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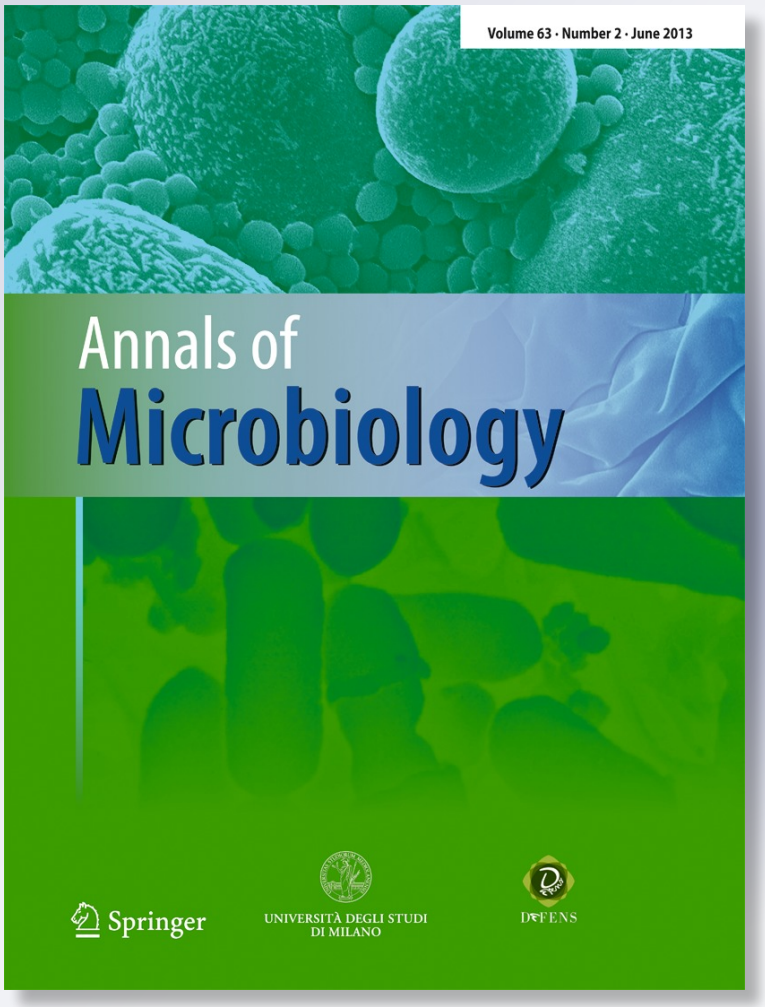


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ORIGINAL ARTICLE

# Polymorphism detection among wild *Saccharomyces cerevisiae* strains of different wine origin

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**Abstract** In this study, wild *Saccharomyces cerevisiae* strains, isolated from spontaneously fermenting grapes of different varieties and origins, were submitted to genetic analysis using different molecular techniques, such as amplification of genes coding for cell wall proteins and containing minisatellite-like sequences, karyotyping, mtDNA-RFLP, and analysis of the $\delta$ region. The lowest discriminative power was obtained by minisatellites analysis, in particular the amplification of *AGA*1 genes. Karyotyping and mtDNA-RFLP analysis yielded the same differentiation among the strains, whereas the PCR amplification of $\delta$ sequences resulted the best method as it was fast and it showed a very high discriminative power. In any case, it has to be underlined that some strains, showing the same delta profiles, exhibited a different mtDNA restriction profile and electrophoretic karyotype, suggesting that more than one molecular marker is required for reliable strain discrimination. Although the techniques used revealed a different resolution power, they all revealed a genetic relationship among strains isolated from spontaneous fermentation of grapes of different origins. In fact, none of the typing methods was able to discriminate some strains isolated from different areas.

