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RESTRICTION PROFILES OF 26S rDNA AS A MOLECULAR APPROACH FOR WINE YEASTS IDENTIFICATION

IDENTIFICAÇÃO DE LEVEDURAS DE INTERESSE ENOLÓGICO POR PERFIS DE RESTRIÇÃO DO ADNr 26S

Geni C. Zanol *, M. Margarida Baleiras-Couto, Filomena L. Duarte

Instituto Nacional de Recursos Biológicos, I.P./ INIA Dois Portos, Quinta d'Almoínha 2565-191, Dois Portos, Portugal
*Corresponding author: geni.zanol@inrb.pt

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SUMMARY

The complex microbial ecosystem existing in grape, must and wine comprises a wide diversity of yeast species. The knowledge of composition and dynamics of yeast biota occurring along vinification process would provide a better control of wine quality. The sequence of D1/D2 domain of 26S ribosomal DNA (rDNA), reflects ascomycetous yeast phylogenetic relationships and enables their separation at the species level. A region of the 26S rDNA, with around 1100 bp comprising domain D1/D2, was amplified by PCR and then digested with restriction endonucleases (*Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I) in order to differentiate yeast species frequently isolated from grape surfaces, wine and cellar equipments. A total of 78 yeast strains (including 36 type strains) belonging to 53 species were used to generate the restriction profiles. Numerical analysis of the profiles generated by the five restriction enzymes enabled to group the strains in 47 different clusters and 42 of them clearly corresponded to different yeast species. The remaining groups comprise closely related species. The enzymes *Mse*I, *Hae*III and *Cfo*I revealed a high discrimination power and the restriction profiles generated were sufficient to clearly identify the 42 species mentioned above. Despite one of the clusters included different yeast genera, with different wine characteristics, the common wine spoilage yeasts *Zygosaccharomyces bailii* and *Z. lentus* could be separated to one distinctive cluster through the use of ApaI restriction profiles. Since the analysis of restriction profiles of amplified 26S rDNA showed to be a valuable method to identify oenological yeast species, a database comprising the majority of wine yeast biota was created to be applied both at research and industrial environment.

RESUMO

O ecossistema microbiano existente nas uvas, no mosto e no vinho é composto por uma grande diversidade de espécies de leveduras. O conhecimento deste biota de leveduras ao longo do processo de vinificação permite um melhor controlo da qualidade do vinho. Para a identificação de leveduras o ADN ribossómico (ADNr) tem-se revelado muito adequado para estimar relações filogenéticas, consideradas pelas correntes mais actuais da taxonomia como estando na base da classificação taxonómica. No presente trabalho, avaliou-se um método baseado na amplificação do ADNr 26S, compreendendo a região D1/D2, seguido de digestão por enzimas de restrição - Perfis de Restrição - para a identificação de espécies de leveduras envolvidas no processo de produção de vinho. Esta avaliação foi efectuada através do uso de 78 estirpes pertencentes a 53 espécies (incluindo 36 estirpes tipo). Utilizaram-se as enzimas de restrição *Apa*I, *Hinf*I, *Mse*I, *Hae*III e *Cfo*I e, análise numérica dos perfis de restrição gerados permitiu agrupar as espécies estudadas em 47 grupos, 42 dos quais correspondendo a uma única espécie. As enzimas de restrição *Mse*I, *Hae*III e *Cfo*I foram as que apresentaram maior poder discriminante ao nível da espécie, permitindo a identificação das mesmas 42 espécies. Apesar da enzyma ApaI ter apresentado o mais baixo grau de polimorfismo, esta enzima poderá ser útil para medidas de controlo uma vez que seu perfil de restrição pôde agrupar em um grupo distinto as leveduras *Zygosaccharomyces bailii* e *Z. Lentus*. O método desenvolvido revelou eficácia, rapidez e facilidade de aplicação na identificação de leveduras de interesse enológico. Com o presente trabalho iniciou-se a construção de uma base de dados de perfis de restrição para posterior aplicação em condições industriais e de investigação.

Key words: 26S rDNA, endonucleases, non-*Saccharomyces* yeasts, restriction profiles, wine yeasts

Palavras-Chave: ADNr 26S, endonucleases, leveduras enológicas, leveduras não-*Saccharomyces*, perfis de restrição

INTRODUCTION

The art of wine making represents one of the oldest technological uses of yeast by man. Only during the last century the scientific knowledge of wine has significantly increased, assisted by newly developed techniques that permitted deeper investigation into the biological and physiological diversity of yeast species associated to the process (Pretorius, 2000). The grape has an incontestable influence in aroma and flavour of wines leading to the creation of distinct products. However, wine has more flavour than the grape juice which is fermented from (Romano *et al*., 2003) and it is the metabolism of grape constituents by yeast that is essential to the development of wine flavour (Bartowsky and Pretorius, 2009).

The wine fermentation is a complex ecological and biochemical process involving the sequential development of different microbiota such as non-*Saccharomyces* yeasts, *Saccharomyces* yeasts and lactic acid bacteria present in must and on surface of cellar equi-

pments (Fleet, 2003). The non-*Saccharomyces* yeasts can produce a diversity of enzymatic activities and fermentation metabolites of oenological importance and may interfere with the growth and/or change the fermentation behaviour of the starter *Saccharomyces cerevisiae* yeast, thus ultimately influence wine quality (Cabrera *et al*. 1988; Romano *et al*., 1997; Ciani and Ferraro, 1998; Ciani and Maccarelli, 1998; Ferreira *et al*., 2001; Romano *et al*., 2003; Ciani *et al*., 2006; Domizio *et al*., 2007; Bely *et al*., 2008; Romano *et al*., 2008). The use of selected strains of *S. cerevisiae* as starters became a widespread practice in wineries. Nevertheless, wine makers have recently returned to spontaneous fermentation as well as to the use of non-*Saccharomyces* in order to obtain wine of distinctive quality and diversified products.

