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International Journal of Food Microbiology 84 (2003) 33–39
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Molecular typing techniques as a tool to differentiate non-*Saccharomyces* wine species

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Received 28 March 2002; received in revised form 3 August 2002; accepted 18 August 2002

Abstract

A total of 32 yeast strains belonging to four non-*Saccharomyces* species associated with winemaking was characterized by different molecular techniques. The PCR amplification of 18S rRNA-coding DNA and nontranscribed spacer, followed by restriction analysis with the endonucleases *Hae*III and *Msp*I, and PCR fingerprinting with microsatellite primers $(GAC)_5$ and $(GTG)_5$ were used. The methods used provided species-specific profiles and proved to be fast and reliable for monitoring the evolution of the four non-*Saccharomyces* yeast populations throughout wine fermentation.

Keywords: Non-*Saccharomyces* wine yeasts; Molecular typing; Microbiological control of fermentation

1. Introduction

The transformation of grape juice into wine by spontaneous alcoholic fermentation is the result of the combined action of several yeast species, which grow more or less in succession throughout the fermentation process. Although *Saccharomyces cerevisiae* is the principal agent in alcoholic fermentation, the non-*Saccharomyces* yeasts can achieve populations of about 10^7 cfu/ml during the initial step of fermentation (Fleet, 1999), and consequently contribute to taste and flavour (Ciani and Picciotti, 1995; Romano et al., 1997; Esteve-Zarzoso et al., 1998). In the grape juice and during the early fermentation phase, apiculate yeasts and *Candida* spp. are dominant and, to a lesser extent, species of *Rhodotorula*, *Pichia*, *Schizosaccharomyces*, *Kluyveromyces* and *Hansenula* can be detected (Querol et al., 1990; Fleet and Heard, 1993; Schütz and Gafner, 1994). Rapid information about the composition and dynamics of yeast flora occurring throughout the vinification process will help to control fermentation and consequently wine quality.

Molecular techniques have been used by numerous authors to discriminate different wine yeast species. Schütz and Gafner (1993) have characterized strains of *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* on the basis of their electrophoretic karyotypes. Quesada and Cenis (1995) have employed random amplified polymorphic DNA using polymerase chain reaction technique (RAPD-PCR) for the differentiation of wine yeasts of *Saccharomyces*, *Candida*, *Pichia*, *Torulaspora*, *Hansenula* and *Rhodotorula*. PCR fin-

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doi:10.1016/S0168-1605(02)00392-6

gerprinting using the simple repeat primers $(GAC)_5$ and $(GTG)_5$ has been applied to discriminate strains of the genus *Saccharomyces* at species level (Lieckfeldt et al., 1993) and non-*Saccharomyces* yeasts (Caruso et al., 2002). PCR ribotyping (ARDRA) has been applied for the identification of some yeast species (Baleiras Couto et al., 1995, 1996; Smole-Mozina et al., 1997), proving its usefulness for the differentiation at species level. The amplification by PCR of spacer region between the ribosomal gene cluster, followed by restriction analysis, has been used successfully to differentiate several wine yeast species (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999; Fernández et al., 1999; Granchi et al., 1999).

The work was performed to establish a suitable molecular method to characterize non-*Saccharomyces* yeasts of wine origin. Due to its complexity, numerous yeast species can grow in grape must and, therefore, the possibility to perform a rapid microbiological control of the dominant yeasts becomes of great oenological importance. Species-specific molecular profiles will be useful to detect and study population dynamics of non-*Saccharomyces* yeasts and will allow constructing a protocol for the rapid identification of these yeast species during wine spontaneous fermentation.

Table 1
List of yeast strains used

No. strains	Code	Origin		
		Grape	Region	
Candida stellata (Cs)				
3	*Cs-AG*	Aglianico	Basilicata	South Italy
3	*Cs-SG*	Sangiovese	Emilia–Romagna	North Italy
3	*Cs-CB* CBS1713	Cabernet Type strain	Veneto	North Italy
Metschnikowia pulcherrima (Mp)				
3	*Mp-AG*	Aglianico	Basilicata	South Italy
2	*Mp-MS/Si*	Malvasia	Sicilia	South Italy
2	*Mp-CB* CBS5833	Cabernet Type strain	Veneto	North Italy
Kloeckera apiculata (Ka)				
3	*Ka-AG*	Aglianico	Basilicata	South Italy
2	*Ka-MS/Si*	Malvasia	Sicilia	South Italy
2	*Ka-MS/Cl*	Malvasia	Calabria	South Italy
2	*Ka-CB* CBS314	Cabernet Type strain	Veneto	North Italy
Schizosaccharomyces pombe (Sp)				
2	*Sp-SG*	Sangiovese	Emilia–Romagna	North Italy
1	*Sp-MS/Si* CBS356	Malvasia Type strain	Sicilia	South Italy