**Keywords** *Saccharomyces cerevisiae* · Wine strains · Minisatellites · Karyotyping · mtDNA-RFLP · Interdelta region

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## Introduction

The efficient fermentative metabolism of *Saccharomyces cerevisiae* allows the predominance of this yeast during the fermentative process, leading to recognition of *S. cerevisiae* as the principal wine yeast. During the spontaneous fermentation, different *S. cerevisiae* strains dominate the process; in fact, over 100 genetically distinct strains of this species have been reported in some fermentations (Pramateftaki et al. 2000; Torija et al. 2001), whereas other studies have reported that only few strains were found to persist (Frezier and Dubourdieu 1992). The distinctive character of many wines can be linked to particular strains of *S. cerevisiae* involved in the fermentation (Romano et al. 2008). Consequently, differentiation of yeasts at the subspecies level is an important requirement. In fact, the *S. cerevisiae* strains differ significantly in their fermentation performance and their contribution to the final *bouquet* and quality of wine, but they cannot be readily distinguished and identified using classical biochemical methods. Genetic variability of wine yeasts has been demonstrated using several methodologies of typing based on DNA polymorphisms. These techniques have enabled the population dynamics of *S. cerevisiae* strains in vineyards or wineries to be studied (Legras and Karst 2003), as well as the control of industrially dried yeast production. Techniques used to discriminate closely related yeast strains are represented by pulsed field gel electrophoresis (PFGE) (Antunovics et al. 2005; Csoma et al. 2010), randomly amplified polymorphic DNA (RAPD-PCR) (Capece et al. 2005), restriction analysis of the mitochondrial DNA (mtDNA-RFLP) (Fernandez-Espinar et al. 2001), amplified fragment length polymorphism (AFLP) (Flores Berrios et al. 2005), and amplification of interdelta regions by PCR (Legras and Karst 2003), among others.

Sometime ago, karyotyping was considered to be the reference method for strain typing. As chromosome karyotyping can be too complex, laborious and time-consuming

for the analysis of numerous yeast isolates, several other molecular methods of typing have been developed for this purpose. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) was simplified (Querol et al. 1992) to render it a fast and easy method. Digestion of mtDNA with restriction enzymes like *Hinf*I or *Rsa*I is associated to a high polymorphism, and has also been used to study the authenticity of commercial wine yeast strains. Recently, PCR amplification of $\delta$ sequences has been improved (Legras and Karst 2003; Le Jeune et al. 2006), and can now be considered as discriminant as karyotyping or mitochondrial DNA restriction analysis (Schuller et al. 2004). Some authors (Marinangeli et al. 2004a, b) found that genes coding for cell wall proteins, containing minisatellite-like sequences, cause length polymorphisms, and minisatellites fingerprinting has been revealed to be very useful to discriminate *S. cerevisiae* wine strains.

The main objective of this research was to evaluate the use of different DNA fingerprinting methodologies as an experimental approach for the study of the biodiversity among *S. cerevisiae* strains of different geographical origin. Wild strains isolated from grapes collected in different geographical areas in Italy were characterized by RFLP-mtDNA, PFGE, amplification of delta region, and evaluation of cell wall gene polymorphisms. The exploitation of the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes and to ensure the conservation of gene pools of technological importance.

## Materials and methods

### Yeast strains

Twenty-three wild strains of *S. cerevisiae*, belonging to the collection of Fermenting Yeasts Laboratory of the Basilicata University, were used. The strains were previously isolated from spontaneously fermented grapes, which were collected aseptically in vineyards cultivated with different varieties in central–southern Italian regions. The strains studied were maintained on YPD medium (1 % (w/v) yeast extract; 2 % (w/v) peptone; 2 % (w/v) glucose; 2 % (w/v) agar).

### PCR amplification of *AGA*1, *SED*1 and *DAN*4 genes

The 23 strains were submitted to amplification of *AGA*1, *DAN*4 and *SED*1 genes, using the protocol described by Marinangeli et al. (2004a, b). The PCR products were analyzed by electrophoresis on a 1.4 % agarose gel in 0.5X TBE buffer (Tris–borate 0.045 M, EDTA 0.001 M, pH 8). Similarities among combined fingerprints were calculated using the Pearson product–moment correlation coefficient. Cluster analysis of the pairwise values was generated using Ward's method by means of the software Statistica for Windows, v.6.0 (Statsoft).

