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Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape

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Abstract

Although the use of starter cultures in winemaking has improved the reproducibility and predictability of wine quality, the main drawback to this practice is the lack of the typical traits of wines produced by spontaneous fermentation. In this study, we identified for the first time the yeast population occurring during spontaneous fermentation of the Catalanesca white grape, a variety from Campania (Italy). Yeasts were identified using molecular tools: PCR-DGGE and partial sequence analysis of the 26S rRNA gene from isolates. Eighteen different species belonging to 11 different genera were identified. *Hanseniaspora* spp., *Issatchenkia* spp. and *Candida* spp. were the dominant yeasts during the early stages of fermentation, while the middle and end phases were dominated by *Saccharomyces cerevisiae*. Four species of *Issatchenkia* spp., rarely isolated from wine fermentation, were found in this study accounting for the 33.5% of the total isolates. The RAPD-PCR screening of the isolates followed by partial rRNA gene sequencing proved to be a very effective approach to first differentiate the isolates and then identify yeast species involved in a wine making procedure. The results show very high yeast diversity in this natural wine fermentation and also highlight the possibility of considering interesting autochthonous strains for starter selection.

Keywords: Non-*Saccharomyces*; *Saccharomyces cerevisiae*; *Issatchenkia hanoiensis*; 26S rRNA gene; PCR-DGGE

1. Introduction

Wine has been produced for years by the natural fermentation of grape juice caused by yeasts that originate from the grapes and winery equipment without deliberate inoculation to start the process. Spontaneous alcoholic fermentation of grape must is a complex process characterized by the presence of a large number of different yeast genera and species (Heard and Fleet, 1988) contributing to the flavour of wines (Lambrechts and Pretorius, 2000; Fleet, 2003). In grape musts and during the early phase of wine fermentation, yeast species with low fermentation activity, such as apiculate yeasts belonging to the genera *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* are dominant (Longo et al., 1991). Sometimes, species of *Pichia*, *Issatchenkia* and *Kluyveromyces* may also grow at this stage. These yeasts grow to about 10^6–10^7 CFU mL-1 but, by mid-fermentation, begin to decline and die off. Under these conditions, strains of *Saccharomyces cerevisiae* and related species, which are more tolerant to ethanol and more competitive for growth in media with high sugar concentration (Querol et al., 1990), become the dominant yeasts and complete the process (Fleet and Heard, 1993). Ethanol production by *S. cerevisiae* is the major factor affecting the growth of non-*Saccharomyces* species during fermentation. Generally, the species of *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia* and *Issatchenkia* are not tolerant of ethanol concentrations exceeding 5–7% (Heard and Fleet, 1988). However, low temperatures decrease the sensitivity of these species to ethanol and, consequently, wine fermentations conducted at temperatures below 15–20 °C may show a greater contribution from *Hanseniaspora* and *Candida* species. On such occasions, these species may predominate along with *S. cerevisiae* at the end of fermentation and, accordingly, can positively influence the overall character of the wine mainly by enhancing aromatic properties and imparting complex and novel flavour profiles (Heard and Fleet, 1988; Erten, 2002). Other yeasts, such as species of *Brettanomyces*,

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Schizosaccharomyces, *Torulaspora* and *Zygosaccharomyces*, may also be present during the fermentation or ageing of the wine and sometimes, due to modifications in the fermentation parameters, they may dominate part of the process and adversely affect wine quality (Pretorius, 2000).

However, the number of species and their presence during fermentation depends on several factors such as production area (Amerine and Kunkee, 1968), climatic conditions (Fleet et al., 1984; Parrish and Carroll, 1985), age of the vineyards, grape variety, health and integrity and harvest technique (Cuinier, 1976; Martini et al., 1980; Rosini et al., 1982), practical winemaking process (Cuinier, 1978) and the type of wine produced (Poulard, 1984), with subsequent wine quality variations from region to region but also from one year to another (Querol et al., 1990).

All this makes the outcome of spontaneous fermentation difficult to predict (Pretorius, 2000). In an attempt to address this problem, many winemakers use pure *S. cerevisiae* cultures which are inoculated into the must after pressing. As a result of this oenological practice, a more rapid and complete grape must fermentation, and a higher degree of reproducibility in the character of specific wines can be achieved (Rankine and Lloyd, 1963; Querol et al., 1992b; Degré, 1993; Pretorius, 2000; Mannazzu et al., 2002; Romano et al., 2003b).

