EVALUATION OF FERMENTING GRAPE MUST YEAST DYNAMICS BY SSCP PROFILES

AVALIAÇÃO DA DINÂMICA DE LEVEDURAS PRESENTES NA FERMENTAÇÃO DE MOSTOS POR PERFIS DE SSCP

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SUMMARY

Alcoholic fermentation of grape must is a dynamic process involving diverse yeast populations which play an important role in wine characteristics. In order to enable a permanent control by the oenologist rapid and low cost methodologies to identify the species of yeasts present during fermentation should be implemented. Single Strand Conformation Polymorphism technique (SSCP), targeting D1 and D2 domains of 26S rDNA, was tested to differentiate wine yeast species. SSCP profiles were produced for 17 collection strains belonging to 15 different wine related yeast species. The technique was further investigated for the identification of yeasts inoculated to sterile grape must as single or in mixtures of two different species. Identical SSCP profiles were obtained from yeasts grown in grape must and in conventional growth media. SSCP allowed obtaining species specific bands, and SSPC profiles from grape must inoculated with mixtures of two strains revealed the presence of specific bands of both species. Direct SSCP yeast analysis of a spontaneous fermenting must carried out after 48 and 192 h of fermentation, enabled to compare the yeast community at two different periods of fermentation. SSCP profiles showed the presence of bands from species associated with the beginning and the end of fermentation, respectively at 48 and 192 h of fermentation. We conclude that SSCP is a quite promising methodology to monitor yeast populations present during grape must fermentation, which revealed to be easy to implement and low-priced.

RESUMO

A fermentação alcoólica do mosto de uva é um processo dinâmico que envolve diversas populações de leveduras que desempenham um papel importante nas características do vinho. A fim de permitir um controle permanente pelo enólogo urge implementar metodologias rápidas e de baixo custo, para identificação das espécies de leveduras envolvidas na fermentação. O método de Polimorfismo de Conformação do DNA de Cadeia Simples (SSCP) visando os domínios D1 e D2 da região 26S do rDNA foi testado para diferenciar espécies de levedura associadas ao vinho. Foram determinados perfis de SSCP para 17 estirpes de coleção pertencentes a 15 espécies diferentes associadas a ambientes vínicos. A técnica foi depois testada para a identificação de leveduras inoculadas em mosto estéril utilizando uma única espécie ou em misturas de duas espécies diferentes. Foram obtidos perfis idênticos de SSCP a partir de leveduras cultivadas em mosto de uva e em meios de cultura convencionais. A análise de SSCP permitiu a obtenção de bandas específicas de cada espécie, e os perfis de SSPC obtidos a partir de mosto de uva inoculado com misturas de duas estirpes de espécies diferentes revelou a presença de bandas específicas de ambas as espécies. A análise direta por SSCP das leveduras presentes numa fermentação espontânea, realizada após 48 horas e 192 h de fermentação, permitiu comparar a comunidade de leveduras em dois períodos diferentes de fermentação. Os perfis de SSCP revelaram a presença de bandas de espécies associadas com o início e com o fim da fermentação, respetivamente às 48 e às 192 h de fermentação. Em conclusão, a análise SSCP é uma metodologia bastante promissora para monitorizar as populações de leveduras presentes durante a fermentação, que se mostrou ser de fácil aplicação e de baixo custo.

INTRODUCTION

Yeasts have a primordial role in wine quality. The presence of different populations of yeast species during grape must fermentation, results in the production of different compounds or different compound proportions, which determines the sensorial characteristics of the produced wine (Ciani and Maccarelli, 1998; Jolly *et al*., 2006; Ciani and Comitini, 2011). The knowledge of which yeast species are involved, as well as their dynamics is thus a key factor to control wine quality.

Molecular biology based methods have been described for the rapid identification of yeast species. Most of these methods such as RAPD, PCR fingerprinting and restriction enzyme analysis of rDNA fragments rely on PCR amplification (Baleiras Couto *et al*., 1995, 1996, 2005). Nevertheless, these methods require previous isolation and cultivation of single cultures which is very laborious and time consuming. When diversity and dynamics of microbial communities are to be studied, culture independent methods are recommended (Callon *et al*., 2006; Mounier *et al*., 2009). Methods such as Single Strand Conformation Polymorphism (SSCP), Real Time PCR (RT-PCR), Denaturing Gradient Gel Electrophoresis (DGGE) or Fluorescence in situ hybridization have been developed in order to allow species identification directly from natural substrates (Cocolin *et al*., 2000, 2002; Peters *et al*., 2000; Prakitchaiwattana *et al*., 2004; di Maro *et al*., 2007; Renouf *et al*., 2007; Andorrà *et al*. 2008, 2012; Mounier *et al*., 2009).