### Restriction analysis of mitochondrial DNA (mtDNA-RFLP)

Total DNA extraction and mtDNA restriction analysis were performed according to the protocol reported by Capece et al. (2010), by using *Hinf*I and *Rsa*I as the most suitable restriction endonuclease. Fragments were separated in 0.8 % agarose gel in 0.5× TBE buffer and the images were captured by Gel Logic 100 system (Kodak). Cluster analysis of the pair-wise values was generated using UPGMA algorithm and Jaccard coefficient by FQWest software v.4.5 (Bio-Rad).

### Electrophoretic karyotype analysis (CHEF)

Chromosomal DNA was prepared from late exponential phase cultures in low-melting-point agarose plugs as described by Miklos et al. (1997). The chromosome-size DNA molecules were separated by contour-clamped homogeneous electric field (CHEF DRII and DRIII) electrophoresis systems (Bio-Rad) in 0.8 % agarose gel (Bio-Rad) in 0.5× TBE buffer at 200 V and 14 °C, using the following program: 60 s switch time for 15 h followed by 90 s switch time for 9 h. The laboratory strain *S. cerevisiae* S288c (Yeast Genetic Stock, Berkeley, CA, USA) was used as the standard for electrophoretic karyotyping.

### Amplified inter $\delta$ sequence DNA polymorphism

Amplification reactions were performed on a Hybaid thermal cycler, using the primers δ2 and δ12, following the protocol reported by Le Jeune et al. (2006), with some modifications. The amplification of $\delta$ region was performed directly from the colony, without previous DNA extraction, by increasing the time and the temperature of initial denaturation.

PCR amplification was carried out in 50-μl reaction volumes containing 5 units of *Taq* polymerase (Promega), 10 μl *Taq* polymerase 5× buffer, 4 μl 25 mM of $MgCl_2$, 1 μl 10 mM of each dNTP, and 2 μl 25 μM for each oligonucleotide primer of the δ2 and δ12 family. Amplification was performed for 30 cycles under the following conditions: after 10 min of initial denaturation at 97 °C, each cycle consisted of 30 s denaturation at 95 °C, 30 s primer annealing at 52 °C and 1.30 min primer extension at 72 °C, followed by a 10-min final extension step at 72 °C.

PCR products were analyzed by electrophoresis in 1.2 % (w/v) agarose gel in 0.5× TBE buffer and detected, after ethidium bromide (10 μg/ml) staining, with a Gel Logic 100 system. Clustering of profiles was done using UPGMA

algorithm and Jaccard coefficient by FQWest software v.4.5 (Bio-Rad).

## Results

### PCR amplification of *AGA*1, *DAN*4 and *SED*1 genes

The 23 *S. cerevisiae* strains analyzed during this study were previously selected, based on interesting enological traits. The wild strains were submitted to the amplification of the cell wall genes containing minisatellites *AGA*1, *DAN*4 and *SED*1 by using primer pairs specific for these genes (Marinangeli et al. 2004b).

In the case of *AGA*1, all the strains produced a single amplification product (Table 1) of the same molecular weight (1,200 bp). In our case, *AGA*1 amplification was not able to differentiate the wild *S. cerevisiae* strains; contrary to these results, Marinangeli et al. (2004b), analyzing a population of wild *S. cerevisiae*, found that the resulting genes *AGA*1 were highly polymorphic in length. The amplification with the primer pair specific for *SED*1 genes yielded a single amplicon for all the strains and two different profiles (Table 1), characterized by two different molecular sizes (1,000 and 1,200 bp). Among the genes analyzed, *DAN*4 proved to be the highest polymorphic one in the wild *S. cerevisiae* analyzed. Also, the amplification of *DAN*4 gene yielded a single amplicon for all the studied strains, characterized by four different molecular sizes (approximately 1,200, 1,310, 1,420 and 1,500 bp). The *DAN*4 PCR profile H was the most common, occurring in strains isolated from almost all the grape varieties analyzed, while the profile P occurred very rarely and was exclusive of *S. cerevisiae* isolated from Inzolia grape variety (Table 1).

