Curr Microbiol (2010) 61:506–514
DOI 10.1007/s00284-010-9645-y

Molecular Relationships Between *Saccharomyces cerevisiae* Strains Involved in Winemaking from Mendoza, Argentina

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Received: 2 October 2009 / Accepted: 26 March 2010 / Published online: 21 April 2010

Abstract Three molecular typing techniques were applied to assess the molecular relationships of *Saccharomyces cerevisiae* strains isolated from winery equipment, grapes, and spontaneous fermentation in a cellar located in "Zona Alta del Río Mendoza" (Argentina). In addition, commercial *Saccharomyces* strains widely used in this region were also included. Interdelta PCR typing, mtDNA restriction analysis, and microsatellite (SSR) genotyping were applied. Dendrograms were constructed based on similarity among different patterns of bands. The combination of the three techniques discriminated 34 strains among the 35 isolates. The results of this study show the complex relationships found at molecular level among the isolates that share the same ecological environment, i.e., the winemaking process. With a few exceptions, the yeast isolates were generally clustered in different ways, depending on the typing technique employed. Three clusters were conserved independently of the molecular method applied. These groups of yeasts always clustered together and had high degree of similarity. Furthermore, the dendrograms mostly showed clusters combining strains from winery and fermentation simultaneously. Most of the commercial strains included in this study were clustered separately from the other isolates analyzed, and just a few of them grouped with the strains mainly isolated from spontaneous fermentation. Only one commercial strain was clustered repetitively with a noncommercial strain isolated from spontaneous fermentation in the three dendrograms. On the other hand, this study has demonstrated the importance of selecting an appropriate molecular method according to the main objectives of the research.

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Introduction

The alcoholic fermentation of grape juice into wine by yeasts is an ecologically complex process [1]. Though many types of yeast species are associated with wine fermentation, *Saccharomyces cerevisiae* is the main species involved. Various studies on wine microbial ecology have elucidated the complex biodiversity involved in the development of the fermentation [2–7], establishing that *Saccharomyces* populations are integrated by multiple strains, even in inoculated fermentations. Consequently, it is important to have simple and appropriate methods that allow for discrimination at the strain level.

Numerous molecular techniques have been proposed to study *Saccharomyces* strain diversity, such as pulsed field electrophoresis [8], mitochondrial DNA (mtDNA) restriction analysis [9], and interdelta element PCR amplification [10, 11]. In recent years, the amplification of polymorphic microsatellite loci (SSRs, simple sequence repeats) has also been proposed as a powerful tool for *S. cerevisiae* strain differentiation [12–15].

Several studies have used molecular characterization of *Saccharomyces* to examine their biodiversity and distribution in vineyards, the geographic origin of the native isolates, and the impact of the enological practices on autochtonous ecosystems [16–18].

The "Zona Alta del Río Mendoza" (ZARM) is an important Argentinean wine region, which has optimum conditions for the growth of Malbec grapes for the production of high-quality wines. Currently, little information is available about the yeast populations involved in the winemaking process carried out in this region [5, 19]. A previous study attempted to identify the native *Saccharomyces* strains associated with winery equipment, grapes, and spontaneous fermentation on Malbec grape must from a cellar located in the ZARM region, during two consecutive years. It was demonstrated that a stable and resident *Saccharomyces* microbiota was present in the winery, which exhibited a dynamic behavior across different seasons and between years. Moreover, low occurrence of *Saccharomyces* on grapes and their limited participation during fermentation was also observed. Fermentations showed strain sequential substitution, and about 30–60% of yeast population at the end of fermentation were from the population already present in the winery. In addition, some commercial yeast strains were found during fermentation and on winery equipment in low percentage [5].

Although these results showed the microbial ecology of alcoholic fermentation in an industrial winery from Mendoza, there is still a lack of information on the genetic relationships among these *Saccharomyces* native populations.

The aim of this study was to assess the genetic relationships among *Saccharomyces* native population isolated from winery equipment, grapes, and spontaneous fermentation in a cellar located in "Zona Alta del Río Mendoza" (Argentina). Three molecular typing techniques (interdelta amplification analysis, mtDNA restriction analysis, and SSR genotyping) were applied for the molecular characterization of *Saccharomyces* isolates. The molecular analysis included the comparison of the polymorphism obtained by each method and the determination of the genetic relationship among the strains from the same ecological origin and selected commercial strains.

