Journal of Engineering and Technology Research Vol.1 (4), pp. 068-071, July 2009
Available online at http:// www.academicjournals.org/JETR
© 2009 Academic Journals

Full Length Research Paper

Restriction analysis of rDNA regions to differentiate non-*Saccharomyces* wine species in mixed cultures

Capece A, Siesto G, Romaniello R and Romano P*

Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy.

Accepted 15 June, 2009

The control of wine quality is related also to the knowledge of composition and dynamics of yeast-flora occurring throughout the vinification process. The restriction analysis of NTS and ITS regions was used to differentiate in mixed fermentation non-Saccharomyces species frequently encountered in the early fermentation phase, such as Hanseniaspora uvarum, Candida stellata and Metschnikowia pulcherrima. PCR-RFLP analysis of ITS region allowed to identify all the species inoculated in the grape must. The novelty of the protocol used is the application of PCR-RFLP analysis on yeast DNA isolated directly from grape must fermentation, without previous yeast colony isolation.

Key words: Non-*Saccharomyces* yeasts, NTS region, yeast mixed cultures.

INTRODUCTION

Although *Saccharomyces cerevisiae* is considered the principal actor of grape must transformation, the growth of non-*Saccharomyces* yeast species during the early fermentation phase plays an important role on wine quality, influencing the chemical composition as well as the organoleptic properties of the wine (Egli et al., 1998; Romano, 2002). Some species are responsible for the formation of fermentation products of oenological interest, whereas others are usually considered as wine spoilage yeasts, being producers of undesirable levels of secondary compounds. To acquire more information about the dynamic of wine yeast populations becomes of oenological interest in order to guarantee a regular fermentation course.

In this perspective, non-*Saccharomyces* yeasts assume a particular importance when, in addition to their wide dominance in the early fermentation phase, they dominate overall the process limiting *S. cerevisiae* activity and, consequently, affecting considerably the final organoleptic quality of wine (Lambrechts and Pretorius, 2000; Romano et al., 2003). Therefore the rapid recognition of the yeast species involved in wine fermentation can avoid the predominance of undesirable species (Pretorius et al., 1999).

The ability to isolate and identify yeasts is fundamental to understanding their occurrence and significance in foods and beverages. Although cultural procedures remain basic to these needs, molecular methods are making the study of yeast ecology much more attractive and convenient than ever before (Beh et al., 2006). Whereas the yeast identification once required the laborious completion of 80 to 100 morphological, biochemical and physiological analyses, this task is now quickly achieved by DNA sequencing. Although sequencing of ribosomal genes is now the accepted method for yeast identification, restriction fragment length polymorphism (RFLP) analysis of the ITS1-ITS2 region (called as 5.8S-ITS) is a less expensive and faster alternative. In addition, databases containing the results of such analyses have been established and are available for food yeasts (Fernandez-Espinar et al., 2006).

The restriction patterns of the 5.8S-ITS region have been used by numerous authors (Egli and Henick-Kling, 2001; Esteve-Zarzoso et al., 1999; Fernàndez- Espinar et al., 2000; Guillamon et al., 1998; Las Heras Vazquez et al., 2003; Sabate et al., 2002; Torija et al., 2001) for wine yeast identification purposes because of its high discriminative capacity, relative ease of manipulation and high reproducibility. The restriction analysis of non-transcribed spacer or NTS region amplified was successfully employed by Capece et al. (2003) to differentiate some wine yeast species. Maoura et al. (2005) analyzed this region, sometimes also known as IGS region, for yeast typing because it has high discriminatory potential, due to the presence of multiple repeats.

In this work the use of the restriction analysis of ampli-

*Corresponding author. E-mail: patrizia.romano@unibas.it. Tel.: +39 0971-205576. Fax: +39 0971-205686.

fied NTS and ITS regions was applied for the identification of a mixed culture of non-*Saccharomyces* wine species, such as *Hanseniaspora uvarum, Metschnikowia pulcherrima* and *Candida stellata*, which are well-known to be the more encountered yeasts in the early fermentation. The rapid identification of these species represents a valid tool for the winemakers allowing the fast individuation of the predominant yeasts during wine fermentation.