The data resulting from the amplification profiles were converted into binary matrices and cluster analysis was carried out. According to the resulting dendrogram, the use of the primer pairs specific for *AGA*1, *DAN*4 and *SED*1 highlighted the existence of 5 clusters (I–V) within the population of 23 strains (Fig. 1). The resulting strains were distributed randomly in the five groups; no correlation between strain origin and grouping was found, except for

**Table 1** Molecular profiles obtained by all DNA typing methods used

| Strain | Grape variety | *AGA* 1 | *SED* 1 | *DAN* 4 | RFLP-mtDNA | | CHEF | δ region |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Rsa* I | *Hinf* I | | |
| CB1-7Sr3 | Nero d'Avola (Sicily) | A | II | M | II | B | B2 | 2 |
| CD7-9Sv2 | | A | II | H | I | A | A | 1 |
| RB7-4Sv3 | | A | I | H | III | C | B1 | 3 |
| SA6-31 | Sangiovese (Tuscany) | A | II | H | I | A | A | 1 |
| SB8-8 | | A | II | H | I | A | A | 1 |
| SC1-8 | | A | II | H | I | A | A | 1 |
| SC5-25 | | A | II | H | I | A | A | 1 |
| SC7-15 | | A | II | H | I | A | A | 1 |
| SC9-11 | | A | II | M | II | B | B2 | 4 |
| SC9-42 | | A | II | H | I | A | A | 1 |
| BA-215 | | A | I | L | XI | L | C | 5 |
| AA5-5 | Inzolia (Sicily) | A | I | P | VI | F | B3 | 6 |
| AB2-6 | | A | I | L | VII | O | B6 | 7 |
| AC3-1 | | A | I | P | VI | F | B3 | 6 |
| RA3-9 | | A | I | H | V | E | B9 | 8 |
| TA4-10 | | A | I | L | IV | D | B4 | 9 |
| E1-6 | Aglianico del Vulture (Basilicata) | A | II | H | I | A | A | 1 |
| E2-20 | | A | II | M | II | B | B2 | 10 |
| AGME-5I | | A | I | H | XII | N | B10 | 6 |
| 4 LBI-3 | | A | I | L | X | J | B7 | 11 |
| GD1-3S | Fiano (Campania) | A | I | L | VIII | G | B5 | 12 |
| VEME-2 | | A | I | H | IX | H | B8 | 13 |
| BNME-6 | | A | I | L | VIII | G | B5 | 12 |