Although inoculation is recommended in modern, industrial wine-making, where rapid and reliable fermentations are essential for consistent wine flavour and predictable quality, there is some controversy about the use of commercial wine yeast due to the lack of some desirable traits provided by natural or spontaneous fermentation (Fleet and Heard, 1993). The use of dried yeasts can influence the natural microflora in must to the extent that certain desired metabolic components, such as higher alcohols and other metabolic by-products, are no longer produced in adequate amounts (Sponholz and Dittrich, 1974; Mateo et al., 1991). Moreover commercial *S. cerevisiae* strains, despite being numerous, possess very ordinary characteristics, producing wines with standard qualities and do not enhance the aromatic traits that characterize many yeasts isolated from specific geographical areas (Rainieri and Pretorius, 2000). Finding strains that possess an ideal combination of oenological capabilities is highly improbable and this has recalled the attention of wine-researchers and wine-makers to the autochthonous strains with the aim of selecting starter cultures that are potentially better adapted to the growth in a specific grape must, whilst emphasising the sensorial and typical quality of the specific wine. The maintenance of biological patrimony is essential both to obtain starter strains that are able to fully develop the typical flavours and aromas of wines originating from different grapevine cultivars (Pretorius, 2000), and to ensure the conservation of gene pools of primary importance for the preservation of productive activities based on yeast-mediated processes.

Recently, several studies have focused mainly on non-*Saccharomyces* indigenous wine yeasts and their potential application in winemaking has been explored. Countless references report the beneficial and detrimental influence of non-*Saccharomyces* yeasts on the volatile composition of musts from varying grape varieties (Ciani and Maccarelli, 1998; Granchi et al., 2002; Romano, 2002; Mingorance-Cazorla et al., 2003; Plata et al., 2003; Romano et al., 2003c; Clemente-Jimenez et al., 2004). These yeasts produce and secrete several enzymes, e.g. pectinase, β-glycosidases, proteases, esterases or lipase, interacting with grape-derived precursor compounds, thus contributing to reveal the varietal aroma and improving the winemaking process (Fleet and Heard, 1993; Esteve-Zarzoso et al., 1998; Fernandez et al., 2000; Cordero Otero et al., 2003).

The present study employs biochemical and molecular biology techniques to identify yeast genera and species and their diversity and dynamics during spontaneous fermentation of Catalanesca (*Vitis vinifera*), an ancient grape variety only cultivated on the north-western slopes of Vesuvius in southern Italy. Although Catalanesca is mainly used as a table grape for direct consumption, many local wine makers also use it for wine fermentation, obtaining wines of appreciated value.

2. Materials and methods

2.1. Catalanesca wine production and sampling

Fermentation of Catalanesca was carried out for 25 days at 16 °C using 50 L of grape must; $K_2S_20_5$ (50 mg L^{-1}) was added at the beginning of fermentation. Pumping over was performed every day according to the traditional process. All the fermentation process was carried out in stainless steel tanks.

Analyses were carried on grape must samples at different stages of spontaneous fermentation of the Catalanesca grape during the 2004 harvest. The process was carried out according to traditional white wine vinification, without inoculation of active dried yeast. Samples were taken in duplicate and collected at the beginning, the middle of fermentation (once the daily practice of pumping-over was completed) and at the end of wine production. The collected samples, their sugar content and pH are summarized in Table 1. The following samples were analysed: must at the beginning of the fermentation (sample 1) and after 24 h (sample 2); must at the middle of fermentation (five days, sample 3); wine at twenty-five days, after the first racking (sample 4) and finally wine after five months (sample 5).

Table 1
Viable counts of yeast population during the fermentation of Catalanesca grapes

Number	Sample	Time	Brix degree	pH	CFU ml^{-1} [a]	
					YPD agar	Lysine agar
1	Grape must	0	21.4	3.27	4.35×10^6	6.70×10^6
2	Grape must after pumping-over	24 h	20.2	3.6	8.40×10^6	1.28×10^7
3	Grape must	5 days	10.8	3.6	1.72×10^8	1.50×10^8
4	Wine after the first racking (alcohol 12.2% v/v)	25 days	6.8	3.8	4.30×10^6	3.90×10^6
5	Wine (alcohol 13.2% v/v)	5 months	6.8	3.8	1.39×10^5	<10

[a] The data are the means based on three replicates; standard deviations were less than 20% of the means.

2.2. Yeast isolation

For each sample, homogenization and dilutions in quarter strength Ringer's solution (Oxoid) were prepared and spread for cultivation on two different media: a non-selective YPD-agar medium (1% yeast extract; 2% peptone; 2% glucose and 2% agar) and lysine–agar medium (Morris and Eddy, 1957). The latter was used to count non-*Saccharomyces* yeasts since it is a synthetic medium with glucose, vitamins, inorganic salts and L-lysine as the sole nitrogen source; *Saccharomyces* spp. are unable to grow on this medium (Angelo and Siebert, 1987). Plates were incubated at 28 °C for 5 days for colony development. After viable counts, the morphologically different colonies from both YPD and lysine medium were purified by repetitive streaking on YPD-agar. All yeast isolates were preserved on YPD agar slants, stored at 4 °C and subcultured every 3 months.

