EVALUATION OF PFGE AND mtDNA RESTRICTION ANALYSIS METHODS TO DETECT GENETIC DIVERSITY OF *SACCHAROMYCES CEREVISIAE* STRAINS ASSOCIATED TO *VITIS VINIFERA*

Mar VILANOVA[1]*, Sol ZAMUZ[1], Antón MASA[1] and Carmen SIEIRO[2]

1: Misión Biológica de Galicia (CSIC), P.O. Box 28, 38080, Pontevedra (Spain)
2: Área de Microbiología. Facultad de Biología, Universidad de Vigo, As Lagoas, Marcosende, 36310 Vigo (Spain)

Abstract

Aims: The aim of this work was realize a comparative study of two different methods of *Saccharomycec cerevisiae* yeast strain characterization.

Methods and results: Pulsed-field gel electrophoresis analysis (PFGE) and mitochondrial DNA (mtDNA) restriction analysis have been carried out to differentiate strains of *Saccharomyces cerevisiae* associated to *Vitis vinifera* musts from different Galicia wineyard (NW Spain). Seventeen strains isolated from wineries from Galicia were used in this study.

Conclusion: The results have showed that although PFGE analysis technique has greater discriminatory power than mtDNA restriction analysis to detect genetic diversity in *Saccharomyces cerevisiae*, some clones with the same PFGE profile can only be differentiated by mtDNA restriction analysis.

Significance and impact of study: Pulsed-field gel electrophoresis analysis of chromosome (PFGE), by its discriminating power, constitute an ideal technique for the differentiation of *Saccharomyces cerevisiae* strains in biotechnological industries, however, mitochondrial DNA (mtDNA) restriction analysis is a rapid, simple and less expensive and time-consuming method. The results obtained demonstrate the value of using molecular genetic methods in taxonomic and ecological surveys.

Key words: characterization, mtDNA, PFGE, *Sacharomyces cerevisiae*, *Vitis vinifera*

Résumé

Objectif : Le but de ce travail était de réaliser une étude comparative de deux différentes méthodes de la caractérisation de souches de *Saccharomyces cerevisiae.*

Méthodes and résultats : On a réalisé une électrophorèse en champ pulsée (PFGE) et une analyse de restriction mitochondrial du DNA (mtDNA)pour différencier des souches de *Saccharomyces cerevisiae* associés au moût de *Vitis vinifera* de différents vignobles de la Galice. Dix-sept souches isolées de vignobles de la Galice ont été utilisées dans cette étude.

Conclusion : Les résultats montrent que bien que l´électrophorèse en champ pulsée PFGE analyse technique ait un plus grand pouvoir discriminant que mtDNA, pour détecter une diversité génétique dans *Saccharomyces cerevisiae*, quelques clones avec le même profil par PFGE peuvent être seulement différenciés par mtDNA.

Signification et impact de l'étude : PFGE analyse technique, par son pouvoir discriminante, constitue une technique idéale pour différencier souches de *Saccharomyces cerevisiae* dans des industries biotechnologiques, mais l´analyse de restriction mitochondrial du DNA (mtDNA) est une méthode rapide, simple et moins coûteux. Les résultats obtenus démontrent la valeur d'utiliser des méthodes de génétique moléculaire dans taxonomie et des études écologiques.

Mots clés : caractérisation, mtDNA, PFGE, *Sacharomyces cerevisiae*, *Vitis vinifera*

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*Corresponding author: ;mvilanova@mbg.cesga.es

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INTRODUCTION

Several species of yeast are predominant in the vineyard environment. Yeast associated with grape skin depends on grape variety, vintage and degree of grape maturation. These species from grape constituted the predominant microbiota in must and they developed during the first stages of the process of fermentation (Pretorius, 2000).

The transformation of must into wine is a complex microbial process in which different *Saccharomyces* spp. strains take part, amongst the yeast and bacterial species (Ribereau-Gayon *et al*., 2000; Fleet, 2003). In spontaneous fermentations, there is a progressive growth pattern of indigenous yeast, with the final stages invariably being dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*, universally known as the « wine yeast » (Pretorius, 2000). *S. cerevisiae* strains during spontaneous alcoholic fermentation contribute to the qualities of the resulting wine (Lurton *et al*., 1995) and brings forth the major changes between grape must and wine: modifying aroma, flavour, mouth-feel, colour and chemical complexity (Swiegers *et al*., 2005). Several studies shown the diversity of metabolite production in wine by different yeast (Lambrechts and Pretorius, 2000; Romano *et al.* , 2003; Fleet, 2003) and by different yeast strain of *Saccharomyces cerevisiae* (Mateo *et al.*, 2001; Nurgel *et al.*, 2002; Vilanova and Masneuf, 2005)

Different molecular methods for wine yeast strain differentiation have been used in enological strains of *S. cerevisiae* characterization. Polymerase chain reaction (Degre *et al*., 1989), pulsed-field gel electrophoresis analysis (PFGE) (Vezinhet *et al.*, 1990; Cocolin *et al.*, 2004), mitochondrial DNA (mtDNA) restriction patterns (Vilanova and Masneuf 2005; Dubourdieu *et al.*, 1984; Blanco *et al.*, 2006) or RiboPrinter® microbial characterization (Arvik *et al.*, 2005) have showed a genetic variability among isolates of the *S. cerevisiae*.

