

Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin

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ABSTRACT

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Aims: The characterization by molecular and physiological methods of wild apiculate strains, isolated from 'Aglianico del Vulture' grape must.

Methods and Results: The restriction analysis of 18S rDNA allowed the identification of strains at the species level, which were predominantly *Hanseniaspora uvarum*. The RAPD analysis and the evaluation of technological traits, such as the metabolic and enzymatic activities, were useful to evaluate the polymorphism of this species.

Conclusions: The RAPD analysis clustered the wild *H. uvarum* strains in four main genetic groups and a very high phenotypic variability confirmed this genetic polymorphism. The technological variables, which determined the strain biodiversity differed significantly, demonstrating that these technological traits are strain dependent. A certain correlation was found between the strain behaviour and its isolation zone, indicating the influence of the environment on the genetic patrimony of the population.

Significance and Impact of the Study: The genetic and technological biodiversity recorded among *H. uvarum* wild strains represents the basis for organizing a collection of apiculate strains exhibiting oenological characteristics at different levels, such as high/low production of secondary compounds, and, therefore, potentially useful for a selection programme.

Keywords: enzymatic and metabolic activities, *Hanseniaspora uvarum*, PCR-RFLP analysis, RAPD-PCR analysis, strain biodiversity.

INTRODUCTION

Although *Saccharomyces cerevisiae* and related wine species are widely considered as the most important agents of alcoholic fermentation, there is increasing knowledge that non-*Saccharomyces* yeasts, which dominate the early fermentation stages, contribute significantly to wine fermentation and quality (Heard 1999; Romano 2003).

Quantitative studies on yeasts participating in grape juice fermentation showed that non-*Saccharomyces* species survive at significant levels for longer periods during fermentation

than previously thought and their growth was not suppressed in inoculated fermentations with selected cultures of *S. cerevisiae* (Schütz and Gafner 1993; Romano 1997; Egli *et al.* 1998). The role of non-*Saccharomyces* yeasts in winemaking has been emphasized only recently, leading to the investigation of the potential effect of different strains of non-*Saccharomyces* species on the organoleptic characteristics of wine (Lambrechts and Pretorius 2000; Romano *et al.* 2003).

Of these yeasts, species of the genus *Hanseniaspora* were often dominant during the early phase of grape must transformation and consequently numerous studies in the last decade have been developed aiming to evaluate the effects of apiculate strains on the quality of the final fermentation product (Romano *et al.* 1997, 2000; Comi *et al.*

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2001). In this context, determining the degree of variability of apiculate strain populations is of practical interest.

Traditionally, the identification of yeast species has been based on conventional physiological identification (Kurtzman and Fell 1998). These methods utilize mostly assimilation tests of different carbon and nitrogen compounds, fermentation tests and morphological traits, such as the difference in the shape and number of ascospores. These techniques are labour-intensive and can give ambiguous results, because of yeast adaptation to the different environments. Another disadvantage of these methods is their inability to discriminate among strains within a species. To avoid doubtful identifications or misidentifications, genetic methods, which generate results independent of environmental conditions, have been applied and several studies reported the usefulness of molecular techniques in identifying wine yeast species and in discriminating strains (Baleiras Couto *et al.* 1996; Cadéz *et al.* 2002; Caruso *et al.* 2002; Capece *et al.* 2003). Esteve-Zarzoso *et al.* (1999) evaluated the use of restriction fragments length polymorphism (RFLP) of rDNA amplified by polymerase chain reaction (PCR) to generate a database of restriction patterns for the routine identification of yeast species most frequently isolated from food; Guillamón *et al.* (1998) used the same methods to identify wine yeast species.

The molecular techniques employed to differentiate strains at intraspecific level include mtDNA restriction analysis (Guillamón *et al.* 1994; Comi *et al.* 2000), comparison of chromosomal DNA profiles (Cardinali *et al.* 1995), and analysis of random amplified polymorphic DNA by PCR (RAPD-PCR) (Baleiras Couto *et al.* 1994; Quesada and Cenis 1995). Most of these techniques have been used to characterize *S. cerevisiae* strains. In the case of *Hanseniaspora* strains, analysis of chromosomal profiles has been used to differentiate *H. uvarum* wine strains (Schütz and Gafner 1993; Vaughan-Martini *et al.* 2000).

