In this study we used two molecular methods, the RAPD (random amplified polymorphic DNA) and ARDRA (amplified ribosomal DNA restriction analysis) to evaluate their usefulness for differentiation among the selected six enological strains *of Saccharomyces* spp.

Materials and Methods

Yeasts strains

All wine yeast strains were isolated from grape must, and classified by morphological and physiological methods as: *Saccharomyces cerevisiae* var. *uvarum* CCMI 885, *Saccharomyces cerevisiae* var. *capensis* CCMI 886, *Saccharomyces cerevisiae* var. *montuliensis* CCMI 887. *Saccharomyces cerevisiae* var. *chevalieri* CCMI 888, *Saccharomyces cerevisiae var. bayanus* CCMI 889, *Saccharomyces cerevisiae* var. *cerevisiae* CCMI 890. The yeast strains are deposited at the Culture Collection of Industrial Microorganisms (CCMI) of INETI, Lisboa, Portugal (*13*).

DNA extraction

The technique used was described in Lopes *et al.* (*2*) with slight modifications. The yeasts were cultured on malt extract agar for two days and transferred to malt extract broth. The cells were spun down and freeze-dried. For DNA extraction the cells were redissolved in 2 mL of extraction buffer (0.2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris, pH=8.0, 1 mM EDTA). After the addition of 10 μL of RNase (20 mM) and 4 μL of proteinase K (20 mg/mL) the suspension was incubated at 37 °C for 1 h. The yeasts were homogenised by vortexing during 5 min, in the presence of 1 mL of phenol-chloroform-isoamyl alcohol mixture. Tris-EDTA buffer (200 μL) was added, and the aqueous layer was collected after centrifugation. The DNA was precipitated with ethanol. DNA concentration was determined by measuring the $A_{260\ nm}$. All extracted DNA exhibited a ratio $A_{260\ nm}/A_{280\ nm}$ between 1.8–2.0.

RAPD assay

From twenty seven tested primers, twenty decamer arbitrary primers tested were from AB1 (Advanced Biotecnologies, Surrey), three decamer arbitrary primers were from OPC (Operon Technologies, California) and four were synthesised according to Couto *et al.* (*9*).

Reactions were performed in a volume of 20 μL containing 100 ng yeasts genomic DNA, 2.5 U Taq polymerase (Pharmacia Biotech, Uppsala), 10x polymerase buffer (670 mM Tris-HCL (pH=8.8, 25 °C), 166 mM $(NH_4)_2SO_4$, 0.1 % Tween-20), 2 mM dNTPs, 15 pg/μL primers and 50 mM $MgCl_2$ (Ecotaq, Ecogen, Barcelona) using Hybaid-OMN-E thermal cycler. Initially, the reaction mixture was heated for 4 min at 94 °C followed by 45 cycles with a temperature profile of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. The amplified DNA fragments were separated on 1 % agarose gel (Pharmacia Biotech, Uppsala) by electrophoresis. The DNA fragments were visualised with ethidium bromide, photographed and analysed. A 100-bp DNA ladder (Pharmacia Biotech, Uppsala) was used as the size standard.

ARDRA assay

A modification of a technique previously described elsewhere was used (*14*). The DNA was isolated and diluted to 250 ng/μL. The rDNA gene region was amplified in a Hybaid-OMN-E thermal cycler. Reactions were performed in a volume of 25 μL containing 250 ng yeasts genomic DNA, 1 U Taq polymerase (Pharmacia Biotech, Uppsala), 10x polymerase buffer (166 mM $(NH_4)_2SO_4$, 670 mM Tris-HCL (pH=8.8, 25 °C) 0.1 % Tween-20), 200 μM dNTPs, 50 pg/μL primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTATTGATATGC-3'), and 2.5 mM $MgCl_2$ (Ecotaq, Ecogen, Barcelona). The thermal cycling parameters were an initial denaturation during 4 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 1min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. 5 μL PCR products were digested in final volume with the restriction endonucleases. A*lu*I, H*ae*II, D*pn*II (Biolabs, New England) and, R*sa*I, M*sp*I, (Boehringer Mannheim, Mannheim) were allowed to cut at 37 °C for 3 h and

T*ru9*I (Boehringer Mannheim, Mannheim) at 65 °C, according to supplier's instructions. Restriction fragments were electrophoresed on 1 % agarose (Pharmacia Biotech, Uppsala) stained with ethidium bromide and photographed. 100-bp DNA ladder marker (Pharmacia Biotech, Uppsala) was used as size standard.