MATERIAL AND METHODS

Three wild non-*Saccharomyces* yeast strains, belonging to the collection of Wine Microbiology Laboratory of the Basilicata University were used. The strains were: *H. uvarum* Ka-AG, *M. pulcherrima* Mp-AG and *C. stellata* Cs-AG.

After 24 h growth in YPD medium (1% yeast extract, 2% bacteriological peptone, 2% glucose), the cells were collected for DNA extraction. The isolated DNA of each strain was mixed in the same ratio and the mixed DNA was submitted to amplification of NTS region, following the protocol reported by Capece et al. (2003). The amplification product was visualized on 0.8% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, Steincheim, Germany, USA). The PCR product was digested with the restriction enzyme *Hae*III (Roche Diagnostics, Milan, Italy). The digestion was performed in a final volume of 20 µl, containing 5 µl of amplified rDNA, 2 µl of specific enzyme buffer and 2 U of enzyme, by adding water until the final volume. The reaction mixture was incubated for about 3 h at 37°C and the restriction fragments were separated on a 1.5% (w/v) agarose gel.

The fermentation assay was performed in autoclaved "Aglianico del Vulture" grape must (100°C for 20 min), the typical red variety of Basilicata region, characterized by 21% (w/v) fermentable sugar, 0.70 titratable acidity, pH 3.15. Each strain was inoculated in grape must at a concentration of 10^4 cells per ml, from a pre-culture grown for 48 h in the same must. During the first two fermentation days, 10 ml of fermenting grape must were sampled and centrifuged at 10.000 rpm for 5 min. The recovered pellet was submitted to numerous washings with demineralised water and used for DNA extraction. The DNA was extracted by using Instagen matrix (Bio-Rad), following the suppliers instruction. The yeast DNA isolated from fermenting grape must was submitted to amplification of NTS region, followed by restriction analysis with *Hae*III enzyme, as previously reported. On the same DNA, restriction analysis of the amplified ITS1-5.8S-ITS2 region was performed. The amplification of this region was carried out in 50 µl of reaction mixture containing 10 µl of PCR buffer (5X) (Promega, USA), 4.0 µl of 25 mM MgCl2, 1 µl of 10 mM dNTP (Promega, USA), 1 µl each of forward and reverse primers (ITS1 /ITS4) (20 µM) (White et al., 1990), 0.25 µl (5 U/µl) of Taq DNA polymerase, 5 µl of template DNA, by adding autoclaved deionised water until final volume. The amplification program was started with an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 53°C for 20 s and elongation at 72°C for 1 min. The PCR was ended with a final extension at 72°C for 5 min and the amplified product was cooled at 4°C (Jeyaram et al., 2008). The amplification product was visualized on 1 % agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, Steincheim, Germany, USA). The PCR product was digested with the restriction enzyme *Hae*III, following the protocol above reported for amplification of NTS region.

RESULTS AND DISCUSSION

Analysis of NTS region on mixed DNA

Analysis of NTS region was used to differentiate the three strains of non-*Saccharomyces* species commonly predominant during early fermentation phase. The amplification of this region yielded a single fragment, characterized by a molecular size characteristic of each yeast species analyzed, as previously reported (Capece et al., 2003). The amplification fragment of each individual species was compared with the fragment obtained by the amplification of mixed DNA from *H. uvarum, M. pulcherrima* and *C. stellata.* The amplification of this region on mixed DNA of the three yeast species yielded a single fragment, characterized by an intermediate molecular size compared to the molecular sizes of the fragments from the amplifications on pure cultures of each species tested (Figure 1A).