Compared to other methods SSCP revealed to be less expensive, does not involve complex equipment nor limits the number of species to be identified. SSCP also enables further analysis of individual bands

which can be excised for identification by sequencing or reverse transcription PCR followed by sequencing when SSCP is performed over RNA (MacGregor and Amann, 2006). SSCP technique has shown high discriminative power in diverse types of studies, enabling the recognition of medically important opportunistic fungi (Walsh *et al.*, 1995) the profiling of microbial communities from estuarine sediment and hypersaline microbial mats (MacGregor and Amann, 2006) and in the differentiation of representative groups of yeast species from ascomycetes and basidiomycetes (Wang *et al.*, 2008). Yeasts diversity and population dynamics in fermentation of diverse food products has also been evaluated by SSCP analysis (Chamkha *et al.*, 2008; Chang *et al.*, 2008; Mounier *et al.*, 2009). Due to the adequacy of ribosomal DNA to yeasts phylogenetic inferences, the above mentioned works rely on fragments from rRNA gene or rRNA to perform SSCP analysis. The sequence of D1/D2 domains of 26S rDNA has been used for yeast identification (Kurtzman and Robnett, 1998). Previous works on the identification of wine yeasts from different environments have shown that sequencing of these domains or restriction analysis of fragments comprising these domains was very accurate (Baleiras-Couto *et al.*, 2005; Zanol *et al.*, 2010; Baleiras-Couto *et al.*, 2012).

The aim of this work was to investigate the use of SSCP analysis to differentiate wine yeast species and to evaluate the adequacy of the method to fermenting must in order to establish a rapid and inexpensive tool to survey yeast population dynamics during fermentation. SSCP analysis of 26S rDNA amplified fragments (D1 and D2 domains) was performed to characterise 17 different collection yeast strains belonging to 15 wine associated species. SSCP analysis was also carried out directly to grape must inoculated with cultures of one or two yeast species. Additionally the applicability of SSCP analysis was tested in grape must after 48 and 192 h of spontaneous fermentation in order to compare the yeast community present at two different periods of fermentation.

MATERIAL AND METHODS

Yeast strains

The yeast strains used in this study are included in Colecção de Microrganismos EVN (EVN) and were obtained from other culture collections, representing 15 wine related species, as indicated in Table I.

Cells culture

To determine the SSCP profiles of each species in study, yeast cells were grown in conventional agar medium (YPD agar - yeast extract, peptone, dextrose, agar) at 25 °C.

For cells growth in grape must a loop full of cells was added to test-tubes with 10 mL sterile grape must and incubated at 25 °C during 48 h without shaking. When cell numbers reached 5×10^6 per mL SSCP profiles of single or mixtures of equal volumes of two cultures were determined.

DNA extraction and PCR amplification

Cells grown on YPD agar were disrupted according to Sampaio *et al.* (2001). DNA was further purified with chlorophorm:isoamyl alcohol (24:1), precipitated with 1/10 volume of sodium acetate (3 M, pH 5.2) and two volumes of absolute ethanol. Further incubation with RNase (100 µg/mL in TE: 10 mM tris-HCl and 1 mM EDTA, pH 8) was performed for 30 min, at 37 °C and additional purification and precipitation steps were carried out as described above. DNA was washed with ethanol (70 %, v/v), dried and ressuspended in TE.

For DNA extraction from cells grown on grape must, 2 mL of must with approximately 5×10^6 cel/mL was centrifuged at 18.200 g at 4 °C during 10 min. The pellet was washed with NaCl solution (9 g/L) and ressuspended in 500 µL lyses buffer (Tris 1 M; EDTA 0,25 M; NaCl 5 M; CTAB 0,5 mM; pH 7,5) added with 120 µL of 5% (w/v) N-lauroylsarcosine sodium solution. Then 200 µL glass beads (425-600 µm Ø) were added, vortexed at maximum speed for 1 min and left for 15 min at 65 °C. DNA was purified with chlorophorm:isoamyl alcohol (24:1), precipitated with isopropanol and washed with ethanol (70 %, v/v). Incubation with RNase was also performed and the DNA was precipitated with sodium acetate and isopropanol at -20 °C overnight. DNA was washed with ethanol (70 %, v/v), dried and ressuspended in TE.