The aim of this work was to study *Hanseniaspora* isolates of the same wine origin, by determining the variability of technological and molecular traits, in order to emphasize the genetic polymorphism within this genus.

MATERIALS AND METHODS

Yeast strains

In this study 57 strains, isolated from 'Aglanico del Vulture' grape must were used. These strains were isolated from two different parts in the production area of this wine: the strains coded from H1 to H21 were isolated from Rionero zone (I), whereas the others (H22–H57) from Ripacandida zone (II).

The isolation was carried out on grapes from different plants in same vineyard for each part. The grapes were

collected aseptically from the vines and then crushed in laboratory; grape must samples were diluted and plated on lysine medium in order to obtain single colonies. The plates were then incubated at 25°C and 10 colonies were isolated from each sample. Only the strains identified as apiculate yeasts by morphological and biochemical analysis, according to Kurtzman and Fell (1998), were selected.

In addition, type strains of *Hanseniaspora* spp. occurring in wine (*H. uvarum*, *H. guilliermondii*, *H. occidentalis*, *H. osmophila* and *H. vineae*) and seven *H. uvarum* reference strains, all obtained from Centraalbureau voor Schimmeltcultures, were used (CBS314, CBS8130, CBS6617, CBS5934, CBS2587, CBS2589, CBS5073 and CBS5074 for *H. uvarum*; CBS2591 for *H. guilliermondii*; CBS2592 for *H. occidentalis*; CBS106 for *H. osmophila*; CBS8031 for *H. vineae*).

Apiculate strains were maintained on slants of YPD (1% yeast extract; 2% peptone; 2% glucose; 2% agar) at 4°C.

Isolation of DNA

DNA isolation was performed according to the method described by Capece *et al.* (2003).

PCR-RFLP analysis of 18S rDNA

The primers used for the amplification of 18S rDNA and ITS regions, including the 5.8 S fragment, were NS3 (GCAAGTCTGGTGCCAGCAGCC) and ITS4 (TCCT-CCGCTTATTGATATGC) (White *et al.* 1990).

The final volume of the PCR reaction mixture was 50 µl containing 100 ng of genomic DNA, 5 µl of 10X reaction buffer (Promega, Madison, WI, USA), 5 µl of MgCl₂ (25 mmol l⁻¹), 0.5 µl of 10 mmol l⁻¹ deoxynucleoside triphosphate mixture, 20 ng of a pair of primers and 0.85 µl of *Taq* polymerase (5 U µl⁻¹, Promega). The PCR conditions were as follows: an initial denaturation step of 5 min at 95°C, followed by 35 cycles at 95°C for 30 s, at 61.5°C for 30 s and at 72°C for 3 min. The last extension step was performed at 72°C for 7 min and the samples were cooled down to 4°C.

The PCR products were analysed on a 1% (w/v) agarose gel in TBE buffer (Tris–borate 0.045 M, EDTA 0.001 M, pH 8) and subsequently submitted to restriction analysis by testing the following five enzymes: *Rsa*I, *Hae*III, *Scr*FI, *Msp*I and *Dde*I. Digestions were performed in a final volume of 10 µl, containing 3 µl of amplified rDNA, 1 µl of specific enzyme buffer and 2 U of each enzyme, by adding water until the final volume. The reaction mixture was incubated overnight at the temperature specific for each enzyme and the restriction fragments were separated on a 1.5% (w/v) agarose gel.

RAPD-PCR analysis

The sequence of the P80 primer used was 5-CGCG-TGCCCA-3. The PCR reaction was performed in 30 μ l reaction mixture containing 50 ng μ l⁻¹ of DNA template, 3 μ l of 10X reaction buffer (Promega), 3 μ l of MgCl₂ 25 mmol l⁻¹, 0.3 μ l of 10 mmol l⁻¹ deoxynucleoside triphosphate mixture, 1.5 μ l of primer 20 μ mol l⁻¹ and 0.5 μ l of *Taq* DNA polymerase (5 U μ l⁻¹, Promega).

The PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min and polymerization at 72°C for 2 min. The polymerization was completed by one additional cycle of 7 min at 72°C. On some strains the amplification was performed in duplicate on the same colonies in order to assess the reproducibility of the patterns obtained.

The PCR products were separated on 1.5% agarose gel in TBE buffer (Tris–borate 0.045 M, EDTA 0.001 M, pH 8) and the DNA bands were sized against the DNA molecular weight marker VI (Roche Diagnostics, Milan, Italy). The RAPD-PCR profiles obtained were analysed using molecular Analyst Fingerprint 2.0 (Bio-Rad, Milan, Italy). Similarities among combined fingerprints were calculated using the Pearson product–moment correlation coefficient. Cluster analysis of the pairwise values was generated using UPGMA algorithm.

Assay for β -glucosidase and β -xylosidase activities

Hanseniaspora uvarum wild strains were tested for enzymatic activities of oenological interest, such as β -glucosidase and β -xylosidase activities.

Yeasts were grown overnight in 25 ml GPY medium (glucose 40 g l⁻¹; peptone 5 g l⁻¹; yeast extract 5 g l⁻¹; pH 5.5) at 25°C in an orbital shaker at 200 rev min⁻¹. Cells were harvested by centrifugation and washed twice with sterile saline solution (0.85%, w/v, NaCl) to removal residual nutrients. The washed cells (10⁶) were inoculated for 2 days at 25°C in a medium specific for the two enzymatic activities. The β -glucosidase activity was determined in a medium containing 1.7 g l⁻¹ yeast nitrogen base (Difco) (without aminoacids and ammonium sulphate), 5 g l⁻¹ ammonium sulphate, 5 g l⁻¹ yeast extract, 5 g l⁻¹ peptone, 10 g l⁻¹ glucose, pH 5.5. For the determination of β -xylosidase activity the same medium was used, substituting glucose with xylose. The ingredients were dissolved in McIlvaine buffer (100 mmol l⁻¹ citrate-phosphate buffer, pH 5.0).

β -Glucosidase activity was measured using *p*-nitrophenyl- β -D-glucosidase (*p*-NPG, Sigma) as substrate, following the method described by Manzanares *et al.* (2000). β -Xylosidase activity was measured using *p*-nitrophenyl- β -D-xylosidase (*p*-NPX, Sigma) as substrate, following the method described by Manzanares *et al.* (1999).

Assay for proteolytic activity

The proteolytic activity of *H. uvarum* wild strains was estimated by digestion of casein, following the method reported by Rosi *et al.* (1987). Yeasts were grown up overnight at 25°C in 5 ml of simulated grape must medium (SGM), composed of 1.7 g l⁻¹ yeast nitrogen base (Difco) without aminoacids and ammonium sulphate, 5 g l⁻¹ tartaric acid, 5 g l⁻¹ D-L-malic acid, 0.2 g l⁻¹ citric acid, 2 g l⁻¹ casamino acids (Difco), 100 g l⁻¹ glucose, pH 3.2. For each strain a preculture of 200 μ l was inoculated in 10 ml SGM medium, supplemented with 1% (w/v) of casein. The samples were incubated at 26°C for 14 days and the proteolytic activity was determined as reported by Charoenchai *et al.* (1997).

Microvinification and analytical determination

Red grape must from 'Aglianico del Vulture' cultivar, originating from a big cellar in the production area, was used to determine the strain capacity to produce secondary compounds. This grape must consisted of 19% (w/v) fermentable sugars, 0.70% (w/v) titrable acidity, pH 3.15.

Fermentations were carried out in 130-ml Erlenmeyer flasks filled with 100 ml of grape must, autoclaved at 100°C for 20 min. Each sample was inoculated with 10⁴ cells per ml from a preculture grown for 48 h in the same must. The grape must surface was covered with a thin layer of sterilized paraffin oil in order to avoid air contact. The fermentation of the inoculated grape must went on for 6 days, without the total consumption of sugars. The determination of weight loss was used as a parameter to follow the fermentation process. The samples were incubated at 25°C until the CO₂ evolution ceased, then refrigerated for 1 day at 2°C, racked and stored at -20°C until required for analysis.