Materials and Methods

Yeast Strains

Saccharomyces strains were previously isolated from winery equipment and spontaneous fermentation of Malbec grape must from ZARM wine region during two consecutive years [5]. Twenty-eight previously isolated strains were selected as representatives of the diversity found on winery equipment and during fermentation (Table 1). Seven commercial strains originally isolated in France and widely used in the ZARM wine region were included in this study; they were named with an alphabetical code to preserve the company's confidentiality.

Interdelta PCR Analysis

Total DNA extraction was performed as previously described by Hoffman and Winston [20]. Oligonucleotides primers delta 1 (5′-CAAAATTCACCTAT[A/T]TCTCA-3′) and delta 2 (5′-GTGGATTTTTATTCAACA-3′), or delta 12 (5′-TCAACAATGGAATCCCAAC-3′) and delta 21 (5′-CATCTTAACACCGTATATGA-3′) were used to amplify the total genomic DNA between the repeated interspersed delta sequences as previously described [10, 11]. PCR products were separated in 1.5% agarose gels in 0.5× TBE buffer. The molecular marker 100-bp DNA ladder (Promega, Madison, USA) was used as the molecular size standard. Electrophoresis gels were stained with ethidium bromide (5 μg ml^{-1}), visualized by UV transillumination, and processed using the Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA).

Mitochondrial DNA Restriction Fragment Length Polymorphism (mtDNA RFLP)

The total DNA was extracted using the Wizard Genomic DNA extraction kit (Promega, Madison, USA) according to the manufacturer's instructions. Digestions were performed with the restriction enzyme *Hin*fI as previously described [9]. Restriction fragments were separated by electrophoresis in 1% agarose gels, stained, and visualized as described earlier for PCR products.

Analysis of SSR Loci

Quick preparation of DNA template for PCR was carried out as described by Jubany et al. [13]. The set of six SSR loci used in this study (Table 2) were chosen from a previous study [13], in which a large group of SSR loci were evaluated. In that study, nine SSRs were selected and six of them showed the highest discriminative power between closely related native strains. SSRs were named following the standardized criteria recently described by Jubany et al. PCR amplifications and electrophoresis were performed as described by Jubany et al. [13].

Cluster Analysis

The patterns of bands obtained after gel electrophoresis were employed for the construction of presence/absence

Table 1 Summary of patterns obtained by all typing methods used on 28 *Saccharomyces* isolated at ZARM wine region and 7 commercial wine strains

Isolate number code	Origin of isolation	Year of isolation	Pattern assigned by each molecular method			
			PCR interdelta	"Improved" PCR interdelta	RFLP ADNmt	SSR
1	Fermentation tank[a]	2001	1	1	1	1
2	Pipe for filling of tank[a]	2002	1	2	1	1
3	Spontaneous fermentation[a]	2002	1	3	1	3
4	Pipe for must transport[a]	2002	2	4	1	4
5	Spontaneous fermentation	2001	3	5	2	5
6	Spontaneous fermentation[a]	2002	1	2	1	1
7	Spontaneous fermentation	2001	4	6	3	6
8	Fermentation tank	2001	4	7	4	7
9	Fermentation tank[a]	2001	4	6	3	8
10	Spontaneous fermentation	2002	5	8	5	9
11	Grape	2002	5	9	5	10
12	Pipe for filling of tank[a]	2002	6	10	6	11
13	Fermentation tank[a]	2002	6	11	7	11
14	Pipe for must transport[a]	2002	6	12	8	12
15	Reception hopper[a]	2002	6	13	8	12
16	Pipe for must transport[a]	2002	7	14	1	13
17	Crusher	2002	6	15	9	15
18	Fermentation tank	2002	6	10	10	11
19	Spontaneous fermentation	2002	6	10	11	15
20	Pipe for must transport[a]	2001	6	16	7	11
21	Fermentation tank[a]	2001	6	12	8	16
22	Crusher	2001	6	17	12	17
23	Spontaneous fermentation	2001	6	18	13	18
24	Fermentation tank	2001	6	6	3	19
25	Crusher[a]	2002	8	19	7	11
26	Fermentation tank	2002	8	20	14	20
27	Spontaneous fermentation	2002	8	21	15	21
28	Spontaneous fermentation	2002	8	22	16	22
29	Commercial strain A	–	9	23	17	23
30	Commercial strain B	–	10	24	18	24
31	Commercial strain C	–	11	25	19	25
32	Commercial strain D	–	12	26	20	26
33	Commercial strain É	–	13	27	21	27
34	Commercial strain F	–	14	28	22	28
35	Commercial strain G	–	15	29	2	29
	Total of different patterns		15	29	22	29