For the amplification of D1/D2 domains of 26S rDNA, PCR conditions were similar to those described by Zanol *et al.* (2010) using 1 ng of total DNA and primers NL1 (5'-GCATATCAATAAGC-GGAGGAAAAG-3') and NL4 (5'-GGTCCGT-GTTTCAAGACGG-3').

SSCP analysis

To denature the amplified fragment, 4 µL of PCR product were mixed with an equal volume of denaturing formamide solution (containing 1 mg/mL xylene cyanol, 1 mg/mL bromophenol blue, 20 µL/mL EDTA 0,5 M). The reaction mixture was denatured by heating at 95 °C for 5 min in a thermocycler (T Gradient 96 cycler, Whatman-Biometra, Gottingen, Germany) and immediately cooled on ice directly before use. Eight µL of the sample were loaded into each well of a non-denaturing 5 % acrylamide:bis (30:1) gel containing 5 % (v/v) glycerol casted on a Mighty Small II apparatus from Hoefer/Pharmacia (8 x 7 cm and 0.75 mm thick). DNA migration occurs from denaturing (loading solution) to non-denaturing (polyacrylamide gel) conditions. A standard DNA

TABLE I
Strains studied and their sources of isolation.
Estirpes estudadas e respetivas fontes de isolamento.

| Species | Acession number [1] | | Substrate of origine |
	EVN [2]	Other collections [3]	
Candida cantarelli	365[T]	PYCC 3073[T]	Must
Candida stellata	387[T]	CBS 157[T]	Grapes
Debaryomyces hansenii var. hansenii	329[T]	PYCC 2968[T]	Beer
Dekkera anomala	330[T]	PYCC 5153[T]	Stout beer
Dekkera bruxellensis	333[T]	PYCC 4179[T]	Lambic beer
Hanseniaspora uvarum	394[T]	CBS 314[T]	Muscatel grape
Metschnikowia pulcherrima	375[T]	PYCC 4830[T]	Berries of *Vitis labrusca*
Pichia kluyveri	307	PYCC 5158	*Tagelus plebeus* (clam)
Pichia membranifaciens	284[T]	PYCC 3796[T]	Ulmus tree exsudates
	285	PYCC 3315	Wine
Saccharomyces bayanus	271[T]	PYCC 4456[T]	Turbid beer
Saccharomyces cerevisiae	252[NT]	PYCC 4455[NT]	Brewer's top yeast
	251	PYCC 4072	Commercial wine yeast
Schizosaccharomyces pombe var. pombe	396[T]	CBS 356[T]	Unknwon
Starmerella bacillaris	367[T]	PYCC 3044	Grapes
Torulaspora delbrueckii	326[NT]	PYCC 2477[NT]	Unknwon
Zygosaccharomyces bailii var. bailii	226[T]	PYCC 5167[T]	Beer

[1] T, type strain; NT, neotype.

[2] EVN, Microorganisms Culture Colection EVN, INIAV, Dois Portos, Portugal.

[3] CBS, Centraalbureau voor Schimmelcultures, Delft. Holland; PYCC, Portuguese Yeast Culture Collection, Univ. Nova de Lisboa, Mte. Caparica, Portugal.

non-denatured marker (1 kb DNA Ladder, MBI Fermentas) was loaded on the first and the last wells of the gel, and used as a reference to determine the relative positioning of the SSCP bands, as well as the double stranded DNA band.

Electrophoresis was performed in 1x TBE (Tris-borate-EDTA) buffer, run at 150 V, at 4 ºC until migration of xylene cyanol dye reached 0.5 cm of the bottom of the gel. The gels were silver stained and photographed. The relative position of SSCP bands, was determined using GelCompar II software, version 5.1 (Applied Maths, Saint-Martens-Latem, Belgium). Gels were repeated at least three times and the bands present on all the gels were attributed to the species.

RESULTS AND DISCUSSION

Analyses of single-strand conformation polymorphism (SSCP) enable DNA fragments with similar size but different sequence to be separated according to their configuration - secondary structure (Hebenbrock *et al.*, 1995). Several regions of the rRNA gene have proved adequate to discriminate yeast species, namely the D1/D2 domains of 26S rDNA, which was selected to be used in the present work (Kurtzman and Robnett, 1998; Esteve-Zarzoso *et al.*, 1999; Zanol *et al.*, 2010).