Higher alcohols (*n*-propanol, isobutanol, isoamyl alcohol), acetic acid, acetaldehyde and ethyl acetate were analysed by injecting 2 μ l of fermented grape must into a 180 cm \times 2 mm glass column packed with 80/120 Carbopak B/5% Carbowax 20 M (Supelco, Manchester, UK). A CARLO ERBA Fractovap series 2350 gas chromatograph, equipped with a flame ionization detector and linked to a software Cromatoplus (Shimadzu, Milan, Italy) was used. The column was run from 60 to 200°C at a rise rate of 6°C per min. The carrier gas was nitrogen at a flow rate of 20 ml min⁻¹. Each sample was preloaded with *n*-butanol at a concentration of 100 mg l⁻¹.

Statistical analysis

Data regarding the metabolic and enzymatic activities exhibited by the *H. uvarum* strains were evaluated statistically, by converting these data into a dimensional values.

Each metabolic and enzymatic activity was considered low, middle or high and each strain was evaluated as a function of its behaviour for all the parameters considered, assigned the value '0' to the parameters exhibited by the strains at the low level, value '1' at middle levels, value '2' at the high level. Similarity values were calculated using the Morisita coefficient and the dendrogram was generated using Paired Group algorithm by SAS software (SAS Institute, Cary, NC, USA, 1990).

RESULTS

PCR-RFLP analysis of 18S rDNA

All the wild strains and the type strains of *Hanseniaspora* wine species were submitted to the amplification and restriction analysis of 18S rDNA, a technique useful for the identification at the species level. The preliminary analysis was performed on the type strains. The size of the amplified fragment was the same (approx. 1900 bp) for all the species tested (data not shown), therefore in order to differentiate the *Hanseniaspora* species, the amplification was followed by restriction analysis with five restriction endonucleases. The restriction patterns obtained are reported in Table 1, where each endonuclease is indicated by the corresponding capital letter and the different patterns obtained are numbered in succession. By analysing these data, only the *DdeI* enzyme generated restriction patterns specific for each *Hanseniaspora* species tested. The restriction analysis with the other endonucleases did not allow to differentiate all the species. In particular, *H. uvarum* and *H. guilliermondii* yielded the same restriction profile with the other four enzymes tested; in addition, the *MspI* enzyme did not have restriction sites on the amplified 18S rDNA fragment for these species.

Figure 1 reports the restriction patterns obtained with the *DdeI* enzyme. This enzyme was used to identify 57 *Hanseniaspora* wine isolates. *DdeI* restriction patterns of 52 strains showed the same profile as the type strain of *H. uvarum*, whereas five strains showed the profile exhibited

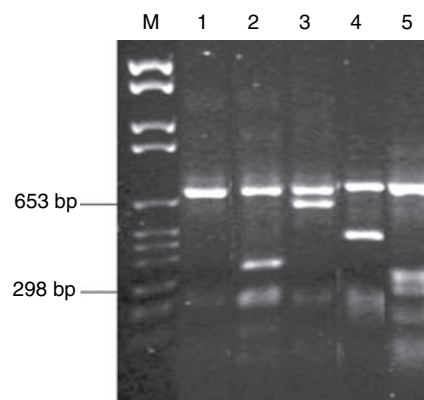


Fig. 1 PCR-RFLP analysis of 18S rDNA region in *Hanseniaspora* type strains with the restriction enzyme *DdeI*. Lane 1: *H. osmophila* CBS 106; lane 2: *H. guilliermondii* CBS 2591; lane 3: *H. occidentalis* CBS2592; lane 4: *H. vineae* CBS 8031; lane 5: *H. uvarum* CBS 314. Lane M: Molecular Weight Marker VI (Roche Diagnostics)

by *H. guilliermondii* type strain. In Fig. 2 the profiles of some *H. uvarum* isolates are presented together with some strains exhibiting restriction patterns typical of *H. guilliermondii*.

RAPD-PCR analysis

As the majority of wild strains (about 90%) belonged to the species *H. uvarum*, the work was focused on the molecular and technological characterization, by evaluating enzymatic activities and production of some secondary compounds.