On the other hand, yeasts can negatively affect wine quality. Spoilage yeasts such as *Brettanomyces/ Dekkera* produce volatile phenols and acetic acid that under uncontrolled conditions can lead to sensorial defects (Renouf and Lonvaud-Funel, 2007). *Zygosaccharomyces* is another yeast genus that is often regarded as synonymous of food spoilage due to their osmotolerance and resistance to food preservatives (Loureiro and Malfeito-Ferreira, 2003). Therefore, the analysis and identification of yeast biota throughout wine fermentation and conservation are currently important driving forces for innovation in wine technology.

Traditionally, yeast taxonomy has been based on morphological, physiological and biochemical characteristics of species and genera which ambiguity due to strain variability has led to errors in classification (Martini, 1992; Kurtzman and Robnett, 1994; Kurtzman and Fell, 1998). Isoenzymes electrophoretic profiles have also been applied and prove to reflect DNA based yeast species delimitation (Smith *et al*., 1990; Duarte *et al*., 1999; Sampaio et al., 2001; Naumova *et al*., 2003; Duarte *et al*., 2004). However, this technique is highly time-consuming.

Several approaches based on nucleic acids polymorphisms have been developed in an attempt to simplify yeast identification, such as electrophoretic karyotyping, temperature gradient gel electrophoresis (TGGE), microsatellite PCR fingerprinting, random amplified polymorphic DNA, ribosomal DNA (rDNA) restriction profiles and partial rDNA sequencing (Török *et al*.,1993; Baleiras-Couto *et a*l., 1995; Baleiras-Couto *et al*., 1996; Guillamón *et al*., 1998; Kurtzman and Robnett, 1998; Esteve-Zarzoso *et al*., 1999; Hernán-Gómez *et al*., 2000; Esteve-Zarzoso *et al*., 2003; Baleiras-Couto *et al*., 2005; Rodriguez *et al*., 2010).

Nowadays, innovative wine yeast identification techniques such as DGGE (Denaturing Gradient Gel Electrophoresis) on PCR amplified rRNA genes, FISH (Fluorescence in situ Hybridization), real time quantitative PCR (qPCR) and next-generation DNA sequencing can enable the quantification and/or to monitor yeast dynamics throughout the fermentation process (Hierro *et al*., 2007; Mardis, 2008; Salinas *et al*., 2009; Tessonniere *et al*., 2009; Zott *et al*., 2010). However, these techniques need sophisticated and expensive equipments which are not commonly available.

The ribosomal genes (5.8S, 18S and 26S), which have as ultimate function the protein synthesis, are grouped in tandem forming transcription units that are repeated in the genome (Fernández-Espinar *et al*., 2006). rRNA genes have a common origin, are present in all cellular organisms and have proved to be adequate to establish taxonomic relationships, namely on yeasts, as it is present in all cellular organisms, have a common origin and are easy to sequence (Kurtzman and Piškur, 2005). Nucleotide sequences of the D1/D2 domains of the large subunit (26S) of rDNA are sufficiently substituted to allow recognition of most individual yeast species. Kurtzman and Robnett (1998) have sequenced D1/D2 domains for all known ascomycetous yeasts thus, initiating a universal database for rapid identification.

Simpler identification methods were developed based on the amplification of specific regions of rDNA followed by restriction of the amplified fragment. The digested fragments are then separated by electrophoresis in agarose gels and their sizes determined by comparison with appropriate markers. White *et al*. (1990) used this methodology to amplify the ribosomal gene 5.8S and the adjacent intergenic regions ITS1, ITS2 and further to digest with restriction enzymes. Another ribosomal region that is very useful to differentiate at species level is the one that includes 18S gene and the intergenic region ITS1 (Baleiras-Couto *et al*., 1996; Dlauchy *et al*., 1999). Since then, this approach has been used for identifying yeast species mainly associated alcoholic beverages and soft drinks (Guillamón *et a*l., 1998; Esteve-Zarzoso *et al*., 1999; Arias *et al*., 2002; Ferreira *et al*., 2009). Restriction profiles generated have been considered reproducible, cheaper, a less-laborious method and frequently used for yeast identification (Fernández-Espinar *et al*., 2006).

Baleiras-Couto *et al*. (2005) started to evaluate the restriction profiles of a PCR amplicon of the large subunit of rDNA (26S rDNA), comprising the D1/D2 region, as a routine methodology to examine wine yeast species. In the present study, we extended the restriction profiles, originated through digestion with five restriction enzymes (*Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I), of the same PCR amplicon, in order to develop an efficient and rapid methodology for oenological yeasts genotyping. The aim of this work was to create a database of restriction profiles, based on certified yeast strains, to be used in wine related yeast identification carried out both at research and industrial level.

MATERIAL AND METHODS

Microorganisms

A total of 78 yeast isolates, comprising 53 species belonging to 22 genera, included in the Colecção de Microrganismos EVN (INRB/INIA Dois Portos), were used in the present study (Table I). Thirty eight strains were originated from other culture collections, 36 of which are type strains. The remaining 40 strains were isolated from grapes, wine and cellar equipments in our Laboratory and identified by DNA sequencing of D1/D2 region of rDNA.

Yeast cells were grown on YPD medium (20 g/L D-glucose, 10 g/L bacto-peptone, 5 g/L yeast extract and 20 g/L agar) for 48 to 72 hours at 25ºC. Two to three loops of yeast culture (from fresh YPD agar plates) were resuspended in 500 µL of ultrapure sterilised water. Yeast cells lysate was obtained by disrupting cells through freezing of cell suspension in liquid nitrogen for 5 min, followed by incubation at 95 ºC for 5 min, accordingly to Baleiras-Couto *et al*. (2005). The cell lysate containing DNA was then used for PCR amplification purposes. When the ribosomal DNA amplification by PCR was not successful, the cells lysate was obtained by cell disruption using glass beads (0.5 mm Ø) in 500 µL lyses buffer (50 mM Tris-HCl, 250 mM NaCl, 50 mM EDTA and 0.3 % SDS). The cell lysate solution was appropriately diluted and then used for PCR amplification.