2. Materials and methods

2.1. Yeast strains

A total of 32 strains of non-*Saccharomyces* yeast species was studied. Of these, 28 yeasts are wild strains of wine origin: nine strains of *Candida stellata*, seven of *M. pulcherrima*, nine of *Kloeckera apiculata* and three of *Schizosaccharomyces pombe*.

Type strains of each species were purchased from Centraalbureau voor Schimmelcultures and used as reference strains: *C. stellata* (CBS1713), *M. pulcherrima* (CBS5833), *K. apiculata* (CBS314) and *S. pombe* (CBS356).

The list of the strains is reported in Table 1.

2.2. DNA extraction

Pure cultures of each strain were grown on a YPD broth (1% yeast extract, 2% bacteriological peptone and 2% glucose) at 25 °C for 24–48 h. DNA isolation from the strains was based on the protocol of Valente et al. (1996) with some modifications.

Samples (1.5×10^8 cell/ml) of each culture were extracted with 300 μl of Lysis buffer (NaCl 0.5 M, EDTA 10 mM, SDS 2%, Tris–HCl 50 mM; pH = 8) and incubated for 1 h at 65 °C with occasional shaking. After the addition of 30 μl of sodium acetate (3 M, pH = 5), the samples were centrifuged at 12,000 rpm for 10 min.

The supernatant was transferred to a clean tube and 5 μl of RNAase (10 mg/ml) (Sigma-Aldrich, Steincheim, Germany, USA) was added. After an incubation of 2 h at 37 °C, DNA was extracted with phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio, washed with chloroform and precipitated with cold ethanol (96%) at −20 °C.

After centrifugation at 12,000 rpm for 10 min, the pellets were washed with 70% ethanol. The dried pellets were resuspended in 50 μl of TE buffer and stored at −20 °C. Quality of DNA extraction was analysed on 0.8% (w/v) agarose mini-gels.

2.3. Amplified ribosomal DNA restriction analysis (ARDRA)

The 18S rRNA genes were amplified using the primers P108 and M3989, described by James et al. (1994). The sequences of the primers were P108 5′ACCTGGTTGATCCTGCCAGT (2-21 *S. cerevisiae* numbering) and M3989 5′CTACGGAAACCT-CTACGGAAACCT TGTTACGACT (1775-1754 *S. cerevisiae* numbering).

The reaction was performed in a final volume of 50 μl using the following amplification mixture: 50 ng/μl of DNA template, 5 μl of 10× Reaction Buffer (50 mM of KCl; 10 mM of Tris–HCl, pH = 9; 0.1% Triton X-100), 4 μl of $MgCl_2$ 25 mM, 1 μl 100 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 2.5 μl 20 μmol each of the two amplification primers and 0.25 μl of *Taq* DNA Polymerase (5 U, Promega, Madison, WI, USA).

The cycling parameters were as follows: an initial penetration step at 92 °C for 2 min followed by 35 cycles at 92 °C for 2 min (penetration), 55 °C for 1 min (annealing), 72 °C for 3 min (extension) and a final extension at 72 °C for 5 min.

Amplification products were separated by electrophoresis in 0.8% (w/v) agarose gel.

After that, PCR products were digested with restriction enzymes *Hae*III and *Msp*I (Roche Diagnostics, Milan, Italy) following the supplier's instructions. The reaction mixture was incubated at 37 °C for 2 h. The restriction fragments were checked by electrophoresis in 1.4% (w/v) agarose gel by electrophoresis, using TBE buffer (Tris–borate 0.045 M, EDTA 0.001 M, pH 8), and adding ethidium bromide (Sigma-Aldrich) at a final concentration of 0.5 μg/ml. DNA fragments were visualized by UV transillumination and documented by Polaroid 667 photography (Polaroid, St. Louis, MO, USA). A 100-bp DNA ladder marker (Gibco BRL, Gaithersburg, USA) was used as the size standard.