In order to evaluate the genetic variability of this species, the 52 wild strains, identified as *H. uvarum*, and seven *H. uvarum* reference strains were submitted to RAPD-PCR analysis with the primer P80. Amplification products obtained by RAPD-PCR are reported in Fig. 3, showing the dendrogram based on numerical analysis of the PCR patterns derived from amplification with this primer. At a similarity level <20%, five groups and one single strain (H53) were identified. The groups C, D and E can be subdivided in further subgroups and the strains in these subgroups were similar in the range around 70%. Clusters A and B included five of the seven *H. uvarum* reference strains; of the other reference strains one were in the cluster C and one in cluster D. Cluster E did not include reference strains. The strains in clusters C, D and E clustered according to their origin. In fact, *H. uvarum* strains from 'Aglianico del Vulture' zone I belonged, for the majority, to group C, whereas strains from 'Aglianico del Vulture' zone II were subdivided in clusters D and E. The D group represented the most homogeneous cluster, including strains which were isolated from the same area, with the exception of the reference strain CBS2587. These results seem to indicate the existence of a high genetic polymorphism among the *H. uvarum* wild and reference strains.

Table 1 Patterns generated by PCR-RFLP analysis on *Hanseniaspora* wine species

Type strains	Restriction enzymes				
	<i>HaeIII</i>	<i>MspI</i>	<i>RsaI</i>	<i>SrfI</i>	<i>DdeI</i>
<i>H. osmophila</i>	H1	M1	R1	S1	D1
<i>H. guilliermondii</i>	H2	M2*	R2	S2	D2
<i>H. occidentalis</i>	H3	M1	R3	S3	D3
<i>H. vineae</i>	H4	M1	R3	S1	D4
<i>H. uvarum</i>	H2	M2*	R2	S2	D5

**MspI* has no restriction sites in 18S rDNA region of these species.

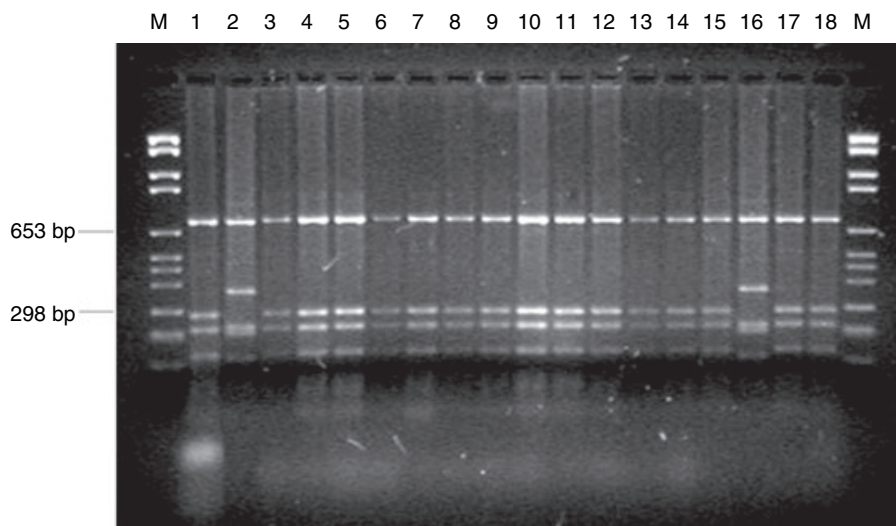


Fig. 2 PCR-RFLP analysis of 18S rDNA region of some apiculate wild strains with the restriction enzyme *DdeI*. Lane M: Molecular Weight Marker VI (Roche Diagnostics); lanes 1–18: wild apiculate strains H1, H19, H3, H8, H9, H11, H12, H13, H16, H17, H18, H21, H24, H28, H32, H55, H45, H50