2.3. Harvesting of bulk cells

After the viable counts, the plates were used for bulk formation according to Ercolini, Moschetti, Blaiotta and Coppola (2001). After the viable counts, all the colonies present on the surface of the plate were suspended in a suitable volume of Ringer's solution, harvested with a sterile pipette and stored by freezing at −20 °C. When necessary, 100 μl of the bulk cells was used for DNA extraction as described below.

2.4. DNA extraction

For the extraction of DNA from must samples the procedure described by Cocolin, Heisey and Mills (2001) was used. Briefly, 2 mL of must were centrifuged at 15,000 ×*g* for 10 min a 4 °C. The cell pellet was resuspended in 1 mL of 8 g/L NaCl solution, transferred to a microfuge tube containing 0.3 g of 0.5 mm diameter glass beads and centrifuged for 10 min at 15,000 ×*g*; the supernatant was discarded. After the addition of 300 μl of breaking buffer and 300 μl chlorophorm-isoamylic alcohol (24:1), the sample was homogenized in a bead-beater instrument (Fast Prep™, Bio101, USA) three times for 45 s each at a speed setting of 4.5. After centrifugation, 200 μl of the supernatant was used for DNA purification using the Dneasy Plant System (Qiagen, Qiagen Italia, Milan, Italy) following the manufacturer's instructions.

DNA extraction from bulk cell suspensions and from yeast pure cultures was performed by a synthetic resin (Instagene Bio-Rad Matrix, 732-6211 Bio-Rad Laboratories, Richmond, CA, USA), according to the supplier's instructions, using 100 μl of the bulk or an isolated colony, respectively.

PCR Amplification of DNA from musts and bulks for denaturing gradient gel electrophoresis (DGGE) analysis.

DNA isolated from grape must samples and bulk cell suspensions was amplified using the polymerase chain reaction (PCR) with a primer set designed by Sandhu, Kline, Stockman and Roberts (1995): 403f, 5′-GTG AAA TTG TTG AAA GGG AA-3′ and 662r, 5′-(*GC*)-GAC TCC TTG GTC CGT GTT-3′. A GC-clamp was added to the reverse primer, according to Muyzer, De Waal and Uitterlinden (1993). PCRs were performed in a programmable heating incubator (MJ Research Inc., Watertown, MA, USA). Each mixture (final volume, 50 μl) contained 20 ng of template DNA, each primer at a concentration of 0.2 μM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM MgCl2, 5 μl of 10× PCR buffer (Invitrogen) and 1.25 U of Taq polymerase (Invitrogen, Milan, Italy). The reactions were run for 30 cycles: denaturation was at 95 °C for 60 s, annealing at 52 °C for 45 s, and extension at 72 °C for 60 s. An initial 5 min denaturation at 95 °C and a final 7 min extension at 72 °C were used. A "touchdown" PCR was performed (Muyzer et al., 1993) to increase the specificity of amplification and to reduce the formation of spurious by-products. The initial annealing temperature was 60 °C for 60 s, which was reduced by 1 °C every cycle for 10 cycles. Finally, 20 cycles were performed at 50 °C for 60 s. The denaturation and extension for each cycle were carried out at 94 °C for 30 s and 72 °C for 3 min, respectively, while the final extension was at 72 °C for 10 min. Aliquots (10 μl) of PCR products were routinely checked on 1.8% (w/v) agarose gels.

2.5. DGGE analysis

PCR products obtained from bulk cell suspension and directly from fermented must, were analyzed by DGGE by using a Bio-Rad Dcode apparatus and the procedure as described by Cocolin et al. (2001). PCR samples were applied directly onto 8% (wt./vol.) polyacrylamide gels in 1× TAE buffer. Optimal separation of the PCR fragments was achieved with a 20 to 50% urea-formamide denaturant gradient (100% denaturing solution contained 40% (wt./vol.) formamide and 7.0 M urea) increasing in direction of electrophoresis. The gels were electrophoresed for 10 min at 50 V and for 4 h at 200 V with a constant temperature of 60 °C. After electrophoresis the gels were stained with ethidium bromide for 5 min, rinsed for 20 min in distilled water and photographed under UV transillumination.

2.6. RAPD-PCR assay of pure cultures

Pure yeast cultures were subjected to Random Amplified Polymorphic DNA (RAPD) for biotype screening (Quesada and Cenis, 1995) among the isolated strains and then to 26S rDNA sequencing for taxonomic identification. The RAPD-PCR screening was performed in order to reduce the number of isolates to be identified at species level.

For RAPD-PCR, XD5 (5′-CTGGCGGCTG-3′) was used as primer. PCR reactions were carried out in 25 μl of reaction mix containing 1× Taq Polymerase-Buffer, 2.5 mM $MgCl_2$, 0.4 mM each dATP, dCTP, dGTP and dTTP, 1.08 μM of the primer, 0.1 U Taq Polymerase and 20 ng of the extracted DNA. PCR was carried out in a programmable heating incubator using an initial denaturation step at 94 °C for 1 min followed by 40 cycles of 1 min at 94 °C, 1 min at 31 °C, 7 min at 72 °C per cycle. Finally, a 7 min extension period at 72 °C was performed.