Amplification of the ribosomal DNA D1/D2 region

Primer sequences for the amplification of 26S rDNA fragments were as follows: NL1 (5'-GCATATCAA-TAAGCGGAGGAAAAG-3') and LR6 (5'-CGC-CAGTTCTGCTTACC-3'). Reactions were performed in a final volume of 50 µL containing 10 mM taq buffer (MBI Fermentas, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 250 µM dNTPs, 0.75 µM of each primer, 2 U taq polymerase (MBI Fermentas) and 2 µL of DNA solution. PCR was performed on a thermocycler (T Gradient 96 cycler, Whatman-Biometra, Gottingen, Germany) with an initial denaturation at 94ºC for 3 min, followed by 36 cycles of 94 ºC at 1 min, 58 ºC for 1 min and 72 ºC for 1.5 min. The final extension was done at 72 ºC for 5 min. Visualization of the PCR amplified fragments was performed by electrophoresis in 1.2 % of agarose in 0.5 X TBE (0.45 M Tris-HCL, 0.45 M boric acid and 10 mM EDTA, pH 8.0) and staining with ethidium bromide (0.5 µM/mL). The amplification efficiency was visualised under UV light and digital images were acquired through a Kodak 290C camera and processed by Kodak 1D Image Analysis software.

Restriction analysis

Aliquots (3-10 µL according to the band intensity) of PCR products were digested with 3 U and 5 U, respectively, of restriction enzymes *Mse*I, *Hinf*I and *Apa*I (MBI Fermentas) and *Hae*III and CfoI (Promega, Madison, WI) in a final volume of 20 µL, following manufacture's instructions. The resulting fragments were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining, as referred above. A standard DNA marker (100 bp DNA Ladder, MBI Fermentas) was used as a reference to determine the size of digested fragments. Restriction fragments were visualised under UV light and digital images were acquired through a Kodak 290C camera and processed as referred above. All restriction profiles obtained were analysed using GelCompar II software, version 5.1 (Applied Maths, Saint-Martens-Latem, Belgium) which determined the molecular sizes of restriction products. Fragments smaller than 100 bp were not included on the analysis because of their low reproducibility. Similarities among banding profiles of the strains in study were based on Dice coefficient and dendrograms were generated by the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm.

RESULTS AND DISCUSSION

Several molecular methods are presently being applied for microbiological identification and classification. Each method has its advantages and disadvantages according to the convenience of applicability, reproducibility, availability of equipments, and resolution level.

In this study, analysis of restriction profiles of NL1-LR6 region of 26S rDNA was used to differentiate wine yeast species associated to wine production. In a total of 78 strains comprising 53 species, the PCR amplification yielded a fragment size of around 1100-1150 bp. The amplified fragment was then digested with five endonucleases (*Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I) and the restriction products were separated by agarose gel electrophoresis. Representative restriction profiles presented by the 53 yeast species analysed, are shown in Figure 1.

Each restriction enzyme generated a large number of digested fragments (19 or 20), with exception of *Apa*I which originated only 10 band classes, allowing the discrimination of only four species (Table II). Indeed, for most analysed yeast species (36), this enzyme was not able to digest the PCR amplified fragment, a fact that was already reported by Baleiras-Couto *et al*. (2005). The profiles generated after digestion with *Apa*I enzyme presented the lowest polymorphism and discrimination power.

On the other hand, the digestion with restriction enzymes *Hae*III and *Cfo*I produced higher number of well-developed bands and higher degree of polymorphism (with 22 and 24 distinct restriction profiles, respectively). The discrimination power of *Hae*III and *Cfo*I was also higher as many restriction profiles were species specific (16 and 14 respectively). The remaining enzymes *Hinf*I and *Mse*I despite the high

TABLE I

Strains used in the present study, their collection number, geographical origin and sources of isolation (when available).
Estirpes de leveduras utilizadas no presente trabalho e respectivos números de colecção, origem geográfica e fonte de isolamento (quando disponíveis).