In order to initiate a wine yeast species SSCP profile database, 17 culture collection strains belonging to 15 yeast species associated with wine environments were studied. A PCR amplified fragment consisting of the D1 and D2 domains of 26S rDNA was used to perform SSCP analyses. After optimization of procedures, SSCP profiles of each strain grown in YPD agar were generated. Figure 1 shows some of the profiles obtained.

Gels were repeated at least three times from independent cultures. All gels were analyzed and the bands relative positioning determined. Table II shows the values of the SSCP specific bands attributed to each species.

Every profile presented three bands, two of them corresponding to forward and reverse single strand DNA (ssDNA) (Callon *et al.*, 2006). The third band corresponded to double strand DNA (dsDNA) as was verified by electrophoresis of denatured and non-denatured amplified DNA (Figure 2). Although this third band is not ssDNA, it appears in SSCP gels (Wang *et al.*, 2008). This band was considered in the profiles since it presented species polymorphism, improving the discriminative power of this methodology.

Results showed that the SSPC profiles obtained for
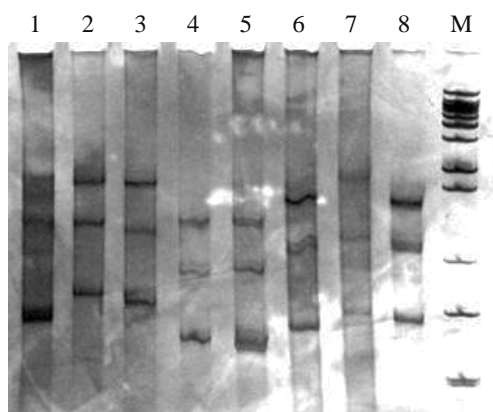
Figure 1 - SSCP profiles of the D1/D2 region of 26S rDNA of EVN culture collection strains. Lanes: 1- *P. kluyveri* (307); 2- *H. uvarum* (394); 3- *Deb. hansenii var. hansenii* (329); 4- *Starmerella bacillaris* (367); 5- *C. stellata* (387); 6- *P. membranifaciens* (284); 7- *Z. bailii var. bailii* (226); 8- *C. cantarelli* (365). M: marker (non-denaturated ds DNA ladder of 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 and 10000 bp).

Perfis de SSCP da região D1/D2 do rDNA 26S de estirpes da coleção EVN. 1- P. kluyveri (307); 2- H. uvarum (394); 3- Deb. hansenii var. hansenii (329); 4- Starmerella bacillaris (367); 5- C. stellata (387); 6- P. membranifaciens (284); 7- Z. bailii var. bailii (226); 8- C. cantarelli (365). M: Marcador (DNA ladder não-desnaturado de 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 e 10000 pb).

TABLE II
SSCP profiles associated to each strain (EVN).
Perfis de SSCP associados a cada estirpe (EVN).

Species	Strain	DNA bands (relative position)
C. cantarelli	365	1300, 1050, 700
P. membranifaciens	284	1300, 1000, 700
P. membranifaciens	285	1300, 1000, 700
C. stellata	387	1200, 900, 600
Starmerella bacillaris	367	1200, 900, 600
Deb. hansenii var. hansenii	329	1500, 1100, 750
D. anomala	330	1400, 1100, 750
D. bruxellensis	333	1400, 1100, 750
H. uvarum	394	1600, 1200, 800
M. pulcherrima	375	1100, 850, 600
P. kluyveri	307	1500, 1200, 700
S. bayanus	271	1500, 1050, 800
S. cerevisiae	251	1800, 1500, 800
S. cerevisiae	252	1800, 1500, 800
Schiz. pombe var. pombe	396	1800, 1200, 750
T. delbrueckii	326	1500, 1050, 750
Z. bailii var. bailii	226	1700, 1100, 750

different strains of the same species, as was tested for *P. membranifaciens* and *S. cerevisiae*, were identical (Table II). A distinct SSCP profile was obtained for the great majority of the species evaluated. Nevertheless, *C. stellata* and *Starmerella bacillaris* presented similar profiles. These two species were for long considered to be conspecific and only recently were clearly differentiated by the sequencing of D1/D2 domains, presenting only 91 % similarity (Duarte *et al.*, 2012). From an oenological point of view this SSCP profile similarity is not a real problem as it has been shown that *C. stellata* is rarely found in wine fermentation unlike *Starmerella bacillaris* which is quite
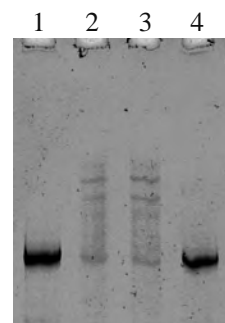

Figure 2 - Gel showing DNA denatured samples (D) side by side with non-denatured samples (ND) from two strains. Lanes: 1- EVN 252 ND; 2- EVN 252 D; 3- EVN 251 D; 4 - EVN 251 ND.