The amplified products (25 μl) were resolved by electrophoresis on 1.5% (w/v) agarose-TBE at 7 V cm-1 for 3 h. 1 Kb DNA Ladder (Invitrogen) was used as molecular weight marker.

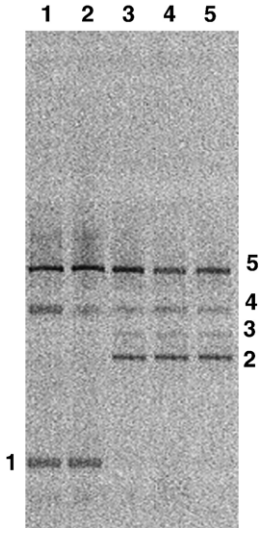

Fig. 1. PCR-DGGE profiles of grape must samples during winemaking obtained according to Sandhu et al. (1995). Lanes: 1, must at time zero; 2, must after 24 h; 3, must after 5 days; 4, wine after 25 days; 5, wine after 5 months. The numbers indicate the sequenced bands, identifications are reported in Table 2.

2.7. 26S rRNA gene amplification

PCR amplification of D1/D2 region of the 26S rDNA was achieved by using primers NL1 5′-GCATATCAATAAGCG-GAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTTCAA-GACGG-3′ (Kurtzman and Robnett, 1998). PCR was performed in a final volume of 50 μl containing 1× Taq Polymerase-Buffer, 2.5 mM MgCl2, 250 μM (each) dNTP Mix, 1.25 U Taq Polymerase, each primer at concentration of 0.2 μM and 20 ng of the extracted DNA. The PCR program consisted of one initial denaturation step at 95 °C for 5 min, 30 amplification cycles (95 °C for 1 min, 52 °C for 45 s, 72 °C for 1 min) and one final elongation step at 72 °C for 7 min. The presence of specific PCR products was checked by agarose 1.5% (w/v)-TBE gel electrophoresis at 7 V cm-1 for about 1 h.

2.8. Sequencing

26S rDNA PCR products, obtained from isolates, were purified by the QIAquick PCR Purification Kit (Qiagen) according to the supplier's instructions. The DNA sequence was determined by the dideoxy chain termination method by using the primer NL4. Research for DNA similarity was performed with the National Centre of Biotechnology Information GeneBank (Altschul et al., 1997).

The abundance of yeast species was calculated as % of isolates belonging to a specific species compared to the total number of isolates.

The DGGE bands of interest (from must samples or bulks), were excised from the gel with a sterile scalpel, disrupted in 20 μl of sterile water and left overnight at 4 °C. One μl of the eluted DNA of each DGGE band was re-amplified by using the appropriate primers and the conditions described above. The success of this procedure was checked by electrophoresing 12 μl portions of the PCR products in DGGE gels as described above together with amplified DNA from grape must samples or bulks as a control. PCR products which gave a single band comigrating with the original band were then purified by QIAquick PCR Purification kit (Qiagen) and sequenced. Sequencing was performed using the primer 403f. The partial sequences were compared with the sequences present in public data libraries (GenBank) using the Blast search program in order to determine their closest known relatives.

3. Results

3.1. Enumeration of yeast population

The viable counts from the selective and non-selective agars are shown in Table 1. At the beginning of the spontaneous fermentation, the fresh grape juice exhibited a total yeast count on YPD medium of 4.35×10^6 CFU/ml, it exhibited a maximum number of 1.72×10^8 CFU/ml on day 5, while viable cells decreased to 1.39×10^5 CFU/ml at the late stages of the process. Viable counts on lysine medium showed a similar general trend to that detected on YPD medium. Initial levels of total non-*Saccharomyces* spp. yeasts were as high as 6.7×10^6 CFU/ml, they increased to 1.5×10^8 CFU/ml after 5 days of fermentation and then decreased to 3.9×10^6 CFU/ml in the late phases. The samples collected after 5 months did not show detectable viable counts. In all, 161 colonies were isolated for subsequent identification by molecular methods.

3.2. Yeast population fingerprinting by PCR-DGGE

Information on yeast diversity and changes at the species level during spontaneous wine fermentation was obtained by extracting DNA directly from samples, followed by the PCR-DGGE of amplicons obtained with the primers 403/662GC (Sandhu et al., 1995). Moreover, in order to obtain a detailed profile of yeasts occurring in the winemaking process, PCR-DGGE analysis was also carried out on DNA extracted from the bulk cells as described above.