Current species name	Original name	Access number EVN	Access number other collections	Substrate of origin	Geographical origin
Aureobasidium pullulans[b]	-	1277		grapes	Portugal
Aureobasidium pullulans[b]	-	1278		grapes	Portugal
Aureobasidium pullulans[b]	-	1279		grapes	Portugal
Brettanomyces custersianus	-	1230		Cellar equipment	Portugal
Brettanomyces naardenensis	*Dekkera naardenensis*	389^{T}	CBS 6042^{T}	lemonade	The Netherlands
Candida cantarellii	*Torulopsis cantarellii*	365^{T}	CBS 4878^{T}, PYCC 3073	must	South Africa
Candida cantarellii	-	1239		cellar equipment	Portugal
Candida fermentati	-	1292		grapes	Portugal
Candida fermentati	-	1293		grapes	Portugal
Candida fermentati	-	1294		grapes	Portugal
Candida norvegica	-	1229		cellar equipment	Portugal
Candida norvegica	-	1233		cellar equipment	Portugal
Candida stellata	*Saccharomyces stellatus, type of Torulopsis stellata*	387^{T}	CBS 157^{T}	Grapes	Germany
Candida vanderwaltii	*Torulopsis vanderwaltii*	368^{T}	CBS 5524^{T}, PYCC 3671	winery equipment	South Africa
Candida zemplinina	-	1148^{T}	CBS 9494^{T}	botrytized wine	Hungary
Candida zemplinina	-	1283		Grapes	Portugal
Candida zemplinina	-	1284		Grapes	Portugal
Cryptococcus albidus	-	1225		cellar equipment	Portugal
Cryptococcus albidus	-	1232		cellar equipment	Portugal
Cryptococcus carnescens	-	1244		cellar equipment	Portugal
Cryptococcus flavencens	-	1243		cellar equipment	Portugal
Cryptococcus flavencens	-	1245		cellar equipment	Portugal
Cryptococcus flavescens	-	1280		Grapes	Portugal
Cryptococcus flavescens	-	1281		Grapes	Portugal
Debaryomyces carsonii	*Pichia vini, Pichia carsonii*	378^{T}	CBS 2285^{T}, PYCC 2757	slime flux of black oak	USA
Debaryomyces hansenii	-	1223		cellar equipment	Portugal
Debaryomyces hansenii	-	1226		cellar equipment	Portugal
Debaryomyces hansenii var. fabryii	*Debaryomyces fabryii*	388^{T}	CBS 789^{T}	interdigital mycotic lesion	Germany
Debaryomyces hansenii var. hansenii	*Debaryomyces tyrocola var. hansenii, type of Saccharomyces*	329^{T}	CBS 767^{T}, PYCC 2968	Beer	Unknown
Dekkera anomala	*Brettanomyces anomalus*	330^{T}	CBS 77^{T}, PYCC 5153	stout beer	UK
Dekkera bruxellensis	-	1231		cellar equipment	Portugal
Filobasidium uniguttulatum	-	1224		cellar equipment	Portugal
Hanseniaspora guilliermondii	*Kloeckera apis*	390^{T}	CBS 2591^{T}	trachea of bee	The Netherlands
Hanseniaspora occidentalis	*Kloeckera javanica*	391^{T}	CBS 282^{T}	Soil	Indonesia
Hanseniaspora osmophila	*Pseudosaccharomyces corticis*	392^{T}	CBS 106^{T}	bark of tree	The Netherlands
Hanseniaspora uvarum	*Kloeckeraspora uvarum*	394^{T}	CBS 314^{T}	muscatel grape	Russia
Hanseniaspora uvarum	-	1290		Grapes	Portugal
Hanseniaspora uvarum	-	1291		Grapes	Portugal
Issatchenkia orientalis	-	338^{T}	CBS 5147^{T}, PYCC 3806	fruit juice	Unknown
Issatchhenkia hanoiensis	-	1285		Grapes	Portugal
Issatchhenkia terricola	-	1286		Grapes	Portugal
Issatchhenkia terricola	-	1287		Grapes	Portugal
Kazachstania exigua	*Saccharomyces exiguus*	381^{NT}	CBS 379^{NT}, PYCC 2543	unknown	Unknown
Kluyveromyces marxianus	*Endomyces pseudotropicalis*	342^{T}	CBS 607^{T}, PYCC 3286	bronchitic patient	Sri Lanka
Kregervanrija fluxuum	*Mycoderma vini, Pichia fluxuum*	370^{NT}	CBS 639^{NT}, PYCC 2597	sour wine	The Netherlands
Lachancea thermotolerans	*Zygosaccharomyces thermotolerans*	372^{T}	CBS 6340^{T} PYCC 4135	mirabelle-plum conserve	Russia
Lodderomyces elongisporus	*Saccharomyces elongisporus*	386^{T}	CBS 2605^{T}, PYCC 4136	Concentrated orange juice	USA
Metschnikowia pulcherrima	-	375^{T}	CBS 5833^{T} PYCC 4830	berries of *Vitis labrusca*	USA
Metschnikowia pulcherrima	-	1296		Grapes	Portugal
Pichia kluyveri var. kluyveri	-	1131		fermenting must	Portugal
Pichia kluyveri var. kluyveri	-	1135		fermenting must	Portugal
Pichia kluyveri var. kluyveri	-	1136		fermenting must	Portugal
Pichia mandshurica	-	294	PYCC 2582	bovine cecum	Unknown
Pichia membranaefaciens	*Saccharomyces -membranifaciens*	284^{T}	CBS 107^{T}, PYCC 3796	ulmus tree exudates	Unknown
Pichia membranifaciens	-	1241		cellar equipment	Portugal
Pichia mexicana	*Candida veronae*	369^{T}	CBS 5815^{T}, PYCC 3664	grape must	Italy
Pichia scaptomyzae	*Pichia alcoholophila*	306^{T}	CBS 1329^{T} PYCC 5294	Soil	Denmark
Rhodotorula mucilaginosa	-	1227		cellar equipment	Portugal
Saccharomyces bayanus	-	271^{T}	CBS 380^{T}, PYCC4456	turbid beer	Unknown
Saccharomyces cerevisiae	-	252^{NT}	CBS 1171^{NT}, PYCC 4455	brewer's top yeast	The Netherlands
Saccharomyces paradoxus	-	279^{NT}	CBS 432^{NT}, PYCC 4570	Unknown	Unknown
Saccharomyces pastorianus var. pastorianus	*Saccharomyces pastorianus*	275^{NT}	CBS 1538^{NT}, PYCC 4601	Unknown	UK
Saccharomycodes ludwigii	-	395^{T}	CBS 821^{T}	Unknown	Unknown
Saccharomycopsis vini	-	1288		Grapes	Portugal
Schizosaccharomyces pombe var. pombe	*Schizosaccharomyces pombe*	396^{T}	CBS 356^{T}	Unknown	Unknown
Torulaspora delbrueckii	*Saccharomyces delbrueckii*	326^{NT}	CBS 1146^{NT}, PYCC 2477	Unknown	Unknown
Torulaspora delbrueckii	-	1129		fermenting must	Portugal
Wickerhamiella domercqiae	*Torulopsis domercqii*	384^{T}	CBS 4351^{T}, PYCC 3067	wine vat	South Africa
Wickerhamomyces anomalus	*Hansenula anomala*	312^{T}	CBS 5759^{NT}, PYCC 4121	Unknown	Unknown
Zigoascus hellenicus	-	1143		fermenting must	Portugal
Zygosaccharomyces bailii	-	1235		cellar equipment	Portugal
Zygosaccharomyces bailii	-	1236		cellar equipment	Portugal
Zygosaccharomyces bailii	-	1242		cellar equipment	Portugal
Zygosaccharomyces bailii var. bailii	*Saccharomyces bailii*	226^{T}	CBS 680^{T}, PYCC 5167	Beer	Unknown
Zygosaccharomyces bisporus	*Saccharomyces bisporus*	242^{T}	CBS 702^{T}, PYCC 5335	Unknown	Unknown
Zygosaccharomyces lentus	-	230	PYCC 5207	spoiled orange juice	UK
Zygosaccharomyces mellis	-	354^{LT}	CBS 736^{LT}	Honey	USA
Zygosaccharomyces rouxii	*Zygosaccharomyces gracilis*	241^{NT}	CBS 732^{NT}, PYCC 5276	grape concentrated must	Unknown
Zygotorulaspora florentinus	*Saccharomyces florentinus, Zygosaccharomyces florentinus*	231T	CBS 746T, PYCC 4169	sulphited grape must	Italy