Analysis of data

Estimation of genetic similarity between the yeasts strains was performed by using the estimator F of Nei and Li: $F=2n_{xy}/n_x+n_y$, where n_{xy} is the number of bands shared by strains x and y, and n_x and n_y are the total number of bands of each strain. A similarity dendrogram was made with the data obtained by using the method UPGMA from the NTSYS software package (*8*).

Results

Two molecular methods, random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA), were used to get strain differentiation amongst six *Saccharomyces* spp. isolates previously, selected as wine yeast strains.

Amplified ribosomal DNA restriction analysis

The ITS4 and ITS5 primers were used to amplify a region of the rDNA gene repeat unit, which includes two non-coding regions designed as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene. Fig. 1 shows that PCR products did not show length variation in this region since all strains displayed only one single band at 800 bp. Moreover, after digestion with six different restriction endonucleases, strain differentiation was not found among the restriction patterns obtained from the six *Saccharomyces* spp. strains (Fig. 2).

Random amplified polymorphic DNA

To carry out the RAPD technique, the genomic DNA of the six *Saccharomyces* spp. strains were isolated yielding an average concentration of 2 μg/μL. From the twenty-seven primers used in this study, only twenty-one of them gave satisfactory results in amplification reactions with all yeasts used. Only four out of twenty-one (AB1-09, AB1-12, AB1-15 and P20 primers) were able to discriminate among the six *Saccharomyces* spp. strains (Fig 3).

Although there is a considerable number of shared bands between the yeast strains, the presence or absence of many other intense bands allowed differentiation of the six strains. Four to fourteen bands of amplified DNA, with sizes ranging from 200 to 2500 bp were usually obtained in PCR reactions with the different primers. The reproducibility of the technique was assessed by repeating the amplification reactions several times and evaluating the patterns obtained.

Similarity of strains

After twenty-one RAPD reactions with the genomic DNA from each of the six *Saccharomyces* spp., a total of 227 bands were obtained. Fig. 4 shows the similarity of the yeast strains under the form of a dendrogram showing several clusters branching at a similarity value of 40 %. One cluster discriminates *Sacch. bayanus* (889) from the remaining five *Saccharomyces* spp. strains. A similarity value of 80 % was obtained for second cluster constituted by *Sacch. capensis* (886) and *Sacch. montuliensis* (887). The strain *Sacch. chevalieri* (888) was differentiated from this cluster for a similarity value of 75 %. *Sacch. cerevisiae* (890) was differentiated from the clusters of *Sacch. capensis*, *Sacch. montuliensis* and *Sacch. chevalieri* with a similarity value of 68 %. The last yeast strain, *Sacch. uvarum* was discriminated from the cluster formed by the later four strains showing a similarity value of 63 %.