2.4. Amplification and restriction analysis of NTS region

The primers used for the amplification of the NTS region were: NTSF, 5′-TGA ACG CCT CTA AGY CAG AAT, and NTSR, 5′-TTA TAC TTA GAC ATG CAT GGC. The reaction mixture was prepared as described by Baleiras Couto et al. (1995). The PCR program utilised was as follows: 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 57 °C and 3 min at 72 °C; after the mixture was heated at 72 °C for 2 min and subsequently cooled at 4 °C.

The products were analyzed onto 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide (Sigma-Aldrich).

The PCR products were digested with restriction enzymes. The digestion took place in a 20-μl volume with 10 μl of processed PCR amplification mixture. The restriction enzymes *Msp*I and *Hae*III (Roche Diagnostics) were applied under the conditions recommended by the manufacturer. Digestion products were analyzed on a 2% agarose gel. The procedure described above was then used.

2.5. Microsatellite PCR

The primers $(GAC)_5$ and $(GTG)_5$ described by Baleiras Couto et al. (1996) were used.

PCR-amplified DNA was separated on a 1.2% (w/v) agarose gel by electrophoresis. After that, the procedure was the same as described for ARDRA.

3. Results and discussion

3.1. Restriction enzyme analysis of the amplified 18S region (ARDRA)

The complete 18S rRNA genes of the different wine yeast species (28 collection strains and 4 type strains) were successfully amplified with P108 and M3989 primers. The PCR amplification products showed differences in size, depending on the yeast species (data not shown).

Fig. 1 shows the products of digestion of the amplified 18S region with the two restriction enzymes: *Hae*III and *Msp*I. Restriction with the endonucleases yielded identical restriction pattern among the strains of the same species, including the type strain. Therefore, in the figure, the profile of one representative strain is reported for each species in comparison to the type strain. *C. stellata* and *S. pombe* strains exhibited three different bands with *Hae*III and four different bands with *Msp*I. The obtained bands

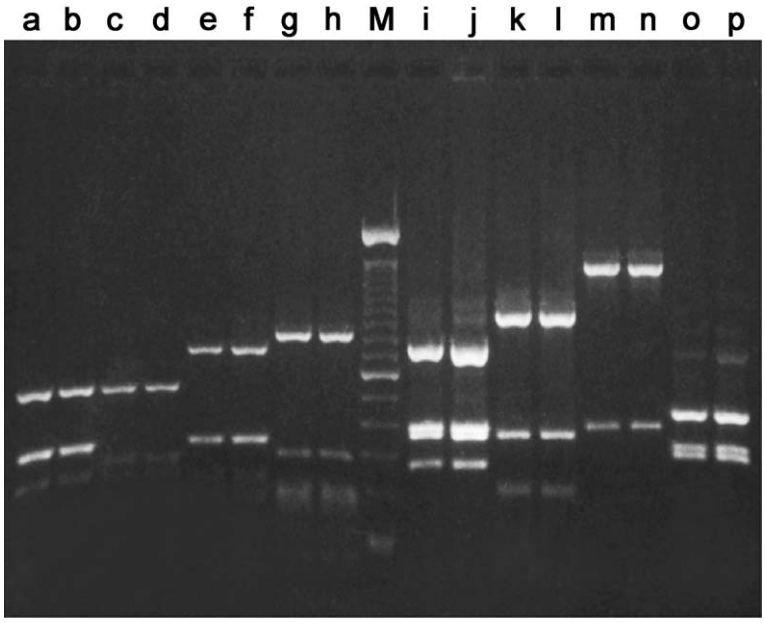

Fig. 1. Amplified ribosomal DNA restriction analysis of yeast strains. Lanes a–h, restriction analysis with *Hae*III; lanes i–p, restriction analysis with *Msp*I; lanes a, b, i and j, *C. stellata*; lanes c, d, k and l, *M. pulcherrima*; lanes e, f, m and n, *K. apiculata*; lanes g, h, o and p, *S. pombe*. Lane M: DNA molecular weight marker, 100-bp ladder.

had approximately the same sizes, except in the case of the first band obtained with *Hae*III (approximately 500 and 780 bp).