[a] Only for type strains; [b] yeast-like fungus
EVN-Colecção de Microrganismos EVN, INRB/INIA Dois Portos, Portugal; CBS-Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; PYCC – Portuguese Yeast Culture Collection, Caparica, Portugal
^{T}Type strain, ^{NT} Neotype strain, ^{LT} Lectotype strain

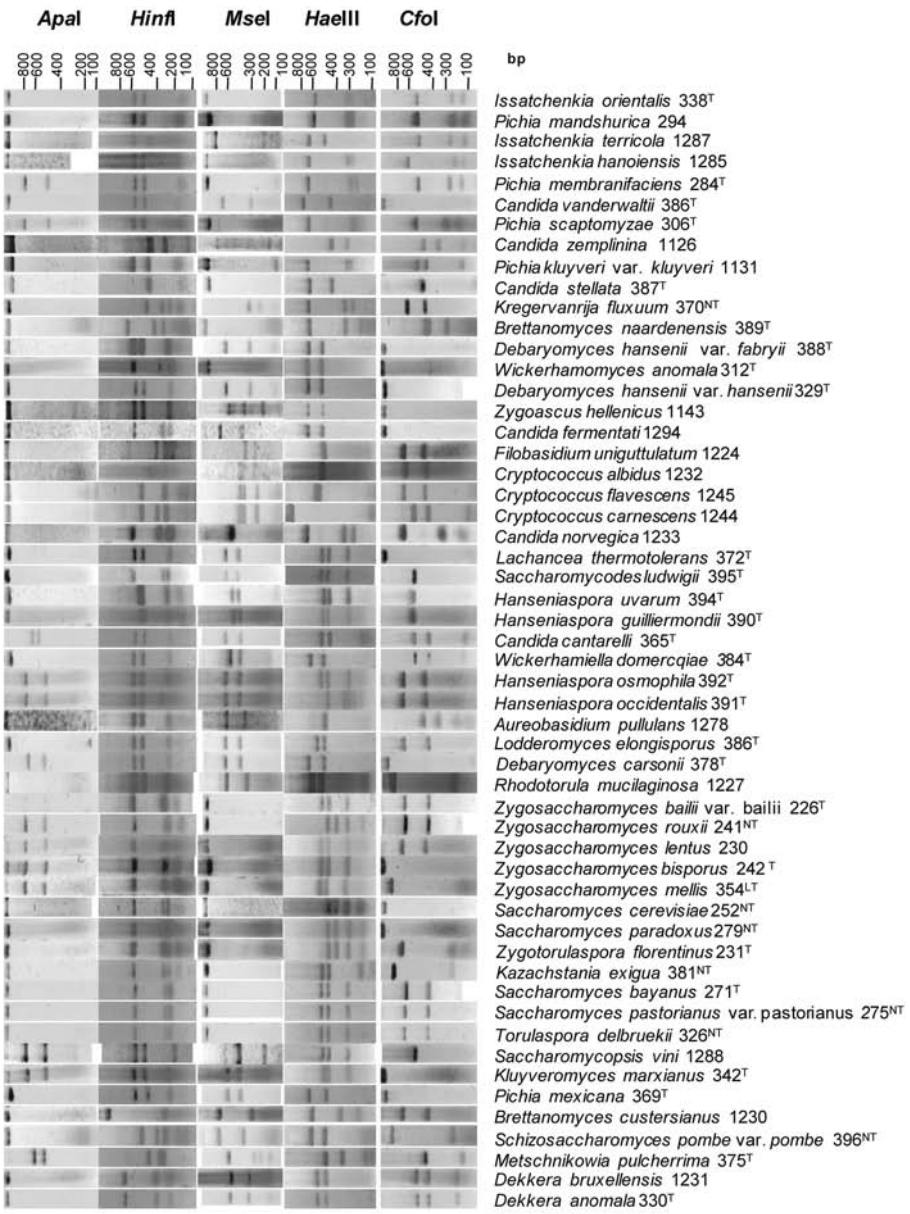

Figure 1 - 53 yeast species representative restriction profiles obtained after digestion with *Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I enzymes of the 26S rDNA region. The number following each species corresponds to the access number of Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T), (NT) and (LT) mean type, neotype and lectotype yeasts, respectively.

Perfis de restrição representativos das espécies de 53 espécies de leveduras obtidos após digestão de uma região do ADNr 26S com as enzimas ApaI, HinfI, MseI, HaeIII and CfoI. O número que segue a espécie de cada estirpe corresponde ao número de entrada na Colecção de Microrganismos EVN (INRB/ INIA Dois Portos); (T), (NT) e (LT) significam leveduras tipo, neotipo e lectótipo, respectivamente.

TABLE I

Characteristics of the restriction fragment length polymorphism profiles of the PCR amplified 26S rDNA region corresponding to each restriction enzymes *Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I.

Características dos perfis de restrição gerados após digestão com cada uma das enzimas ApaI, HinfI, MseI, HaeIII and CfoI do produto amplificado por PCR da região 26S do ADNr.

Restriction Enzyme	Nº of digested fragments (band classes)	Nº of restriction profiles	Nº of species differentiated	Nº of strains without digestion
***Apa*I**	10	7	4	36
***Hinf*I**	20	22	10	0
***Mse*I**	20	16	9	20
***Hae*III**	19	24	16	0
***Cfo*I**	19	22	14	11

degree of polymorphism (with 22 and 16 restriction profiles, respectively) showed an intermediate discrimination power presenting high number of profiles shared by many of the studied species.