Table 2
Identities of bands obtained from DGGE analysis of wine samples

Band[a]	Origin	PCR-DGGE reference	Closest relative	% identity	Closest relative accession number
1	GMS pattern	Sandhu et al. (1995)	*Aureobasidium pullulans*	99	AJ507454
2	GMS pattern	Sandhu et al. (1995)	*Saccharomyces cerevisiae*	98	AY497669
3	GMS pattern	Sandhu et al. (1995)	*Botryotinia fuckeliana*/*Sclerotinia sclerotiorum*	97 97	AY544651/ AY789347
4	GMS pattern	Sandhu et al. (1995)	*Candida stellata*	97	AY160761
5	GMS pattern	Sandhu et al. (1995)	*Hanseniaspora clermontiae*	100	AY953954

[a]DGGE pattern obtained after PCR amplification of 26S rDNA. Band numbers are indicated in Fig. 1.
[b]GMS: Grape must sample.

Table 3
Identification of yeast isolates by 26S rDNA gene sequence analysis

Strain number	RAPD pattern (number of isolates)	Sample[a]	Medium	Closest relative	% identity	Closest relative accession number
1Y32a	B (2)	1	YPD	*Candida stellata*	100%	AY394855
1Y36a	J (4)	1	YPD	*Pichia kluyveri*	99%	AJ746339
1Y43a	K (1)	1	YPD	*Hanseniaspora occidentalis*	100%	AJ973101
1Y44a	AA (14)	1	YPD	*Hanseniaspora uvarum*	99%	U84229
1Y44b	Y (1)	1	YPD	*Issatchenkia terricola*	99%	U76345
1Y47a	BA (2)	1	YPD	*Candida diversa*	99%	U71064
1Y51a	GA (1)	1	YPD	*Pichia kluyveri*	99%	AJ746339
1Y52a	Q (1)	1	YPD	*Issatchenkia terricola*	99%	U76345
1Y57a	EA (3)	1	YPD	*Hanseniaspora uvarum*	99%	U84229
1L41b	U (1)	1	LM	*Issatchenkia terricola*	99%	U76345
1L44a	M (1)	1	LM	*Candida sp.*	99%	AY520361
1L45b	D (14)	1	LM	*Issatchenkia terricola*	99%	U76345
1L51b	FA (2)	1	LM	*Candida sp.*	99%	AY242304
1L52b	T (1)	1	LM	*Candida sp.*	98%	AY452050
2Y54b	N (1)	2	YPD	*Hanseniaspora uvarum*	94%	AF257273
2L44a	V (1)	2	LM	*Candida sp.*	98%	AY452050
2L51a	W (1)	2	LM	*Issatchenkia orientalis*	100%	AY707865
2Y59a	Z (1)	2	YPD	*Hanseniaspora uvarum*	99%	U84229
2Y55b	OA (2)	2	YPD	*Candida stellata*	100%	AY394855
2L41a	PA (1)	2	LM	*Issatchenkia occidentalis*	99%	U76348
2Y55a	HA (1)	2	YPD	*Hanseniaspora uvarum*	99%	U84229
2L55b	MA (1)	2	LM	*Issatchenkia hanoiensis*	99%	AY163900
3Y41b	F (1)	3	YPD	*Pichia kluyveri*	99%	AJ746339
3Y43a	E (2)	3	YPD	*Issatchenkia occidentalis*	100%	U76348
3Y44a	NA (2)	3	YPD	*Hanseniaspora uvarum*	99%	U84229
3Y43b	A (31)	3	YPD	*Issatchenkia occidentalis*	100%	U76348
3L42a	H (5)	3	LM	*Candida sp.*	97%	AY452050
3L42b	L (1)	3	LM	*Candida sorboxylosa*	99%	U62314
3L46b	DA (9)	3	LM	*Candida sp.*	99%	AY242304
3L47b	O (1)	3	LM	*Torulaspora delbrueckii*	99%	AJ508558
3L51b	P (1)	3	LM	*Metchnikowia pulcherrima*	97%	AJ745115
3L52a	IA (1)	3	LM	*Candida sp.*	99%	AY242304
3L54b	LA (1)	3	LM	*Saccharomycodes ludwigii*	99%	U73601
3L55b	JA (1)	3	LM	*Candida sp.*	99%	DQ104729
4Y52b	C (18)	4	YPD	*Saccharomyces cerevisiae*	100%	AJ746340
4L11a	R (2)	4	LM	*Kluyveromyces thermotolerans*	99%	U69581
4L23a	A (5)	4	LM	*Issatchenkia occidentalis*	100%	U76348
4L12a	CA (1)	4	LM	*Zygosaccharomyces bailii*	99%	U72161
5Y31a	X (4)	5	YPD	*Saccharomyces cerevisiae*	100%	AJ544259
5Y35a	KA (1)	5	YPD	*Dekkera bruxellensis*	99%	AF113890
5Y38a	I (2)	5	YPD	*Saccharomyces cerevisiae*	100%	AJ544259
5Y41a	S (2)	5	YPD	*Saccharomyces cerevisiae*	100%	AJ544259
5Y42b	G (18)	5	YPD	*Dekkera bruxellensis*	99%	AF113890

[a] 1, grape must at t=0; 2, grape must after pumping-over at 24 h; 3, grape must at 5 days; 4, wine after the first racking at 25 days; 5, wine after 5 months.