Technological characterization of *H. uvarum* strains

The 52 strains of *H. uvarum* were further characterized for some parameters of technological interest in oenology, in order to ascertain the existence of a significant phenotypic variability, as a result of the genetic polymorphism of strains studied. The strains were tested for proteolytic, β -glucosidase and β -xylosidase activities, as well as for the capacity to produce higher alcohols, acetic acid, acetaldehyde and acetoin during grape must fermentation. The data obtained by technological characterization were elaborated and submitted to cluster analysis. In Fig. 4 the resulting dendrogram can be seen. At a similarity level <0.5 two main groups, PA and PB, were identified; these groups could be further subdivided in subgroups PA1 and PA2 and PB1 and PB2. Table 2 reports the average and range values for enzymatic and metabolic activities, calculated on all the strains and separately for the different groups phenotypically individuated. By analysing the data reported in Table 2, the variables which differentiated the strains in groups were acetic acid levels and β -xylosidase activity. The majority of the strains grouping in PA were characterized by high acetic acid production levels and low β -xylosidase activity. In particular, the subgroup PA1 included strains exhibiting the highest production levels of acetic acid and the lowest values for β -xylosidase activity. The subdivision into the subgroups PA1 and PA2 was determined by β -glucosidase activity: the strains belonging to the subgroup PA1 did not exhibit β -glucosidase activity (except strain H35, which resulted separated from the others by the cluster analysis in Fig. 4), whereas the subgroup PA2 consisted of strains possessing the highest activity of this enzyme.

Conversely, the strains belonging to the group PB were characterized by a low production level of acetic acid and

high β -xylosidase activity, in particular PB2 grouped the apiculate strains exhibiting the highest level of this enzymatic activity.

By the comparison between strain genetic and technological characterization, a significant correlation was found between genetic and technological characterization: the strains possessing the biotype C (Fig. 3) were included, for the majority, in the phenotype PB (Fig. 4), especially in the group PB2, whereas the major part of the strains exhibiting the biotypes D and E were included in the phenotype PA.

DISCUSSION

The aim of this study was to evaluate the natural genetic and technological variability of apiculate wild strains, isolated from 'Aglanico del Vulture' grape musts of different zones in the production area of this wine.

In the first step, the restriction analysis of 18S rDNA was used in order to identify, among the isolates, the most encountered apiculate species, which resulted in most of the strains tested being *H. uvarum*. By using PCR-RFLP analysis of this region with the restriction enzyme *DdeI* all the *Hanseniaspora* wine species were discriminated, whereas by using the others restriction enzymes different species exhibited identical restriction patterns. In particular, *H. uvarum* type strain showed the same profile of *H. guilliermondii* with all the endonucleases tested, except *DdeI*. This result indicates that these two species are closely related, as reported by other authors: Esteve-Zarzoso *et al.* (2001) found that *H. guilliermondii* and *H. uvarum* revealed an identical chromosomal profile, whereas other authors (Cadéz *et al.* 2002) found a high relatedness between these two species by means of ITS-RFLP analysis.

The application to the wild apiculate strains of a polyphasic approach based on analysis of genetic and