Cluster analysis of the strains in study were performed considering the fingerprints of all restriction enzymes, their relationship was calculated by applying the Dice coefficient, and a dendrogram was generated using UPGMA clustering algorithm. The 26S rDNA-based restriction analysis generated 47 clusters 42 of them corresponding to a single yeast species and only five clusters not species-specific (Figure 2). The calculated cophenetic correlation coefficient (0.83) indicates a good fit for the cluster analysis. The species-specific restriction profiles generated by the five endonucleases used in this study allowed the identification of the most predominant non-*Saccharomyces* yeast genus found in grape surfaces or winery environments such as *Hanseniaspora*, *Candida*, *Pichia*, *Rhodotorula*, and *Kluyveromyces* (Longo *et al*., 1991; Fleet and Heard, 1993; Schütz and Gafner, 1993; Torija *et al*., 2001; Clemente-Jimenez *et al*., 2004; Zott *et al*., 2008). This identification is of major importance as non-*Sacharomyces* yeasts might influence wine fermentations both directly, through production of off-flavors, and indirectly by modulating the growth

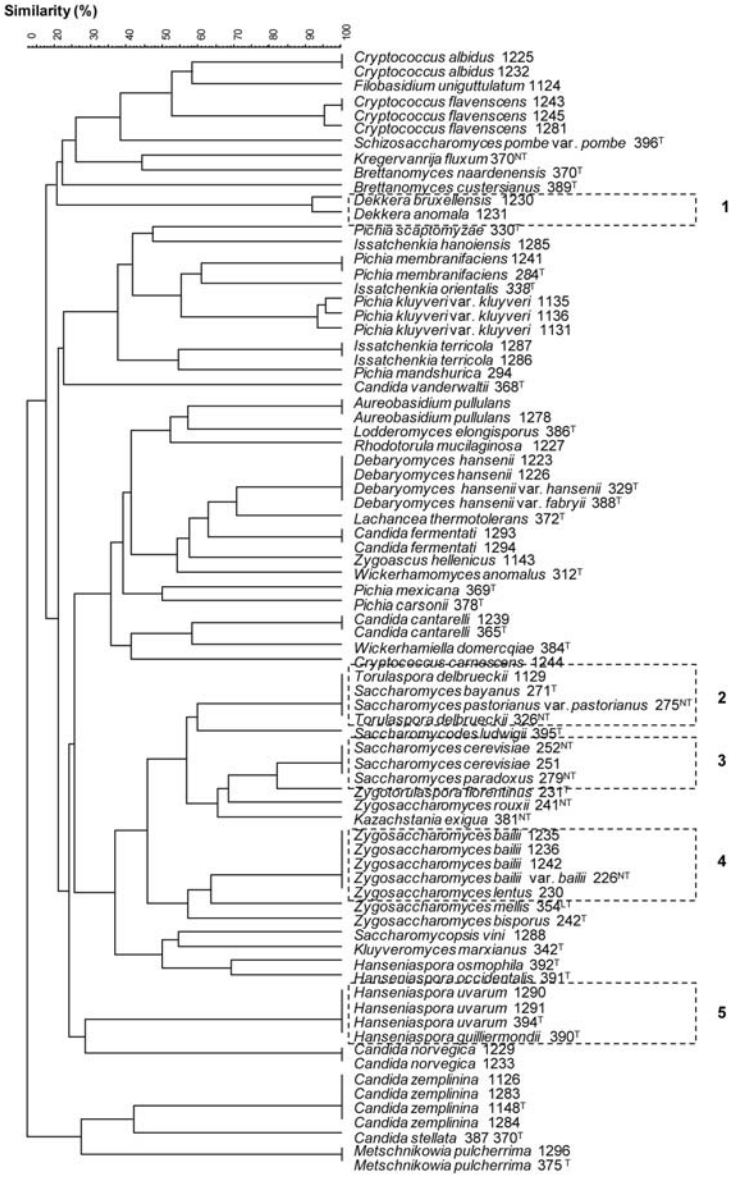

Figure 2 - Dendrogram of restriction profiles fingerprint, obtained after digestion with *Hinf*I, *Mse*I, *Apa*I, *Hae*III and *Cfo*I enzymes, presented by the 78 yeast strains. Dendrogram was generated by the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm, calculated by using GelCompar II (version 5.1), cophenetic correlation coefficient = 0.83. The five clusters that could not be solved at species level are shown in dotted lines. The number following each species corresponds to the access number of Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T), (NT) and (LT) mean type, neotype and lectotype yeasts, respectively.

Dendrograma representando a semelhança entre as 78 estirpes com base nos perfis de restrição de ApaI, HinfI, MseI, HaeIII and CfoI obtidos de uma região do ADNr 26S. O dendrograma foi criado usando o coeficiente de Dice pelo método de agrupamento UPGMA (GelCompar II, versão 5.1), coeficiente de correlação cofenética = 0,83. Os cinco grupos em que não foi possível a identificação ao nível da espécie estão indicados por linhas pontilhadas. O número que segue a espécie de cada estirpe corresponde ao número de entrada na Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T), (NT) e (LT) significam leveduras tipo, neotipo e lectótipo, respectivamente.

or metabolism of the dominant *Saccharomyces* population (Fleet, 2003).

The non-*Saccharomyces* yeast species belonging to *Metschnikowia*, *Kluyveromyces*, *Cryptococcus*, *Rhodotorula*, *Aureobasidium*, *Issatchenkia*, *Debaryomyces*, *Lachancea*, *Zygoascus* and *Saccharomycodes* genera were all well assigned by presenting distinctive restriction profiles (Figure 2). These yeast species although in a lower extent, are normally present during wine fermentation (Mills *et al*., 2002; Baleiras-Couto *et al*., 2005; Nisiotou *et al*., 2007; Bisson and Joseph, 2009).

Schizosaccharomyces pombe, characterized by its special mode of vegetative reproduction and a certain degree of osmophily, can cause food spoilage (Esteve-Zarzoso *et al*., 1999). This species presented a unique restriction profile and, therefore, could be clearly separated.

In the present study, unique species-specific restriction profiles for the five studied *Pichia* species were obtained (Figure 2). Some *Pichia* species are present at high levels at the beginning of fermentations and have been associated with the development of surface flora in wines exposed to air or incompletely filled tanks or barrels (Fleet, 1993). The *P. membranifaciens* species may also present killer property by producing toxins that could inhibit the growth of some spoilage yeast such as *Brettanomyces bruxellensis* (Santos *et al*., 2009).