DGGE fingerprints obtained by analyzing the amplified rRNA gene fragments (Sandhu et al., 1995) from the DNA directly extracted from the samples collected throughout the fermentation process are shown in Fig. 1, while the results of the band sequencing are reported in Table 2. The fingerprints obtained by direct sample analysis consisted of 5 different bands (Fig. 1B). Bands 5 and 4 were visible throughout fermentation and their closest relatives were *Hanseniaspora clermontiae* and *Candida stellata*. The closest relative corresponding to band 1 was *Aureobasidium pullulans* and appeared only in samples 1 and 2. Bands 2 and 3, corresponding to *S. cerevisiae* and *Sclerotinia sclerotiorum*, respectively, were detected only after five days of fermentation and occurred until the end of the fermentation. The sequence analysis was extended to the profiles obtained from PCR-DGGE analysis of the DNA extracted from bulk cells from the countable plates of the two different media employed. The profiles obtained were no different from those obtained using the DNA from must as template (data not shown).

3.3. RAPD-PCR assay

One hundred and sixty one (161) colonies isolated from must samples in different fermentation stages were subjected to RAPD-PCR analysis for biotype screening among all the strains, in order to characterize the identical biotypes and thus narrow the number of isolates to identify by sequence analysis of the D1/D2 region of the 26S rRNA gene. From RAPD-PCR analysis we recognized 42 different electrophoretic profiles (Table 3).

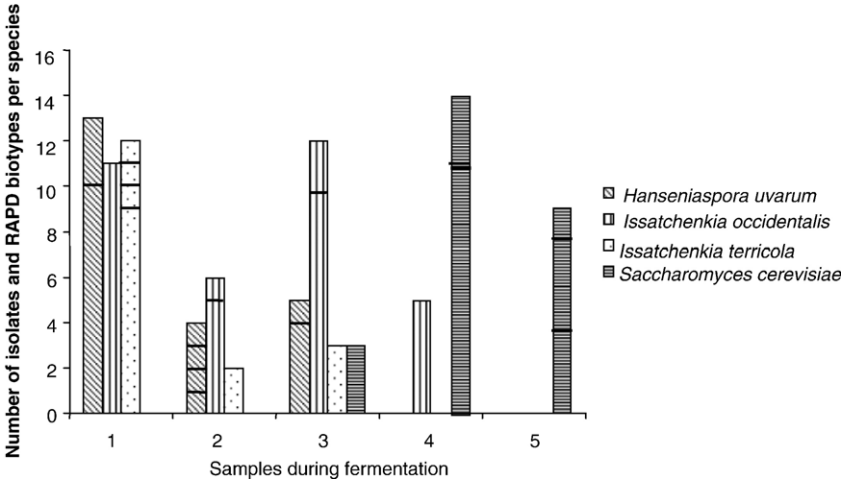

Fig. 2. Number of isolates of representative yeast species revealed during winemaking. Samples: 1, must at time zero; 2, must after 24 h; 3, must after 5 days; 4, wine after 25 days; 5, wine after 5 months. The histograms are interrupted to show the number of RAPD biotypes detected for each species.

3.4. Identification of pure yeast cultures by sequencing the D1/D2 domains of the 26S rDNA

One isolated colony of strains belonging to each of the RAPD patterns was chosen as representative and used for sequencing the D1/D2 region of the 26S rDNA. The results are reported in Table 3. Eleven different genera and 18 different yeast species were identified. The non-*Saccharomyces* yeasts represented most of the total yeast population: *Issatchenkia* spp. were 33.5% of the total isolates, representing the main yeast found in the must samples, followed by *Candida* spp., with 16.8% of the total and *Hanseniaspora* spp. with 14.3%; the other species belonging to the genera *Metchnikowia, Pichia, Torulaspora, Kluyveromyces* were less frequently isolated. However, this percentage dropped sharply as fermentation progressed, and *S. cerevisiae* became the predominant species at the end of the fermentation. The greatest variability at species level was detected in samples 1 and 3, namely at the start of the process and after five days.

For some species, a differentiation at strain level could also be discerned by using RAPD-PCR fingerprinting. The species occurring with multiple biotypes were *Hanseniaspora uvarum* with six different biotypes, followed by *Issatchenkia terricola*, *S. cerevisiae* with four biotypes and *Issatchenkia occidentalis* with three biotypes. The most frequently isolated species and their corresponding number of RAPD-PCR biotypes are summarized in Fig. 2.

4. Discussion

To date, several studies have focused on monitoring spontaneous fermentation of wines due to the increasing interest of winemakers to produce wines with distinct sensorial traits, generally recognized as having a fuller and rounder palate structure. Knowledge of dynamics and occurrence of yeasts in wine fermentation is very important, especially as wine quality is a consequence of their diversity and attitudes.