The other two species, *K. apiculata* and *M. pulcherrima*, showed two bands after restriction with *Hae*III, whereas with *Msp*I, *M. pulcherrima* strains showed three bands and *K. apiculata* strains with two bands, which were characterized by very different sizes (approximately 1400 and 380 bp).

3.2. Restriction enzyme analysis of the amplified NTS region

The primer pair NTSF and NTSR allowed the amplification of the complete NTS region. Only in the case of the *S. pombe* strains was the amplification of the NTS region unsuccessful, although numerous attempts were performed.

No size variation of NTS fragments was found among the strains of the same species. The results of restriction enzyme analysis with *Hae*III and *Msp*I are shown in Fig. 2, which reports the profile of one representative strain for each species in comparison to its type strain. From this figure, three patterns could be identified for each enzyme, one for each species considered.

The species considered yielded two fragments with both enzymes, only *K. apiculata* strains produced three fragments with enzyme *Hae*III.

3.3. PCR fingerprinting

PCR fingerprinting with the microsatellite oligonucleotide primers $(GAC)_5$ and $(GTG)_5$ is shown in Fig. 3, where two profiles are reported for each species: one for a representative wild strain and the other for the type strain.

In the case of microsatellite primer $(GAC)_5$, five different bands were obtained for the species *C. stellata* and *K. apiculata*, while *S. pombe* and *M. pulcherrima* strains exhibited four bands. Band sizes ranged from 230 to 1200 bp (lanes a–h).

As regards the primer $(GTG)_5$, six bands were exhibited by *C. stellata*, *M. pulcherrima* and *K. apiculata* strains, whereas only four bands by *S. pombe* strains.

The amplification and the restriction sizes of the major bands of the different genetic profiles are

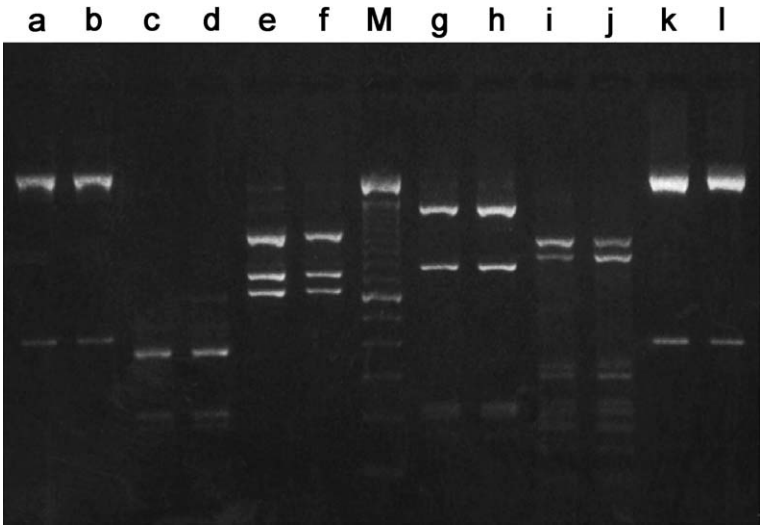

Fig. 2. Restriction digests of the PCR-amplified NTS region of yeast strains. Lanes a–f, restriction analysis with *Hae*III; lanes g–l, restriction analysis with *Msp*I; lanes a, b, g and h, *C. stellata*; lanes c, d, i and j, *M. pulcherrima*; lanes e, f, k and l, *K. apiculata*. Lane M: DNA molecular weight marker, 100-bp ladder.

reported in Table 2. In all cases, strains belonging to the same species (including type strains) showed the same amplification size, differing from the other species in at least one intense band. Four different genetic patterns, resulting from the comparison of the three molecular techniques, were identified and designated by a different capital letter of the alphabet. These genetic patterns are species specific, i.e. each