Gel apresentando amostras de DNA desnaturadas (D) lado a lado com amostras não-desnaturadas (ND) provenientes de duas estirpes. 1- EVN 252 ND; 2- EVN 252 D; 3- EVN 251 D; 4- EVN 251 ND.

common in wine associated environments (Csoma and Sipiczki, 2008; Duarte *et al.*, 2012). Similar SSCP profiles were produced by the strains of *D. anomala* and *D. bruxelensis*, although the sequence of D1/D2 domains of 26S rDNA of these two strains presents a similarity of only 93% (Kurtzman and Robnett, 1998). Nevertheless they could be clearly differentiated from the remaining species. Equivalent results were obtained using restriction profile analysis of 26S rDNA (Zanol *et al.*, 2010). The SSCP showed to be quite discriminative for 11 out of 15 species, which can be clearly differentiated from each other and from the species belonging to the *Dekkera* genus and the *Starmerella* clade, *C. stellata* and *Starmerella bacillaris* (Lachance *et al.*, 2011).

The applicability of the methodology was also tested upon yeast growth on grape must. Another DNA extraction procedure had to be optimized as no PCR amplification was obtained with the previous one, due to the presence of PCR inhibiting compounds. After optimizing the DNA extraction methodology as described in Material and Methods, SSCP analysis was performed without further modification. The profiles obtained were identical to those obtained after growth in conventional medium (Figure 3).

SSCP analysis of mixtures of two yeast strains independently grown in grape must was also performed and the results analyzed using GelCompar II software. The SSCP profiles produced revealed that the assayed mixtures presented a combination of bands corresponding to the specific bands of both species. In Figure 4, SSCP profiles from single and mixed cultures of *C. cantarelli* plus *Starmerella bacillaris* and *C. stellata* plus *D. anomala* are presented, and bands from both species can be visualized. The extra bands present might be due to the use of rDNA fragments as markers (Wang *et al.*, 2008). In order to test the applicability of this methodology to real conditions, SSCP analyses were also performed using DNA directly extracted from grape must after

1 394Y 394M 375M 375Y 365M 365Y 387Y 387M
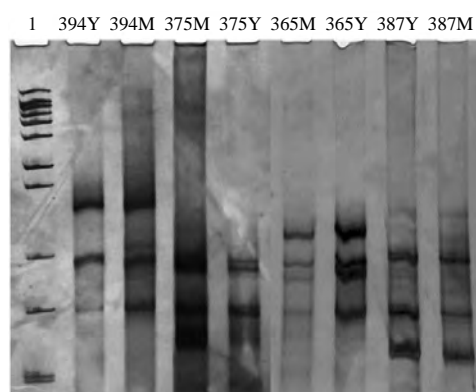
Figure 3 - SSCP profiles of the D1/D2 region of 26S rDNA of EVN culture collection strains grown on YPD agar solid medium (Y) and collected from grape must (M). Lanes: 1 - marker (non-denaturated ds DNA ladder of 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000, 10000 *pb*); 394 – *H. uvarum*; 375 – *M. pulcherrim*; 365 - *C. cantarelli*; 387 - *C. stellata*.

Perfis de SSCP da região D1/D2 do rDNA 26S de estirpes da coleção EVN a partir de cultura em meio convencional (Y) e em mosto (M). 1- Marcador (DNA ladder não-desnaturado de 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 e 10000 pb); 394 – H. uvarum; 375 – M. pulcherrima; 365 – C. cantarelli; 387 – C. stellata.