The use of active dry yeast in fermentation is one of the most common practices in winemaking (Querol et al., 1992a,b; Fleet and Heard, 1993). Although ensuring a reproducible product, it leads to wines of average quality and does not allow enhancement of the aromatic traits of the wines from specific geographical areas. Hence, the need to characterize autochthonous yeasts isolated from spontaneous wine fermentations that are able to optimize the typical attributes of the vine variety (Mannazzu et al., 2002; Jolly et al., 2003; Rementeria et al., 2003; Clemente-Jimenez et al., 2004; Paraggio, 2004).

In this context, the present work aimed to obtain a complete picture of the dynamic changes in the main yeast population involved in the spontaneous fermentation of Catalanesca grape.

As result of this research some new knowledge to the field has been added. The Catalanesca grape had never been studied before and its microbial population during fermentation had never been described. Non-*Saccharomyces* yeast species such as *Issatchenkia* spp. are rarely isolated from wine fermentation while four species of this genus were found in this study accounting for the 33.5% of the total isolates. Moreover, the RAPD-PCR screening of the isolates followed by partial rRNA gene sequencing proved to be a very effective approach to first differentiate the isolates and then identify yeast species involved in a wine making procedure.

In this study, PCR-DGGE was used to obtain profiles of the yeast community in must samples during fermentation. In addition, 26S rDNA sequencing of the isolates was performed in order to achieve species identification after screening of the isolates by RAPD-PCR biotyping. In agreement with other ecological studies on spontaneous wine fermentations (Nurgel et al., 2002; Combina et al., 2005), viable counts showed a significant presence of total yeasts and total non-*Saccharomyces* spp. yeasts. The latest reached populations of up to 10^6–10^7 cells mL^{-1} and some of them (*I. occidentalis, Kluyveromyces thermotolerans, Zygosaccharomyces bailii*) occurred even at the end of the process, although this phase was dominated by *S. cerevisiae*.

It is known that high non-*Saccharomyces* populations can influence wine chemical composition as well as the kinetics of growth and metabolism of *Saccharomyces* spp. (Lema et al., 1996; Mills et al., 2002).

The identification of yeasts by the sequencing of D1/D2 region of the 26S rDNA showed a great population diversity of non-*Saccharomyces* species during fermentation. Although these yeasts lack competitiveness under oenological conditions mainly because they do not have high fermentative power and display a lower stress resistance when compared to *S. cerevisiae*, their presence in this fermentation could be due to the well-known factors that influence the diversity, composition and evolution of yeast flora in grape must: geographic location, climatic conditions (Fleet et al., 1984; Parrish and Carroll, 1985), and age of the vineyard and grape variety (Martini et al., 1980; Rosini et al., 1982). Indeed, *S. cerevisiae* involved in spontaneous fermentations has been recently demonstrated to originate from both vineyard and cellar environments (Le Jeune et al., 2006). Fermentation temperature also plays an important role: indeed, wine fermentation conducted at temperatures less than 15–20 °C may show a greater contribution from *Hanseniaspora* and *Candida* species, which would have a greater impact on wine flavour (Heard and Fleet, 1988; Erten, 2002). In this work the fermentation process was carried out at 15–16 °C, which could justify the abundant presence of these species. Nevertheless, a more exhaustive study of other climatic and cultural conditions should be carried out if their influence on the development of these species is to be understood. These species were also the most frequently described in fresh musts from different fermentations places (Cocolin et al., 2000; Pramateftaki et al., 2000; Jolly et al., 2003).

In this study, the species dominating the early stages of fermentation were *Hanseniaspora uvarum, I. terricola, I. occidentalis* and *Candida* spp. It is widely reported that *H. uvarum* is the most frequently encountered species in fresh must and some strains of this apiculate yeast showed positive oenological properties; in addition to producing ethanol, they are able to produce many secondary compounds and low amounts of acetic acid, and it is suggested that they could be used in grape must fermentations to enhance the aroma and flavour profiles of the wines (Romano et al., 1992, 1993, 1997a,b; Comi et al., 2001; Romano, 2002; Paraggio, 2004). However, a significant strain variability was noted, allowing identification of strains suitable to be used as starter cultures in mixed fermentation with *S. cerevisiae* (Herraiz et al., 1990; Zironi et al., 1993; Romano, 2002; Romano et al., 2003b). Our results also showed 6 biotypes of *H. uvarum* as the most abundant non-*Saccharomyces* yeast at the beginning of fermentation and revealed great strain diversity (Fig. 2). Thus we suppose that this species contributed significantly to the global fermentation rate and influenced the organoleptic qualities of the wine.