In order to emphasize the presence of each species in the amplified product, NTS fragment from mixed DNA was submitted to the restriction with endonuclease *Hae*III, in comparison to the restriction profile exhibited by the three strains in pure culture. As reported in Figure 1B, it is possible to note that the profile obtained from the mixed DNA is a composite molecular profile, resulting from the combination of the molecular profiles of the three individual species.

Analysis of NTS and ITS regions on yeast DNA isolated from grape must

In order to confirm the results obtained by mixing DNA template of non-*Saccharomyces* species also in the case of mixed yeast cultures, mixed inoculated fermentation with *H. uvarum*, *C. stellata* and *M. pulcherrima* was performed. During the process, yeast DNA isolation method, based on DNA extraction directly from fermenting grape must, without previously colony isolation on plates, was pointed out. Different extraction protocols were tested, but only the extraction by Instagene matrix yielded DNA suitable to amplification. Isolated DNA was submitted to amplification of NTS region, producing a single band characterized by a molecular size of about 2100 bp (Figure 2, lane 1).

This amplification product was very similar to the fragment obtained from NTS amplification of mixed DNA template. The amplified NTS fragment from mixed cultures was submitted to restriction analysis with *Hae*III enzyme (Figure 2, lane 2). This restriction profile was composed by numerous bands. Table 1 reports the molecular size of fragments obtained both from the single species and from yeast DNA extracted from the fermenting grape must. By comparing the restriction profile of yeast DNA from mixed inoculated fermentation with the restriction profiles of the three individual species (Figure 1B), this pattern resulted comprising the restriction profile of *H. uvarum, M. pulcherrima,* whereas the bands composing the restriction profile of *C. stellata* were absent (Table 1).

The restriction analysis of NTS region of yeast DNA isolated from grape must didn't allow to detect the inoculated *C. stellata* strain. In the case of PCR-RFLP of NTS region, the results obtained by mixing artificially the isolated DNA, were not confirmed by PCR-RFLP analysis on

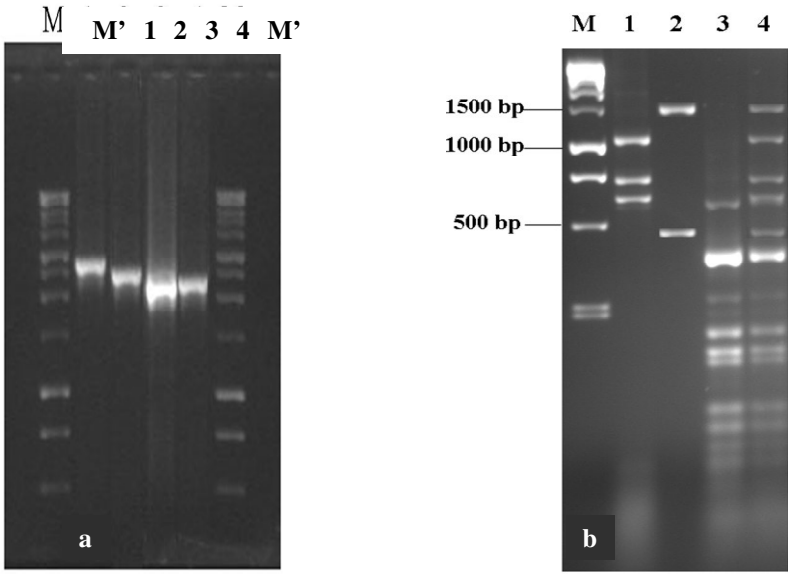

Figure 1. A: PCR-amplified NTS region of individual and mixed DNA, M' = molecular marker 1 kb DNA ladder (Promega); B: Restriction analysis with *Hae*III of NTS amplicons obtained from individual and mixed DNA, M = molecular marker, 100 bp DNA ladder (Promega). Non-*Saccharomyces* species: 1: *C stellata*; 2: *M. pulcherrima*; 3: *H. uvarum*; 4: mixed DNA.