In this study, we have carried out a comparative study of two different methods of yeast strain characterization (pulsed-field gel electrophoresis analysis (PFGE) and mitochondrial DNA (mtDNA) restriction patterns in order to study the genetic diversity of the isolated strains and to determinate the discriminatory power to differentiate among *S. cerevisiae* yeast strains obtained from Galicia (NW Spain).

MATERIAL AND METHODS

1. Yeast strains

Samples were taken aseptically in musts from different Galicia wineyard (NW Spain). After adequate dilution in sterile water, samples were spreaded on plates containing YEPD (1 % yeast extract, 2 % peptone, 2 % glucose and 2 % agar). Plates were incubated at 28 ºC for 2-3 days. Lysine medium was used to distinguish *S. cerevisiae* from non-*Saccharomyces* strains (Heard and Fleet, 1986; Briones *et al*., 1996). The yeast strains were grown in YEPD medium and the colonies obtained were replicated in Lysine agar medium. A total of seventeen clones of *S. cerevisiae* were analysed in this study.

2. Pulsed-field gel electrophoresis analysis (PFGE)

After eight hours of growth at 30 ºC on YEPD medium, chromosomal DNA was prepared in agarose plugs following the method of Bellis *et al.* (1988). Yeast chromosomes were separated by PFGE. A Pharmacia-LKB (Pulsaphor) apparatus based on the CHEF principle (Dubourdieu and Frezier, 1990) was used in the following conditions: 0.8 % agarose gel, migration at 10 ºC, 1xTBE (Tris Sigma 7-9 90 mM, boric acid 90 mM, EDTA 2mM pH 8) as the migration buffer, 165 volts, and a pulsed time program of 90 sec for 20 h, 100 sec for 12 h, 120 sec for 12 h, 30 sec for 4 h.

3. Mitochondrial DNA restriction patterns

DNA extraction and determination of mtDNA restriction patterns of the strains were carried out as previously described by Querol *et al.* (1992) with modifications. Yeast cells were grown in 5 ml YEPD culture (1 % yeast extract, 2 % peptone, 2 % glucose). Cells were centrifuged and suspended in 0.5 ml of 1 M sorbitol, 0.1 M EDTA pH 7.5. They were then transferred to a 1.5 ml microcentrifuge tube, with the addition of 0.02 ml of Zymolyase 20T (7.5 mg/ml). Tubes were incubated at 37 ºC for 45 min. Spheroplasts were pelleted for 5 min in a microfuge and suspended in 0.5 ml of 50 mM Tris-HCl, 20 mM EDTA pH 7.4. After suspension, 0.05 ml of 10 % SDS was added, and the mixture incubated at 65 °C for 10 min. Immediately, 0.2 ml of 5 M potassium acetate was added and the tubes were placed on ice for 15 min. Then, they were centrifuged at maximum speed in a microfuge for 10 min. The supernatant was transferred to a fresh microfuge tube, and the DNA was precipitated by adding 1 vol of isopropanol, incubating at room temperature for 5 min, then centrifuging for 10 min. The DNA was washed with 70 % ethanol, vacuum dried and redissolved in 40 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) pH 7.4. DNA samples of 3-7 µg were digested 2 h at 37 °C with restriction endonucleases: HinfI (to stablish the different *Saccharomyces cerevisiae* strains) and RsaI to confirm the specie of *Saccharomyces* (Boehringer Mannheim). DNA restriction fragments were separated on 1 % agarose gel for 2 h at 100 V in 1xTris-sodium borate-EDTA buffer (TBE).

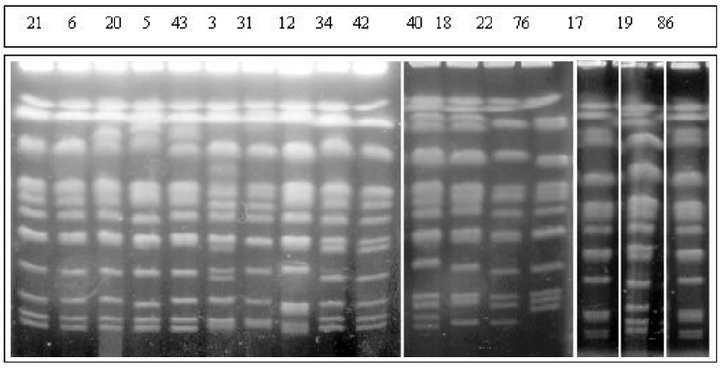

Figure 1 - Karyotype patterns by pulsed-field gel electrophoresis (PFGE) of 17 *Saccharomyces cerevisiae* yeast strains from Galicia.