Microbial species such as *I. terricola, I. occidentalis, Issatchenkia orientalis* and *Issatchenkia hanoiensis* that are unlikely to be related to the wine-making process were detected in this study. Some have been rarely isolated previously from wine fermentations, albeit with different frequencies (Pallmann et al., 2001; Sabaté et al., 2002; Clemente-Jimenez et al., 2004; Combina et al., 2005; Di Maro, Ercolini & Coppola, unpublished results). In their study on wine yeasts during spontaneous fermentation of six varieties of grape must, Clemente-Jimenez et al. (2004) noted that *I. orientalis* showed the best profile of higher alcohols after *S. cerevisiae* and *H. uvarum* and the lowest value of acetaldehyde, and proposed its use at an appropriate stage of fermentation. By contrast, *I. terricola* showed low fermentative power and could not be considered for mixed fermentation due to its high ethyl acetate production (Clemente-Jimenez et al., 2004). *I. hanoiensis* is a new yeast species that has only been described recently. It was discovered by Thanh, Hai and Lachance (2003) in the grass of the litchi fruit borer *Conopomorpha cramerella* Snellen and since then has not been isolated from other substrates; only Hierro, González, Mas and Guillamón (2006) found this species among the colonies of non-*Saccharomyces* identified in their study.

Although the above species were predominant, other non-*Saccharomyces* yeast such as *Candida sorboxylosa*, *Dekkera bruxellensis*, *Hanseniaspora occidentalis*, *K. thermotolerans*, *Metchnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbrueckii*, were also isolated in this study. Their occurrence was also reported in other studies (Pardo et al., 1989; Longo et al., 1991; Pramateftaki et al., 2000; van Keulen et al., 2003). In agreement with previous reports (Constantì et al., 1997; Sabaté et al., 1998; Torija et al., 2001), our results showed that *S. cerevisiae* could be detected in the middle phase (Fig. 2, sample 3), dominated at the end of alcoholic fermentation (Fig. 2, sample 4) while four different biotypes were detected in mature wine. However, only one biotype was most frequently found and occurred increasingly at the end of fermentation probably due to resistance to growth conditions caused by the lack of nutrients and high alcohol contents. The *S. cerevisiae* strain diversity was observed because no commercial starters had been previously used in the cellar and traditional methods of winemaking were adopted. It is known that the use of active dry yeasts reduces the number of indigenous *Saccharomyces* strains in favour of the starter, although there is still a significant development of natural strains during the early stages of fermentation (Querol et al., 1992a; Romano et al., 2003a). This reduction in diversity is observed even when spontaneous fermentations are allowed in cellars that have been previously inoculated (Constantì et al., 1998).

The viable counts showed higher values for non-*Saccharomyces* compared to the *Saccharomyces* population. In addition, only a few isolates were identified as *S. cerevisiae*, while most of the strains belonged to non-*Saccharomyces* species. Therefore, *S. cerevisiae* represented a significant part of the microbiota of the fermentation but only a small contribution to the overall broad species diversity found in this study. This is in agreement with the opinion that *S. cerevisiae* is rarely isolated from natural surfaces, including grapes and vineyard soils, and their presence in spontaneous fermenting grapes is in direct association with artificial, man-made environments such as wineries and fermentation plants (Martini, 1993; Vaughan-Martini and Martini, 1995; De La Torre et al., 1999; Pretorius, 2000) and it is considered an important component of a so-called “residential” or “winery” yeast flora (Fleet and Heard, 1993).

Wine yeasts occurring in the Catalanesca grape must were also identified by sequencing of the DGGE fragments belonging to regions of the 26S rDNA. In our work, this

protocol was employed as a culture-independent method in combination with conventional culture-dependent methods to study the species diversity of the dominant components of the ecosystem during fermentation. Several studies have demonstrated that PCR-DGGE is a valuable alternative to standard plating methods for qualitative assessment of the microbial constituents in model wine fermentations (Cocolin et al., 2000; Cocolin et al., 2001; Mills et al., 2002). This method is usually employed to assess the structure of microbial communities in environmental samples without cultivation, thus avoiding the problems often associated with microbial enrichments (Ercolini, 2004). The PCR-DGGE analysis provided evidence of species that were not isolated by culture methods: *H. clermontiae, A. pullulans* and *S. sclerotiorum*; the latter two are considered fungi by the latest taxonomic criteria (Barnett, Payne and Yarrow, 1983; Kreger van Rij, 1984). However, the microbial diversity detected by PCR-DGGE was significantly lower in respect to the high diversity found by isolation and further identification of yeast strains as shown in Table 3.

In conclusion, the results of the present study provided an overview of the yeast community of Catalanesca, which was shown to be complex and rich in different microbial species and strains. The wide diversity found in this work, both at species and strain level, shows that spontaneous wine fermentation is driven by a complex microbiota, that is difficult to control but is probably responsible for the development of typical wine flavours. We also show that the growth of non-*Saccharomyces* yeasts is significant and thus their use could be hypothesized in combination with *Saccharomyces* yeasts, promoting the selection of species conferring typical wine traits.

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