For each method, a different number/letter was assigned to distinct patterns

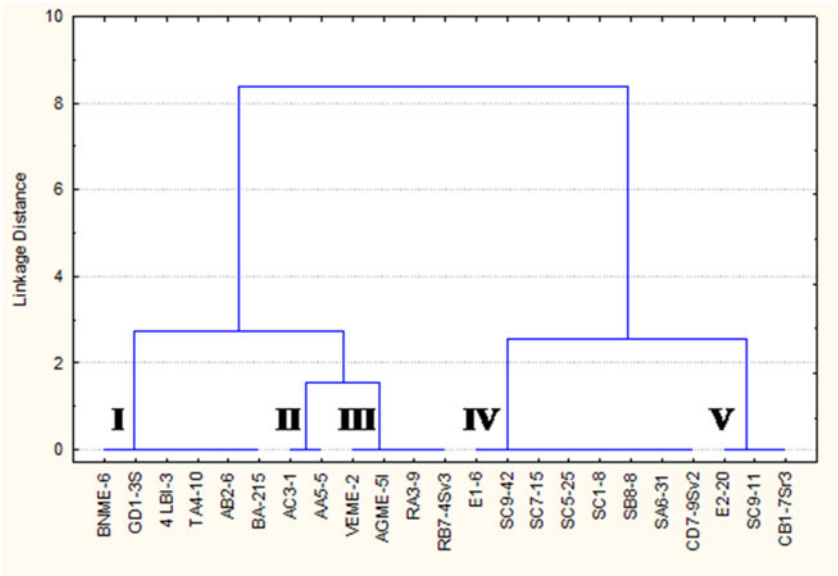


**Fig. 1** Cluster analysis of combined results obtained by amplification of *AGA*1, *DAN*4 and *SED*1 genes from 23 *S. cerevisiae* strains

strains included in cluster II (both isolated from Inzolia variety) and some (6/8) of strains included in the group IV (isolated from Sangiovese).

## Restrictions fragments length polymorphism of mitochondrial DNA (mtDNA-RFLP) and electrophoretic karyotyping analysis (CHEF)

The restriction analysis of mtDNA by using two different endonucleases (*Rsa*I and *Hinf*I) yielded the same results. In addition, mtDNA restriction analysis and electrophoretic karyotyping showed the same degree of polymorphism between the strains. These analyses revealed the presence of 12 different patterns among the 23 *S. cerevisiae* strains (Table 1). Each strain isolated from Nero d'Avola and Aglianico del Vulture exhibited a specific mtDNA restriction pattern and chromosomal profile. On the contrary, among the 8 *S. cerevisiae* from Sangiovese variety only 3 different mtDNA restriction patterns and chromosomal profiles were found.

The patterns obtained by CHEF were conserved among the strains tested, but slight differences were detected, in particular in the number and molecular weight of the chromosomes.

Comparison of strain chromosomal patterns (Table 1) revealed a high degree of polymorphism that affected almost all bands. Three classes of patterns (A, B and C) were clearly distinguished with only slight differences within each class. The profile A differed from B and C essentially for the absence of a band at chromosome IX (450 kb). Within the profile B, 10 different molecular patterns (named B1–B10, Table 1) were identified, on the basis of the differences in the region of the highest chromosomes (680–2,200 kb). The profile C, shown only by the strain BA-215, isolated from Sangiovese, differed from the profile B for the presence of 2 bands instead of 3 in the region of the smallest chromosomes (225–370 kb). In general, in all the strains tested, the chromosomes in this region were completely separated (3 bands), whereas the reference strain S288c yielded only 2 bands.

The relationship among strains according to their mtDNA-RFLP and chromosomal patterns was evaluated using cluster analysis. The same strain distribution was achieved with both the techniques. Figure 2 reports the dendrogram obtained by cluster analysis of the mtDNA-RFLP profiles with *Hinf*I enzyme. Using a similarity coefficient of about 90 %, it was possible to differentiate four clusters (I, II, III and IV) and eight single-strain clusters.

It is worth noticing that some strains, grouped together (cluster I, II and III), came from different grape varieties, whereas cluster IV exclusively grouped strains isolated from the same variety (Inzolia).

## Amplified inter δ sequence DNA polymorphism

The amplification of inter-δ sequences with the improved primer pair δ2 and δ 12 (Le Jeune et al. 2006) was used for further strain discrimination. Delta primers produced 13 different PCR profiles, thus yielding the highest discrimination among the wild *S. cerevisiae* strains tested. The dendrogram resulting from PCR analysis with delta primers, by considering a similarity coefficient of about 90 %, revealed the presence of three clusters (I, II and III) and ten single-strain clusters (Fig. 3). The strain distribution was not related to strain origin; in fact, only the main percentage of strains from Sangiovese (6/8) grouped together (cluster III), although other two strains of different origin (Nero d'Avola and Aglianico del Vulture) were included in this group.