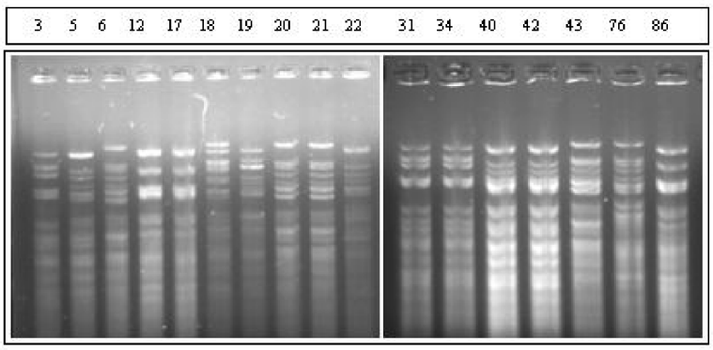

Figure 2 - mtDNA patterns of 17 *Saccharomyces cerevisiae* yeast strains from Galicia. DNA was digested with *Hinf*I.

RESULTS AND DISCUSSION

A comparative study of two different methods of yeast strain characterization was carried out in order to determine the more appropriate to detect the genetic diversity between the *S. cerevisiae* strains isolated from Galician vineyard. Seventeen clones, isolated from a Galician winery were the grapes came from different vineyards separated between them at least 20 km, were used in this study. These isolates were subjected to preidentification by evaluating their inability to use lysine as sole nitrogen source. The strains studied concern to genus *Saccharomyces* because it doesn't assimilate lysine (Briones *et al.*, 1996). A mtDNA restriction analysis was carried out with endonuclease RsaI to confirm the specie of the genus *Saccharomyces*. According to Guillamon *et al.* (1994), the mtDNA restriction patterns obtained with this enzyme reveal species-specific restriction fragments. From these results, all the isolates analysed were identified as *S. cerevisiae*.

In order to differentiate among the seventeen *S. cerevisiae* clones isolated from Galician must, two molecular methods were used. PFGE separates the most part of chromosomes of *S. cerevisiae*. The application of this technique allows differentiating each strain by its specific chromosomal pattern. The figure 1 shows the chromosomal pattern of the seventeen strains used in this study. The electrophoretic karyotyping analysis showed nine different karyotype patterns.

A second method was used to compare the discriminatory power, the mtDNA restriction patterns. The results of the restriction analysis of the mtDNA purified according Querol *et al.* (1990) and digested with restriction endonucleases HinfI are showed in figure 2. The mtDNA showed seven different restriction patterns for the seventeen strains analysed.

Both methods have been applied by different authors (Nadal *et al.*, 1996; Esteve-Zaroso *et al.*, 2001) to detect the genetic diversity of *S. cerevisiae*. mtDNA restriction analysis is a rapid, simple and less expensive and time-consuming method, also very appropriated for monitoring selected strains in controlled fermentations. PFGE is more complex and time-consuming technique requiring

Table 1 - mtDNA restriction patterns and karyotypes by PFGE for 17 *Saccharomyces cerevisiae* yeast strains.

Strain	Karyotype (PFGE)	mitDNA pattern
RBX-3	**A**	**I**
RBX-31	**A**	**I**
RBX-34	**B**	**I**
RBX-5	**C**	**II**
RBX-6	**D**	**III**
RBX-20	**E**	**III**
RBX-21	**D**	**III**
RBX-43	**F**	**III**
RBX-76	**G**	**III**
RBX-12	**H**	**IV**
RBX-17	**H**	**IV**
RBX-86	**H**	**IV**
RBX-18	**G**	**V**
RBX-19	**B**	**VI**
RBX-22	**A**	**VII**
RBX-40	**I**	**VII**
RBX-42	**B**	**VII**
Number of different strains	***9***	***7***

expensive equipment, which however, shows greater discriminating power, making it better suited for the detection of genetic diversity in wine strains. Other authors (Arvik *et al.*, 2005) have developed a different others method to detect yeast diversity (RiboPrinter® microbial characterization system for use with *Saccharomyces cerevisiae* yeast). They use alternative probes based on specific multi-copy gene families. However, polyploidy in industrial yeast strains was the main limitation in the use of most probes.

In this study, the karyotypes obtained by PFGE and mtDNA restriction patterns were used to study the genetic diversity of *S. cerevisiae* isolated from different vineyards of Galicia. Table 1 shows the comparative results between both methods. PFGE grouped the isolated strains into nine different profiles (A-I). When the 17 isolates were differentiated by and mtDNA restriction patterns 7 different types were found (I-VII). The karyotyping technique therefore has greater discriminatory power (it can detect a greater diversity of strains). Similar results were showed by Martínez (2004) among wild isolates of wine-associated *S. cerevisiae* yeast obtained from three South American countries. However, in our case some isolates sharing the same PFGE profile have showed a different mtDNA restriction analysis pattern (table 1). Taking this into account we can conclude that the two methods should be used with the purpose of detecting more diversity.

CONCLUSION

PFGE and mtDNA restriction analysis were two efficient techniques to differentiate between *Saccharomyces cerevisiae* yeast clones isolated from Galician vineyards (NW Spain). PFGE analysis technique showed greater discriminatory power than mtDNA restriction analysis. However, some clones with the same PFGE profile could only be distinguished by mtDNA restriction analysis. So both techniques should be used in order to detect more diversity between *S. cerevisiae* clones.

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