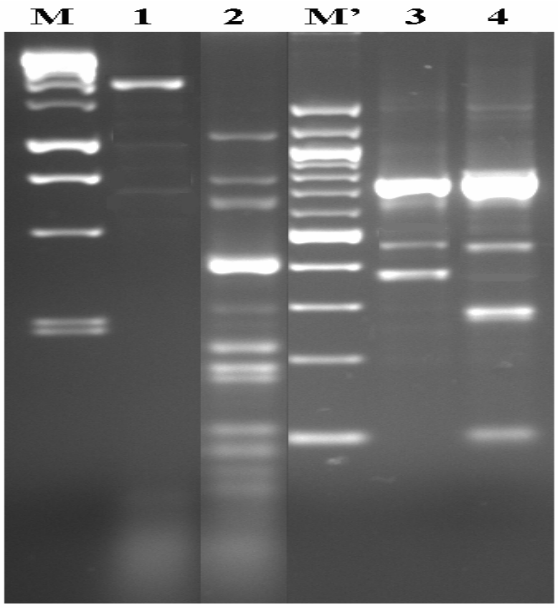

Figure 2. PCR-amplified and restriction analysis with *Hae*III of NTS region (lanes 1, 2) and ITS region (lanes 3,4) of yeast DNA isolated from fermenting grape must. M = molecular marker 1 kb DNA ladder (Promega); M' = molecular weight marker, 100 bp DNA ladder (Promega).

yeast DNA isolated from fermenting grape must inoculated with the three non-*Saccharomyces* species. Therefore, in an attempt to overcome this problem, another fragment of yeast rDNA region was isolated from grape must and submitted to amplification of ITS region, followed by restriction with *Hae*III. The amplification of ITS region from grape must DNA yielded three individual bands, characterized by the molecular sizes typical of the three inoculated species. In fact, the molecular sizes of each band were: 740, 470 and 380 bp approximately (Figure 2, lane 3), typical of *H. uvarum, C. stellata* and *M. pulcherrima* respectively (Esteve-Zarzoso et al., 1999). This result was confirmed by the restriction of ITS fragment with *Hae*III enzyme (Table 1).

In fact, when restriction analysis was applied to PCR product derived from mixed yeast species, a composite pattern was obtained, clearly derived from the combination of the individual species patterns. Figure 2 lane 4 shows the pattern resulted by restriction on ITS fragment of yeast DNA from grape must. This profile is composed by 4 bands, resulting by the combination of restriction profile of each inoculated species. As a result, PCR-RFLP analysis of ITS region allowed to identify all the species inoculated in the grape must. The amplification of yeast DNA isolated from grape must yielded three distinct ITS amplicons, each corresponding to the expected size (Table 1), indicating that no interference occurred during amplification of the target sequences of the three yeast species. Other authors reported similar results (Granchi et al., 1999), regarding the effectiveness of RFLP-ITS region in the detection of mixed colonies, although their results were obtained by amplification of mixed pure colonies, after isolation on plates. In our case, the same technique was applied on yeast DNA isolated from grape must inoculated with three non-*Saccharomyces* species, which imitates the early stages of spontaneous grape must fermentation.

The novelty of this protocol for PCR-RFLP analysis is

Table 1. Size in bp of fragments obtained with *Hae*III restriction of NTS and ITS regions of single yeast species and yeast DNA extracted from fermenting grape must.

Yeasts	Restriction fragments (bp)	
	NTS	ITS
M. pulcherrima	620,400, 280, 240, 235, 210, 175, 165, 100	280, 100
C. stellata	1700, 500	470
H. uvarum	1100, 740, 645	740
Yeast DNA from grape must	1100, 740, 645, 620, 400, 280, 240, 235, 210, 175, 165, 100	740, 470, 280, 100

the application on yeast DNA isolated directly from grape must fermentation, without previous yeast colony isolation. The species-specific profiles obtained from the restriction analysis represent an useful technique for the rapid identification of *H. uvarum, C. stellata* and *M. pulcherrima*, the most frequently non-*Saccharomyces* species, encountered in the early stages of grape must fermentation. Further experiments are in progress at pilot scale fermentation in order to evaluate the applicability of this method in real conditions, also testing other minor non-*Saccharomyces* yeast species. Taking into account that the persistence and dominance of non-*Saccharomyces* yeasts during the vinification process can cause stuck or sluggish fermentation, the availability of a rapid method to detect these species is of practical interest for the wine industry.