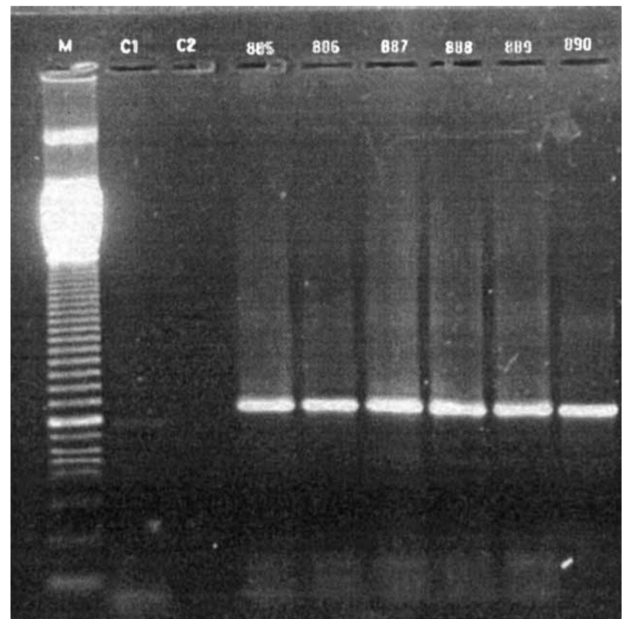

Fig. 1. Amplified DNA of the region ITS1, ITS2 and 5.8S gene rDNA; M-Size marker, 100 bp (Pharmacia Biotech, Uppsala); C1-negative control without DNA, C2-positive control without Taq polimerase; 885-*Saccharomyces uvarum*, 886-*Saccharomyces capensis*, 887-*Saccharomyces montuliensis*, 888-*Saccharomyces chevalieri*, 889-*Saccharomyces bayanus*, 890-*Saccharomyces cerevisiae*

Discussion

The difficulty in the identification of yeasts by the usual microbiological methods has prompted development of different taxonomic approaches (*8*). PCR amplification and restriction analysis of the non-coding region of ITS1 and ITS2 and the gene 5.8S rRNA is a technique normally used to determine the genetic variability among strains of *Saccharomyces* genus (*10,11*). The internal transcribed spacers have been shown to be much less evolutionarily conserved than rRNA coding genes (*10*) so, they are normally claimed to differentiate strains of the same species. Indeed, this technique was sucessefully used for discrimination among nine strains from three species of *Saccharomyces* spp.: *Sacch. cerevisiae*, *Sacch. carlsbergensis* and *Sacch. pastorianus* (*11*). Other researchers have reported the discrimination between one strain of *Sacch. pastorianus* and the type strain of *Sacch. cerevisiae* but not from the type strain of *Sacch. bayanus*