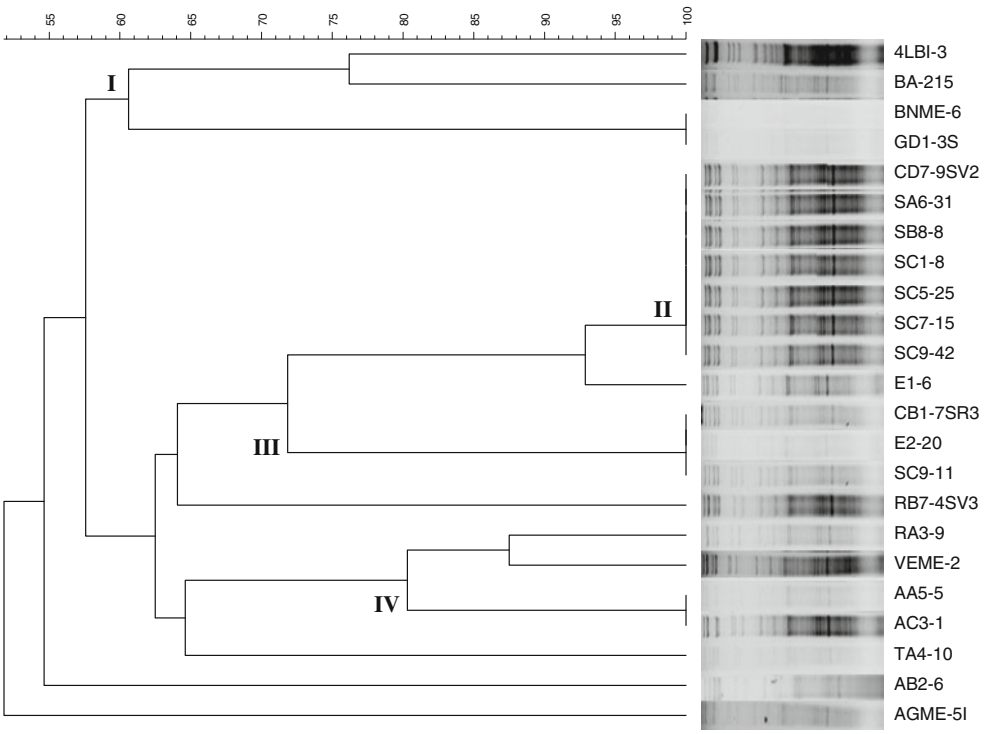


**Fig. 2** Dendrogram illustrating the similarity of 23 *S. cerevisiae* strains based on mtDNA restriction profiles generated by *Hinf*I digestion

Combined analysis of data obtained by molecular methods

For each strain and for each molecular method, a matrix value of one or zero was assigned to denote the presence or absence of different fingerprintings. The data resulting from profiles obtained by different molecular tools (amplification of minisatellites and delta regions, mtDNA-RFLP and CHEF) were combined to perform cluster analysis. The obtained dendrogram (Fig. 4) showed 2 main groups, indicated with I and II. The cluster I includes strains exhibiting