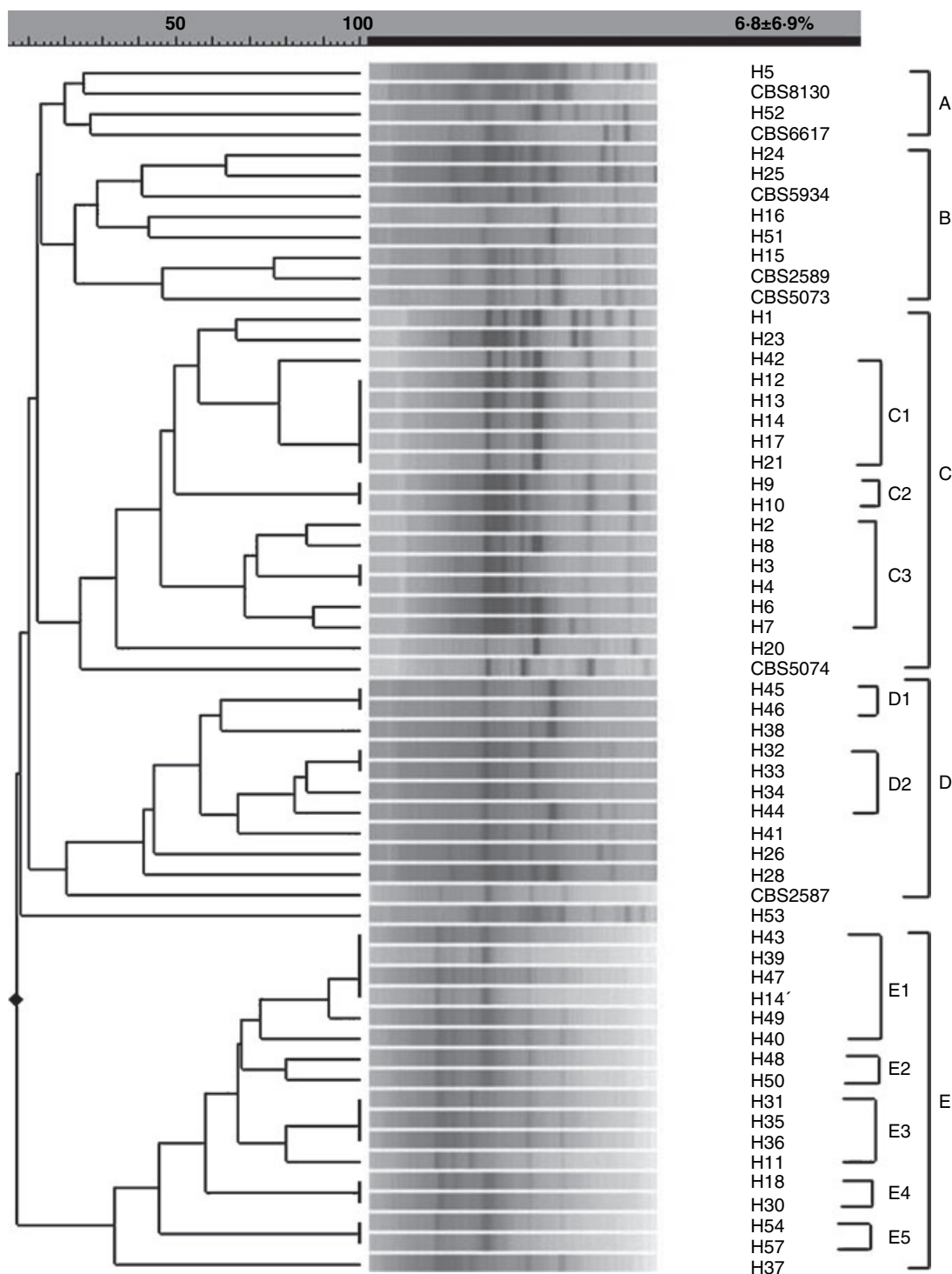


Fig. 3 UPGMA cluster analysis of 59 *Hanseniaspora uvarum* strains based on RAPD-PCR fingerprints

technological traits, has provided reliable information about the polymorphism of the species *H. uvarum*, also in function to the origin.

The cluster analysis of the RAPD-PCR fingerprints revealed five distinct groups characterized by a very low degree of similarity among them. A certain correlation was