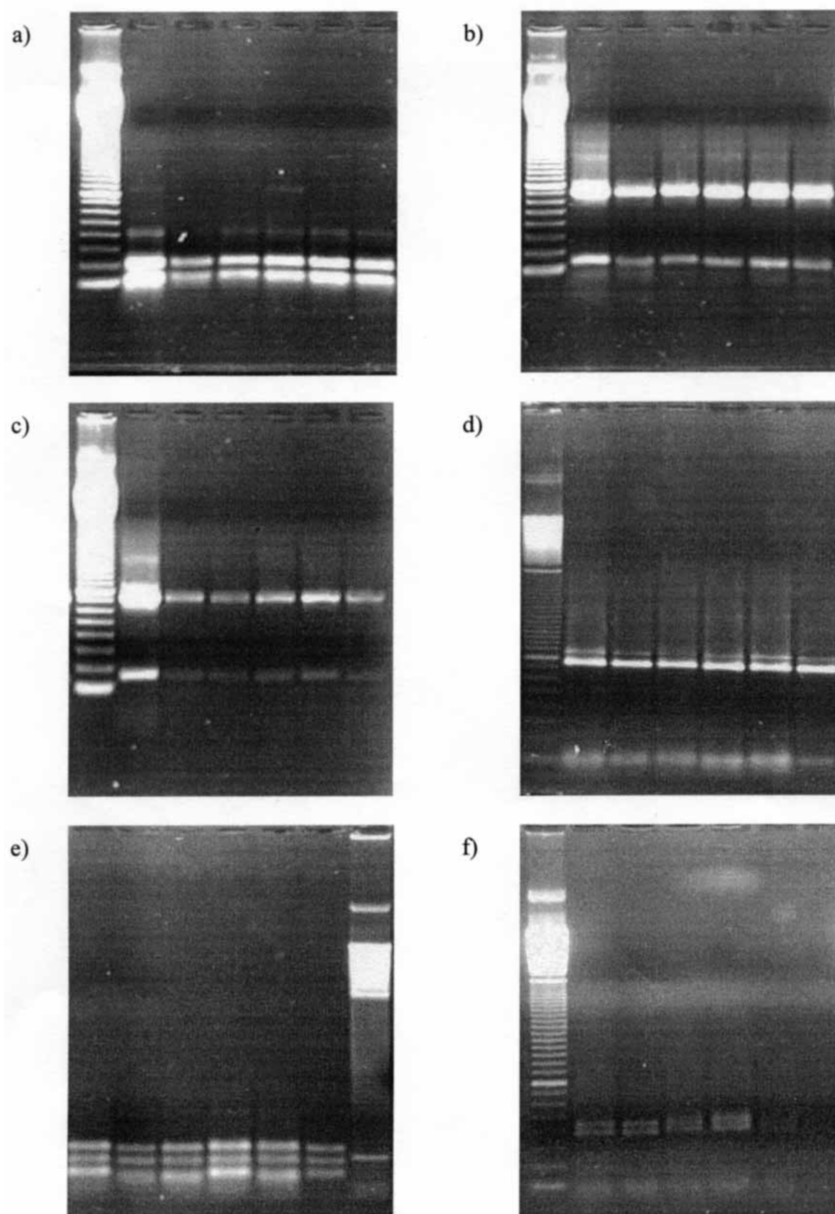

Fig. 2. Restriction pattern of the six strains originated from: a) T*ru*I9; b) R*sa*I; c) M*sp*I; d) A*lu*I; e) H*ae*III; f) D*pn*II

(*10*). In this work restrictions endonucleases were selected in order to recognise the possible four types of sequences but no variations in the length of DNA fragments were observed. We conclude that the ARDRA technique seems to be highly dependent of polymorphism degree in the intergenic region studied. Most probably, the six *Saccharomyces* spp. strains studied have a low polymorphism in that region. On the contrary, the differentiation among the six *Saccharomyces* spp. strains tested in this work was obtained by the RAPD assay. In fact, four distinct banding patterns for all strains were observed with each of the following primers AB1-09, AB1-12, AB1-15 and P20. These results are favourably compared with others, previously reported by Quesada and Cenis (*8*), that were unable to discriminate among five strains of *Saccharomyces* using a single reaction with one primer.

The analysis of genetic similarity among the six *Saccharomyces* spp. strains revealed a low similarity value (45 %) between *Sacch. cerevisiae* and *Sacch. bayanus*. These results confirm other taxonomic studies that consider the latter strains as different species (*12,15*).

Conclusions

A rapid molecular identification technique of different *Saccharomyces* strains, often used as yeast starters for production of white wines from Alentejo region, was shown in this work. Comparing the two methods described it is evident that RAPD assay is a more sensitive technique than ARDRA. The RAPD assay enables the studies on all polymorphic sequences along the genome of the species whereas the ARDRA technique is limited