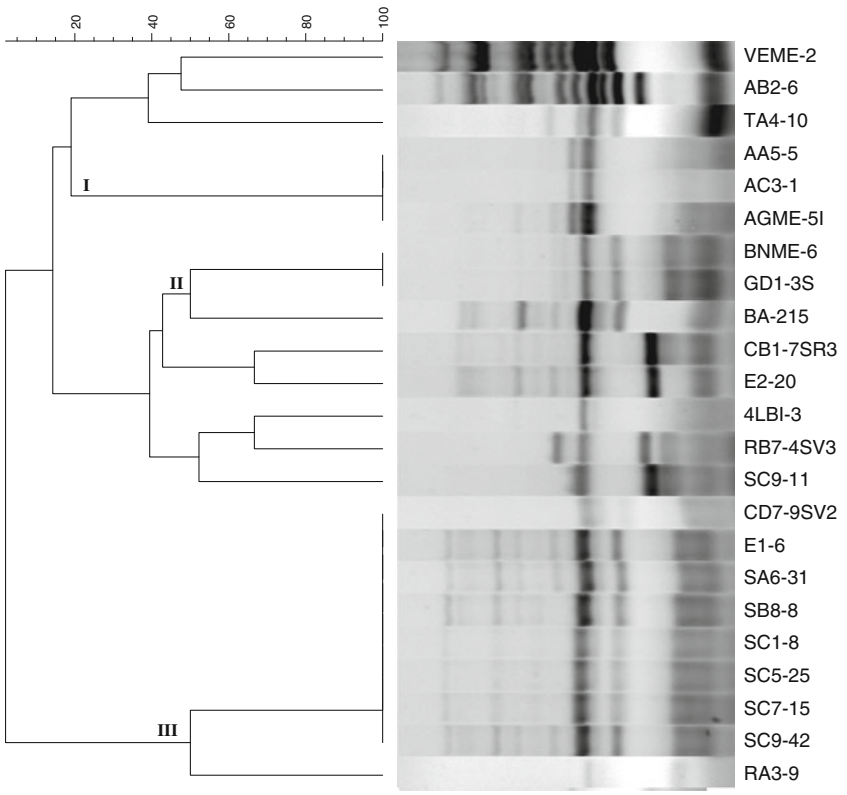


**Fig. 3** Cluster analysis of profiles obtained by amplification with delta primers from 23 *S. cerevisiae* strains

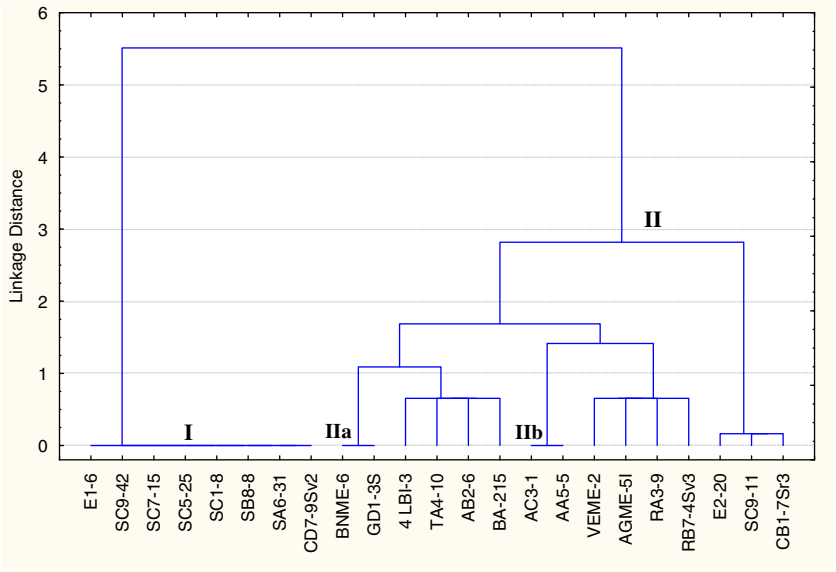


**Fig. 4** Cluster analysis of combined results obtained by all DNA typing methods from 23 *S. cerevisiae* strains

an identical biotype with all tested technique, such as the strains grouped in the IIa and IIb clusters. Furthermore, in group II, 11 single-strain clusters are included. As previously reported, no strict correlation between strain origin and grouping was found, except for most of the Sangiovese strains, which represented the main percentage of strains included in group I. The combination of all the molecular techniques used allowed the differentiation of 14 different biotypes among the 23 wild *S. cerevisiae* strains analyzed.

## Discussion

The high genetic diversity of *S. cerevisiae* wine strains has been shown through multiple analyses at the molecular level (Schuller et al. 2004), and, recently, diversity in yeast populations was demonstrated by genome sequencing of yeasts from different geographic origins (Liti et al. 2009).

In this study, 23 wild *S. cerevisiae* strains, isolated from spontaneous fermentation of grapes collected in vineyards of different varieties and locations, were analyzed by applying different methods to genetically differentiate these wild strains. As summarized in Table 1, depending on the technique used, distinct levels of discrimination were obtained, varying from 1 to 14 different patterns.