48 and 192 hours of spontaneous fermentation. The SSCP profiles showed in Figure 5 were compared with those presented by the culture collection strains. The relative position of the detected bands is also presented at Table III. The SSCP profile of the sample collected after 48 h of fermentation showed bands associated to *C. stellata/Starmerella bacillaris* and to *H. uvarum.* After 192 h of fermentation the SSCP analysis generated 7 bands (Table III) which matched the specific bands of *S. cerevisiae, S. bayanus, T. delbrueckii, Deb. hansenii* and *Schizo. pombe* species. From these results, it can be inferred that grape must at the beginning of fermentation was dominated by *Starmerella* clade and *Hanseniaspora* yeasts as observed by others (Fleet, 2003; Baleiras-Couto *et al*., 2005). Grape must sampling after 192 h pointed out to the presence of bands corresponding to species associated with late grape must fermentations (Fleet, 2003; Baleiras Couto *et al*., 2005).

In view of the results obtained, the performance of SSCP to detect wine related yeast species in grape must fermentation was appropriate, leading to its future applicability to study the diversity and dynamics of yeast communities. Likewise, other works using SSCP have been done featuring the study of microbial diversity and population dynamics in various food products such as olive fermentation brine (Chamkha *et al*., 2008), commercial cheeses (Mounier *et al*., 2009), or a Korean food fermented from vegetables (Chang *et al*., 2008). This methodology presents the advantages of overcoming the amount of work associated with the identification of several isolates and the potential biases inherent in microbial enrichment and of detecting viable non-culturable microorganisms (Wang *et al*., 2008). The SSCP can also be used to evaluate the implantation of inoculated non-*Saccha-*

1 2 3 4 5 6 7
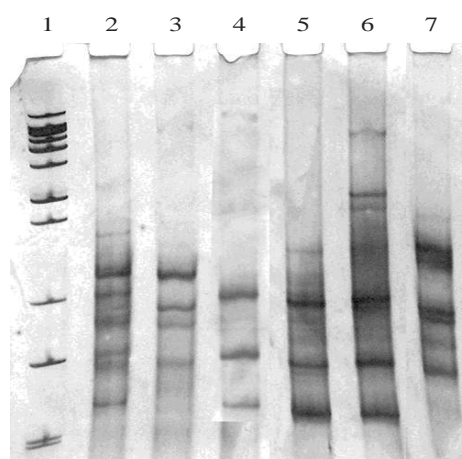
Figure 4 - SSCP profiles obtained from single and mix yeast cultures in grape must. Lanes: 1 - marker (nondenaturated ds DNA ladder of 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 and 10000 bp); 2 – C. cantarelli (365) + Starmerella bacillaris (367); 3 - C. cantarelli (365); 4 - Starmerella bacillaris (367); 5 - C. stellata (387); 6 - C. stellata (387) + D. anomala (330);7 – D. anomala (330).

Perfis de SSCP obtidos por cultura em mosto de uma ou misturas de duas estirpes. 1 - Marcador (DNA ladder não-desnaturado de 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 e 10000 pb); 2 - C. cantarelli (365) + Starmerella bacillaris (367); 3 - C. cantarelli (365); 4 - Starmerella bacillaris (367); 5 - C. stellata (387); 6 - C. stellata (387) + D. anomala (330);7 – D. anomala (330).

1 2 3

Figure 5 - SSCP profiles obtained from grape must during spontaneous fermentation. Lanes: 1 - marker (non-denatured ds DNA ladder of 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 and 10000 bp); 2 – after 48 h; 3 – after 192 h.

Perfis de SSCP obtidos durante a fermentação espontânea de mosto. 1 - Marcador (DNA ladder não-desnaturado de 500, 750, 1000, 1500, 2000, 3000, 3500, 4000, 5000, 6000, 8000 e 10000 pb); 2 – após 48 h; 3 – após 192 h.

TABLE III
SSCP profiles obtained during spontaneous grape must fermentation.
Perfis de SSCP obtidos durante a fermentação espontânea de mosto.

Sample	DNA bands (relative position)
48 h	1600, 1200, 900, 600
192 h	1800, 1500, 1200, 1100, 1050, 800, 750

romyces yeasts which is a promising technology yet at the beginning (Ciani and Comitini, 2011).

CONCLUSIONS

SSCP analysis targeting D1/D2 domains of 26S rDNA proved very discriminative for the yeast species in study. The applicability of this methodology directly to fermenting grape must samples was quite good, revealing similar band definition, thus being appropriate for studying the diversity and dynamics of wine yeasts during fermentation. To our knowledge this is the first time that SSCP was used to compare yeast community during grape must fermentation. Future work is envisaged to enlarge the SSCP profile database that expedites the identification process. This technique presents the advantage of obtaining fast results with less expensive equipment required.

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