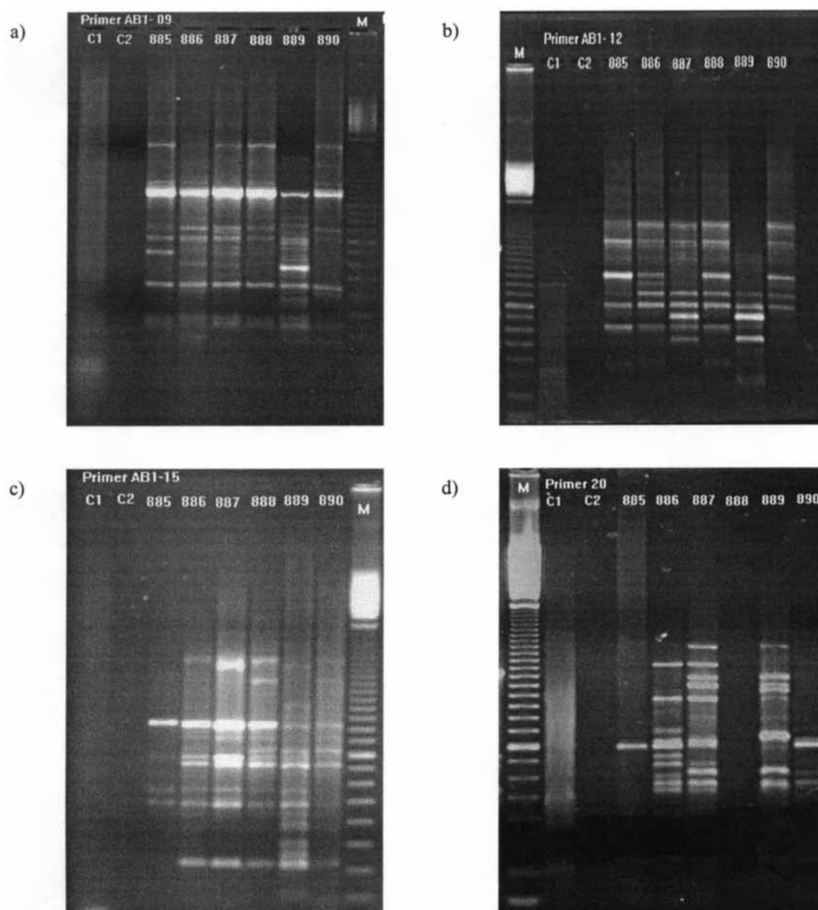

Fig. 3. Patterns of amplified yeast DNA using different primers; a) Amplified DNA with the primer AB1-09 (5'- TGGGGGACTC-3'), b) Amplified DNA with the primer AB1-12 (5'- CCTTGACGCA-3', c) Amplified DNA with the primer AB1-15 (5'- GGAGGG TGT T-3'), d) Amplified DNA with the primer 20 (5'- AGGAGAACGG-3')

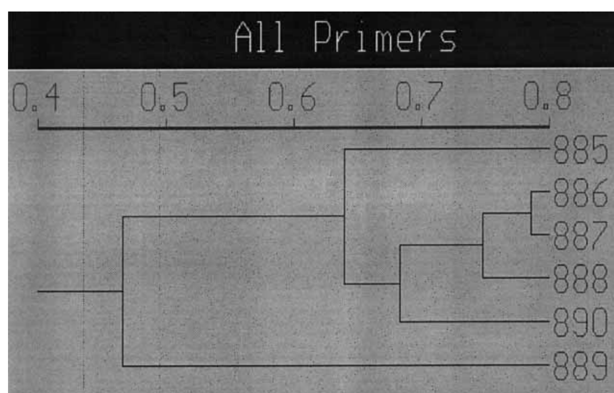

Fig. 4. UPGMA dendrogram with all tested primers showing the similarity values between the six Saccharomyces spp.: 885-*Saccharomyces uvarum*, 886-*Saccharomyces capensis*, 887-*Saccharomyces montuliensis*, 888-*Saccharomyces chevalieri*, 889-*Saccharomyces bayanus and* 890-*Saccharomyces cerevisiae*

by the polymorphic sequences of intergenic spacer. Based on RAPD assay, we were able to type each of the six selected enological *Saccharomyces* spp. strains using a single reaction with one primer. This allows further studies on dynamics of yeast populations during wine fermentation inoculated with a mixture of these selected strains.

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Primjena RAPD analize za razlikovanje šest sojeva vinskih kvasaca *Saccharomyces*

Sažetak

Primijenjena su dva postupka: restrikcijska analiza amplificirane ribosomske DNA i postupak s nasumce amplificiranom polimorfnom DNA (RAPD) kako bi se utvrdila razlika između šest sojeva prethodno utvrđenih kao *Saccharomyces cerevisiae*, prema njihovim klasičnim fiziološkim značajkama i enološkim kriterijima. U prvom je postupku primijenjen restrikcijski model dobiven amplifikacijom interno transkribiranih nekodirajućih područja (ITS1 i ITS2), a ribosomski gen 5,8S nije omogućio razlikovanje između šest sojeva. Postupkom RAPD dobiveni su reproducibilni otisci amplikona za *Sacch.* spp. koristeći 21 različitu klicu (engl. primer). Među njima samo su četiri klice omogućile diskriminaciju između šest *Sacch.* spp. Postotak sličnosti između sojeva iznosio je od 40 do 80 %. Razmatrana je mogućnost RAPD analize za otkrivanje različitosti sojeva vinskih kvasaca i razvoj klica za PCR kako bi se omogućila njihova karakterizacija.