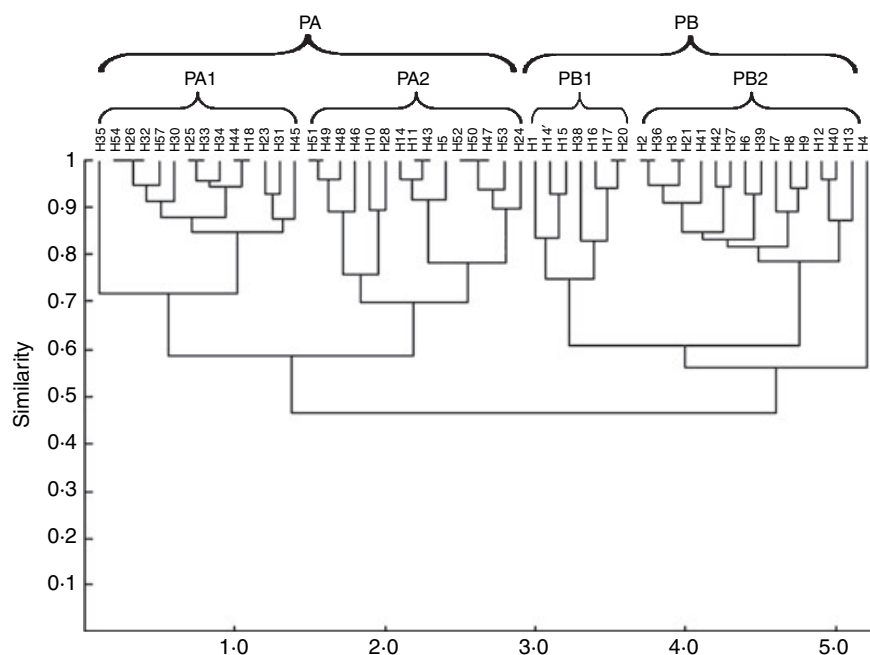


Fig. 4 Dendrogram obtained by cluster analysis based on data of technological characterization of 52 *Hanseniaspora uvarum* wild strains

Strains	Enzymatic activities			By-products (mg l ⁻¹)			
	PROT	β -GLU	β -XYL	Σ HA	ACE	ACET	ADE
All strains							
Average	1.25	5.61	0.71	84.92	158.88	2211.67	25
Range	0.38–1.73	0.28–27	0.2–32	38–173	49–279	649–7736	10–93
Group PA							
Average	1.30	8.72	0.37	81.03	167.21	2793.66	28
Range	1–1.73	0.28–27	0.1–28	53–109	88–251	1322–7736	13–93
Subgroup PA1							
Average	1.39	2.19	0.06	81.79	175.43	3536.57	23.07
Range	1.04–1.73	0.8–67	0–0.86	53–109	106–251	2100–7736	15–32
Subgroup PA2							
Average	1.22	14.80	0.65	80.33	159.53	2100.27	32.60
Range	1–1.58	7–28.27	0.1–28	62–102	88–222	1322–5119	13–93
Group PB							
Average	1.20	1.69	1.14	89.83	148.39	1477.87	21.22
Range	0.38–1.53	0–6.71	0.2–32	38–173	49–279	649–2954	10–32
Subgroup PB1							
Average	1.33	1.20	0.52	81.86	172.43	1405.29	18.86
Range	1.15–1.53	0–3.96	0.1–27	50–106	117–279	905–1817	12–29
Subgroup PB2							
Average	1.14	1.90	1.41	93.31	137.88	1509.63	22.25
Range	0.38–1.53	0–6.71	0.81–2.32	38–173	49–202	649–2954	10–32

PROT = proteolytic activity on casein ($\mu\text{mol tyrosine ml}^{-1}$); β -GLU = β -glucosidase activity ($\mu\text{m p-NP h}^{-1} \text{ ml}^{-1}$); β -XYL = β -xylosidase activity ($\mu\text{m p-NP h}^{-1} \text{ ml}^{-1}$); Σ HA = higher alcohols (*n*-propanol, isobutanol, amyl alcohols); ACE = acetoin; ACET = acetic acid; ADE = acetaldehyde.

found between the groups and the strain isolation origin, indicating the influence of the environment on the genetic patrimony of the population.

A very high phenotypic variability among the *H. uvarum* wild strains corresponded to the genetic polymorphism. The evaluation of the expression of some parameters of techno-

logical importance in oenology, such as the production level of some by-products and enzymatic activities, revealed the existence of a wide phenotypic biodiversity in *H. uvarum* strains. Yeast strains possessing proteolytic activity are of interest in winemaking because this activity could improve the wine clarification, whereas the glycosidasic activities contribute to aroma formation. The technological variables, which determined the strain biodiversity, differed among the different groups, demonstrating that these technological traits are strain dependent. The strains belonging to the biotype C and phenotype PB can be considered as more suitable for winemaking because these apiculate yeasts are characterized by a general low production level of acetic acid. About 70% of the strains of this group produced $<1.5 \text{ g l}^{-1}$ of acetic acid, whereas only 12% of the strains included in the groups D and E assessed the ability to produce low level of acetic acid.

Taking into account that apiculate strains, as dominant yeasts of early phase of grape must fermentation, can influence positively or negatively the sensory quality of the wine, the selection of suitable strains to use in mixed or sequential fermentation with *S. cerevisiae* becomes of practical interest. In this context, the wide strain biodiversity found in *H. uvarum* represents the basis for organizing a collection of apiculate strains possessing oenological characters at different levels and, therefore, potentially useful for a selection program.

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