The lowest discriminative power was obtained by minisatellites analysis. In contrast, Marinangeli et al. (2004a, b), by analyzing a population of wild *S. cerevisiae*, demonstrated that the genes *SED*1, *AGA*1, *DAN*4 and *HSP*150 are highly polymorphic in length and represent a source of unexplored genetic variability. These authors suggested the use of these genes as preferential targets for PCR-based typing of *S. cerevisiae* wine strains. In our case, only *DAN*4 resulted high polymorphic in the population analyzed, and the combination of the results of all the genes analyzed highlighted the existence of only 5 different biotypes among the 23 strains (Table 1). Both mtDNA restriction analysis and electrophoretic karyotyping have been used in numerous studies related to the yeast ecology of spontaneous fermentations and biodiversity (Beltran et al. 2002; Schuller et al. 2004, 2005; Torija et al. 2001; Valero et al. 2005). In our study, both methods had a very similar resolving power at the strain level. In addition, as reported by other authors (Jeyaram et al. 2008; Martínez et al. 2007; Versavaud et al. 1995), for both the mtDNA restriction and electrophoretic karyotype analyses, there was no evident correlation between the similarity of the electrophoretic patterns and strain geographic origin, although some clusters of strains with a common origin were observed (cluster IV and some strains included in cluster II; Fig. 2).

The discrimination power of *S. cerevisiae* strains by PCR-based inter-δ typing depended on the primer pairs used. Amplification with the initially described primer pair δ1–δ2 has been reported to be very useful for the typing of commercial strains (Ness et al. 1993). However, for the delimitation of genetically closely related indigenous yeast strains, this method has a low discrimination power, whereas the inter-δ typing with an optimized primer pair, δ2 and δ12, had almost the same level of discrimination as pulsed field karyotyping (Legras and Karst 2003; Martínez et al. 2007). Our results are consistent with the ones previously described; in addition, of the four methodologies evaluated, the PCR of delta sequences presents the lowest value of similarity among the 23 strains. By considering that this technique is easy to use and highly discriminative, we suggest this technique as the most appropriate method for fast and reliable strain typing of *S. cerevisiae*. However, it has to be underlined that the strain AGME-5I, showing the

same delta profiles of AA5-5 and AC3-1, exhibited a different mtDNA restriction profile and electrophoretic karyotype (Table 1). This result suggests that more than one molecular marker is required for reliable strain discrimination (Fernandez-Espinar et al. 2001; Schuller et al. 2004).

Even though the techniques used have a different power of resolution, they all reveal a genetic relationship among strains isolated from spontaneous fermentation of grapes of different origins. In fact, none of the typing methods was able to discriminate some strains isolated from different areas; for example, the strains included in group I (Fig. 4) were isolated from different grape varieties (Sangiovese, Aglianico del Vulture and Nero d'Avola) and grouped together with all the techniques used. At least two hypotheses can be raised to explain this result. One could be that the strains are very similar, although having different origins, or the explanation could be related to the techniques used, which are not sufficiently accurate to discriminate between them. Concerning the first hypothesis, there is a possibility that commercial *S. cerevisiae* strains used in the wine cellar can disseminate in the vineyards or colonize the vineyards, when oenological practices that facilitate the dispersion of these yeasts are carried out. This dissemination can reduce the biodiversity and homogenize the genetic background of the autochthonous yeast population. In addition, this practice can allow commercial strains to be erroneously recollected and selected as native strains. Other possibility can be related to yeast dissemination by natural agents, such as migratory birds, wind, or by grape movement around different regions during grape harvesting.

In conclusion, an extensive genetic characterization, by using different molecular tools, should be carried out as the first step during selection program of wine yeast starters. This would ensure that the analysis of strains or isolates that correspond to commercial strains is avoided and the selection of yeasts through their physiological, enological and organoleptic properties will be performed only on wild yeasts.

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