The very heterogeneous genus *Candida* includes all yeast species that cannot be classified in any other assexual ascomycetous yeast genera (Esteve-Zarzoso *et al*., 1999). Some *Candida* species have become very interesting for oenology due to their highly fructophilic nature allowing their use along with *S. cerevisiae* which is highly glucophilic (Mills *et al*., 2002). In this study, we analysed six *Candida* species that are frequently isolated in food and beverages. Through the restriction profiles generated with the five endonucleases, all these species could be clearly assigned (Figure 2). In some cases, *Candida* species have been shown to be able to complete the alcoholic fermentations (Clemente-Jimenez *et al*., 2004). The species *C. stellata* was found to be present at high level in musts (Hierro *et al*., 2006; González *et al*., 2007). However, in a recent work Csoma and Sipiczki (2008) have proposed that most isolates from grapes and wine are *C. zemplinina* rather than *C. stellata*. In this work, both species were evidently separated (Figure 2).

The unsolved group, constituted by *Dekkera anomala* and *D. bruxellensis*, which presented identical restriction profiles, was clearly separated from all other studied species constituting a reliable approach for *Dekkera* genus identification (cluster number 1, Figure 2). While Esteve-Zarzoso and co-authors (1999) clearly separated these two species using the 5.8S-ITS region restriction profiles, these authors could not separate *D. anomala* from *H. uvarum* and *H. guilliermondii*. In an industrial perspective, the methodology under study enabled the identification of the genus *Dekkera* which includes dangerous wine spoilers as they negatively modify physical and sensorial properties of wine provoking severe economical losses (Loureiro and Malfeito-Ferreira, 2003). The closer *Brettanomyces* species (*B. naardenensis* and *B. custersianus*) were also separated from each other and from *Dekkera* species.

The species *Torulaspora delbrueckii*, *Saccharomyces bayanus* and *S. pastorianus* were grouped in one cluster whereas *S. cerevisiae* and *S. paradoxus* were separated from them forming another cluster (clusters number 2 and 3, Figure 2). *T. delbrueckii* can produce positive effects on the taste and aroma of wines (Ciani and Maccarelli, 1998) whilst *Saccharomyces* complex (*S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*) is the most strongly fermenting and ethanol-tolerant yeast group which takes over the wine fermentation (Fleet and Heard, 1993). In an early study, James and co-authors (1997) reported that the four species of the *Saccharomyces sensu stricto* were found to be closely related, displaying sequences similarity of the 18S rDNA higher than 99.9 %. Indeed, formerly the separation of *Saccharomyces sensu stricto* species could be achieved through isoenzyme analysis (Duarte *et al*., 1999) and more recently by an extensive and combined gene analysis (Kurtzman and Robnett, 2003). The restriction profile of the 26S rDNA enabled the separation of the *Kazachstania exigua* (formerly named as *Saccharomyces exiguus*), a species member of Saccharomycetaceae family.

The grouping of *Zygosaccharomyces bailii* and *Z. lentus*, in one cluster allowed separating these species which can be very important for quality control purposes (cluster number 4, Figure 2). According to phylogenetic data of the 18S rRNA gene and the ITS region some strains that were previously identified as *Z. bailii* were reclassified as new species *Z. lentus* (Steels *et al*., 1999). This new species also showed some physiological differences when compared to *Z. bailii*. The remaining studied *Zygosaccharomyces* species (*Z. bisporus* and *Z. mellis*) and *Zygotorulaspora florentinus* (formerly named as *Z. florentinus*) presented species-specific restriction profiles.

Hanseniaspora species (anamorph *Kloeckera sp*.) are common yeast constituents on grapes and often dominate the early stages of wine fermentations (Romano *et al*., 1993). Growth of these apiculate yeasts may contribute to the final wine quality through production of esters, glycerol and acetoin (Gil *et al*., 1996). On the other hand, *Hanseniaspora sp*. may also negatively affect wine fermentations (du Toit and Pretorius, 2000). High levels of this yeast have been found in damaged grapes and might be associated with stuck fermentations (Bisson, 1999). The last unsolved cluster was constituted by *H. uvarum* and *H. guilliermondii* which present a very close

relatedness (cluster number 5, Figure 2). These two species showed an insignificant D1/D2 sequence divergence which did not exceed 1% (Kurtzman and Robnett, 1998; Cadez *et al*., 2003), a value that is considered the borderline of species separation (Kurtzman and Robnett, 1998). Indeed, recent results have showed that *D. anomala* presented a high similarity with these two *Hanseniaspora* species in restriction profile 5.8S-ITS region, after the digestion with *Hinf*I, *Hae*III and *Cfo*I enzymes (Barata *et al*., 2008). These authors only achieved the differentiation of *H. uvarum* from *H. guilliermondii* and *D. anomala* by using physiological and biochemical tests. In this work, a separation of *Hanseniaspora* and *Dekkera* genus was achieved, highlighting the advantage of using 26S rDNA instead of 5.8 S- ITS region. Nonetheless, for an accurate identification of *Hanseniaspora* species, sequencing of the ITS regions might be needed (Cadez *et al*., 2003). In this study, *Hanseniaspora occidentalis* and *H. osmophila* presented species-specific restriction profiles.

In order to simplify wine yeast identification using the generated restriction profiles database, cluster analysis was also performed to all possible combinations of three restriction enzymes. The combination of the profiles obtained with the restriction enzymes *Mse*I, *Hae*III and *Cfo*I revealed the highest discrimination power. A total of 46 distinct clusters were formed, from which 42 were assigned to a single species (Figure 3). The main difference from the separation achieved with the five restriction enzymes is that *S. bayanus, S. pastorianus, T. delbrueckii* were grouped together with *Z. bailii* and *Z. lentus*. The very close relationship between *Zygosaccharomyces*, *Saccharomyces* and *Torulaspora* genera has already been suggested based on the phylogenetic trees deduced from 18S rDNA (James *et al*., 1996; 1997) and 26S rDNA (Kurtzman and Robnett, 1998). The closeness between these three genera regarding their response similarity to several physiological tests has also been reported (Esteve-Zarzoso *et al*., 2003). However, the *Apa*I enzyme enabled the generation of a distinctive profile for the two *Zygosaccharomyces* species, therefore allowing their separation from *S. bayanus*, *S. pastorianus* and *T. delbrueckii* (Figure 4). This additional restriction enzyme would be used only if it is necessary to clarify this situation. For example, in wine quality control might be necessary to identify *Zygosaccharomyces* species which are considered dangerous wine spoilage yeasts as they can produce off-flavors, are osmotolerant, fructophiles, highly-fermentative, tolerant to high ethanol levels and extremely preservative-resistant (Steels *et al*., 2000; Loureiro and Malfeito-Ferreira, 2003).