It must be underlined that nowadays innovative techniques able to give amplification results at quantitative level and in real time, such as PCR Real Time, are available, but their application needs expensive instruments and specialized operators. Conversely, the method pointed out during this study represents a fast and cheap technique, which might be used also at cellar level.

ACKNOWLEDGEMENT

This research was supported partly by funds from Italian Agricultural Ministry, project D.M.35/7303/06 .

REFERENCES

Beh AL, Fleet GH, Prakitchaiwattana C,Heard GM (2006). Evaluation of molecular methods for the analyses of yeasts in foods and beverages. In: Advances in Food Mycology, Hocking AD, Pitt JT, Samson RA, Thrane U (eds), Springer pp. 69-106.

Capece A, Salzano G, Romano P (2003). Molecular typing techniques as a tool to differentiate non-*Saccharomyces* wine species. Int. J. Food Microbiol. 84: 33-39.

Egli CM, Henick-Kling T (2001). Identification of *Brettanomyces/ Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. Am. J. Enol. Vitic. 52: 241–247.

Egli CM, Edinger WD, Mitrakul CM and Henick-Kling T (1998) Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J. Appl. Microbiol. 85: 779-789.

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999).Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49: 329–337.

Fernandez-Espinar JT, Martorell P, de Llanos R, Querol A (2006). Molecular methods to identify and characterise yeasts in foods and beverages. In: Yeasts in Food and Beverages, Querol A, Fleet GH (Eds), Springer pp.55-82.

Fernàndez-Espinar MT, Esteve-Zarzoso B, Querol A, Barrio E (2000). RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and differentiation of flor yeasts. Antonie van Leeuwenhoek 78: 87–97.

Guillamon JM, Sabate J, Barrio E, Cano J, Querol A (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Archiv. Microbiol. 169: 387-392.

Jeyaram K, Singh WM, Capece A, Romano P (2008). Molecular identification of yeast species associated with 'Hamei'--a traditional starter used for rice wine production in Manipur, India. Int. J. Food Microbiol. 124:115-25.

Lambrechts MG, Pretorius IS (2000).Yeast and its importance to wine aroma-A review. S. Afr. J. Enol. Vitic. 21: 97-128.

Las Heras-Vazquez FJ, Mingorance-Cazorla L, Clemente-Jimenez JM, Rodriguez-Vico F (2003). Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. FEMS Yeast Res. 3: 3-9.

Maoura N, Mbaiguinam M, Nguyen HV, Gaillardin C, Pourquie J (2005). Identification and typing of the yeast strains isolated from bili, a traditional sorghum beer of Chad. Afr. J. Biotech. 4 (7): 646-656.

Pretorius IS, Van der Westhuizen TJ, Augustyn OPH (1999). Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. S. Afr. J. Enol. Vitic. 20: 61-74.

Romano P (2002) Role of apiculate yeasts on organoleptic characteristics of wine. In: Ciani M (Ed.) Biodiversity and Biotechnology of Wine Yeasts, Research Signpost, Kerala India, pp. 99–109.

Romano P, Fiore C, Paraggio M, Caruso M, Capece A (2003). Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. 86: 169-80.

Sabate J, Cano J, Esteve-Zarzoso B,Guillamo´n JM (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiol. Res. 157:1-8.

Torija MJ, Roze´s N, Poblet M, Guillamo´n JM, Mas A (2001). Yeast population dynamics in spontaneous fermentation: Comparison between two different wine-producing areas of a period of three years. Antonie van Leeuwenhoek 79: 345-352.