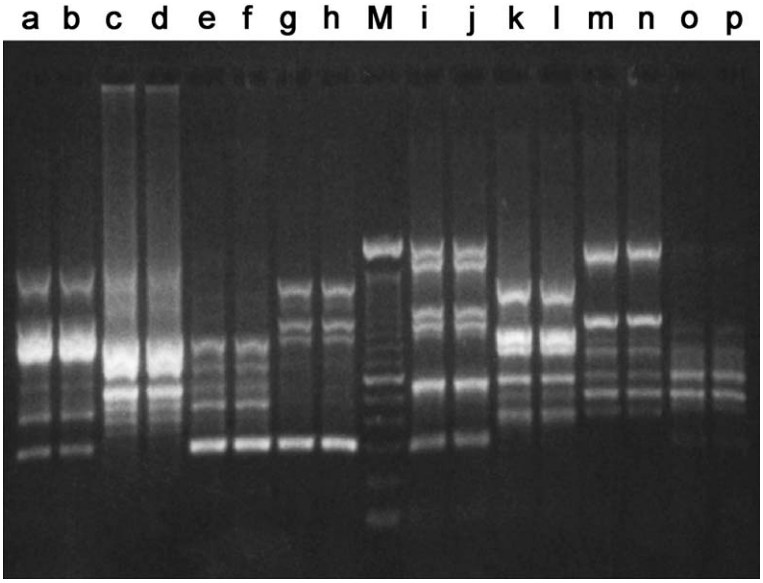

Fig. 3. PCR fingerprinting of yeast strains primed with the microsatellite oligonucleotide primers $(GAC)_5$ (lanes a–h) and $(GTG)_5$ (lanes i–p). Lanes a, b, i and j, *C. stellata*; lanes c, d, k and l, *M. pulcherrima*; lanes e, f, m and n, *K. apiculata*; lanes g, h, o and p, *S. pombe*. Lane M: DNA molecular weight marker, 100-bp ladder.

Table 2
Summary of yeast species molecular profiles resulting in different techniques

Species	No. of strains	Profile type	PCR amplification		ARDRA		NTS amplification	
			$(GAC)_5$	$(GTG)_5$	*Hae*III	*Msp*I	*Hae*III	*Msp*I
			I	I	II	II	II	II
C. stellata	10	A	1150, 650, 500, 300, 190	1950, 1500, 1000, 900, 600, 240	500, 260, 190	700, 380, 300, 230	2000, 360	1600, 800
M. pulcherrima	8	B	600, 520, 380, 290	1150, 860, 760, 600, 460, 330	530, 245	880, 310, 175	350, 180	940, 810
K. apiculata	10	C	780, 650, 540, 400, 230	1700, 920, 700, 600, 460, 340	690, 300	1400, 380	1000, 740, 660	2072, 360
S. pombe	4	D	1200, 920, 800, 240	840, 730, 600, 460	780, 260, 175	670, 360, 260, 250		

I = amplification size (bp).
II = size of the major restriction fragments (bp).

genetic pattern corresponds to a different yeast species.

The recognition of the oenological role played by non-*Saccharomyces* yeasts in the early stages of winemaking has stimulated increasing interest on studies regarding the presence and evolution of wine yeasts during the fermentation process. Thus, it has been demonstrated and accepted that the growth of these non-*Saccharomyces* yeasts influences the analytical composition of the wine (Lema et al., 1996), due to their species-specific characteristics, being high or low producers of secondary compounds, and contributing to the organoleptic quality of the final product (Romano, 1998). In this context, the evaluation of the wine yeast presence assumes a technological interest both in order to guarantee a suitable microbiological course of the process and also to individuate the microbiological pattern, which can be recognized as typical of each wine. Thus, the recent methods, based on molecular techniques, are becoming a useful tool for the identification of yeast species involved in grape must fermentation. Most of these studies have been focused on strains of *Saccharomyces* sensu stricto, mainly on the principal wine species, *S. cerevisiae*, whereas a few data are available on non-*Saccharomyces* yeast of wine origin.

In our study, the molecular techniques used allowed to differentiate between the non-*Saccharomyces* species on the basis of species-specific profiles, characterized by distinct and easily detectable bands, which allow the association of each pattern to each yeast species. It is important to underline that all the strains within the same species yielded a molecular pattern, which is completely different from each other. Therefore, the results obtained could be considered as a useful and fast procedure to characterize and differentiate wine yeast species. The negative results obtained for *S. pombe* strains (non-amplified fragments of NTS region) could be justified with the nonspecific site primers in the DNA structure of this species. In fact, the amplification of other regions with specific primers (e.g. P108 and M3989) showed good amplified products. The analysis of the 18S region allowed discriminating all the yeast species analyzed.

The correlation found between yeast species and specific genetic pattern can facilitate a fast and reliable recognition of these wine yeast species, resulting in a useful tool for the control of wild yeast evolution and, consequently, of wine quality.

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