CONCLUSIONS

The analysis of the restriction profiles obtained from the PCR amplified NL1-LR6 region of the 26S rDNA allowed the discrimination of 42 species among the 53 yeast species analyzed in this study. The remaining groups comprise closely related species both at taxonomic and wine making levels. The method pointed out in this study represents a fast, less laborious and less expensive technique when compared to sequencing besides it does not require sophisticated equipment. This method is a very useful tool when there is a large number of isolates to be identified. Another practical applicability of the method relies on the capacity to clearly assign the common wine spoilage yeasts *D. anomala* and *D. bruxellensis* to one cluster and *Z. bailii* and *Z. lentus* to another distinctive cluster. This is an important result in terms of the applicability of the method for quality control purposes. This study allowed the establishment of a restriction profile database based on certified yeast strains that can be used in yeast identification carried out both at research and industrial level.

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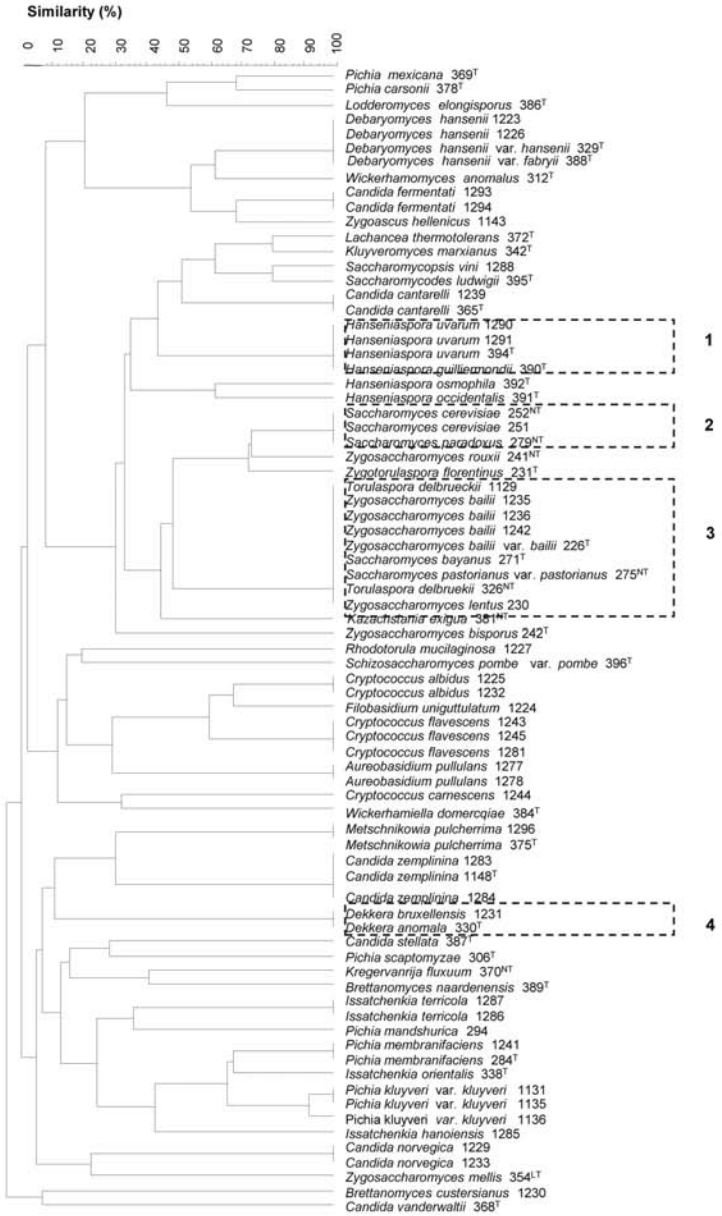

Figure 3 - Dendrogram of restriction profiles presented by the 78 yeast strains by after digestion with *Mse*I, *Hae*III and *Cfo*I, generated by using Unweighted Pair Group Method using Arithmetic Average (UPMGA) clustering algorithm (GelCompar version 5.1). The four clusters that could not be solved at species level are shown in dotted lines. The number following each species corresponds to the access number of Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T), (NT) and (LT) mean type, neotype and lectotype yeasts, respectively.

Dendrograma representando a semelhança entre as 78 estirpes de leveduras com base nos perfis de restrição de MseI, HaeIII and CfoI de uma região do ADNr 26S. Os quatro grupos em que não foi possível a identificação ao nível da espécie estão indicados por linhas pontilhadas. O número que segue a espécie de cada estirpe corresponde ao número de entrada na Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T), (NT) e (LT) significam leveduras tipo, leveduras neotipo e levedura lectótipo, respectivamente.

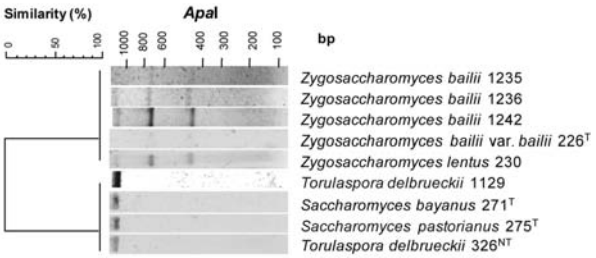

Figure 4 - Dendrogram of restriction profile presented by nine yeast strains after the digestion with *Apa*I enzyme. The number following each species corresponds to the access number of Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T) and (NT) means type and neotype yeasts, respectively.

Dendrograma relativo aos perfis de restrição de nove estirpes de leveduras, obtidos após a digestão com a enzima ApaI. O número que segue a espécie de cada estirpe corresponde ao número de entrada na Colecção de Microrganismos EVN (INRB/INIA Dois Portos); (T) e (NT) significam leveduras tipo e neotipo, respectivamente.

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