[a] Perennial strains defined by Mercado et al. [5]

matrix, taking into account the total number of different bands observed for each method. A total of 25 bands were obtained with improved interdelta PCR, 53 bands with mtDNA RFLP, and 82 fragments with the combination of the six SSR markers used. The Dice coefficient was calculated to estimate the similarity between the strains and a dendrogram was developed with the UPGMA method using NTSYSPC1.1 software (Exceter software, Setauket, USA). Cophenetic correlation as an estimator of goodness of fit of clustering was calculated by applying the same software package. The *S. cerevisiae* laboratory reference strain, S288c, was used as the out-group for the development of the dendrograms.

Results

Molecular Strain Characterization

About 28 wine *Saccharomyces* isolates from a commercial winery from ZARM (Argentina) and 7 commercial yeast strains were analyzed by applying three different molecular

Table 2 Oligonucleotide primers used for the SSR loci amplification

SSR loci	Forward primer 5′–3′	Reverse primer 5′–3′
YPL009C	CTGCTCAACTTGTGATGGGTTTTGG	CCTCGTTACTATCGTCTTCATCTTGC
YOR267C	ATGACTGCAGCAATGAATCG	TCCTCTGTGCTGTTGACTCG
TTA_XXIII	AAGCGTAAGCAATGGTGTAGA	AAGCCTCTTCAAGCATGACC
TG_VI	GACACAATAGCAATGGCCTTCA	ATATTGGCACCAGAAAGTGTCG
GT_X	TGCGCAGCTTAGTATGACCA	GATGGGCTTTCACTCCACTT
C4_XV	GAGAAAAATGCTGTTTATTCTGACC	CCTCCGGGACGTGAAATAAC

typing techniques. This group of yeasts was selected considering the results of a previous study developed in this wine region [5], in which the yeast populations analyzed were obtained from winery equipment, Malbec grapes, and musts during spontaneous fermentation. These latter samples were obtained at different stages of the process carried out in the same winery according to their own standard protocols, including the lack of inoculation with selected commercial strains, which is a current practice of this company for the production of red wines. Among the strains analyzed in this study, 13 yeasts were selected because they represent the "perennial" strains of the winery [5]. These yeasts were the isolates that were able to survive in the cellar, including the isolates found on winery equipment that were not used since the previous year, as well as those recovered from the winery in successive years. Some of these isolates were also found at the end of the spontaneous fermentation conducted in the same cellar (Table 1).

The results presented in Table 1 clearly indicate that according to the technique used, distinct levels of discrimination were obtained. Interdelta PCR with primers delta 1 and delta 2 was the least discriminative technique, distinguishing 15 patterns among the 35 strains analyzed (Table 1). When improved primers, delta 12 and delta 21, were used, the discrimination power was increased, allowing for the discrimination of 29 patterns of the *Saccharomyces* strains analyzed. On the other hand, the analysis of genetic variability of 35 *Saccharomyces* isolates using mtDNA RFLP allowed for the differentiation of 22 patterns. This technique showed a good level of polymorphism with an intermediate discriminating power, when compared with the other molecular markers used (Table 1).

The discrimination power obtained by combining the information from six SSR loci was very high, similar to that of the improved interdelta PCR (Table 1). Out of the 35 *Saccharomyces* isolates analyzed, 29 genotypes had a total of 87 alleles, ranging from 10 to 20 alleles and 12 to 24 genotypes per locus. However, the frequency of appearance of different alleles at each locus was very variable (Fig. 1). TG_VI and TTA_XIII showed a great discriminating power, differentiating 20 and 17 genotypes, respectively. Some alleles were detected more frequently, being more characteristic of the population under study. On the other hand, alleles found less frequently could indicate genotypes rarely detected. In summary, the combination of results obtained with the three applied molecular typing techniques allowed for the differentiation of 34 strains among the 35 isolates. Only two yeast isolates showed identical profile with all the molecular methods applied, and they could be considered as the same strain (Table 1).

Molecular Relationships Among the Strains

Similarities based on the Dice coefficient and UPGMA clustering from improved interdelta PCR, mtDNA RFLP, and SSR are presented in Figs. 2, 3, and 4, respectively. The dendrograms showed different degrees of relationship among the strains, according to the molecular method used. Interdelta PCR showed a superior similitude among the strains (Fig. 2), mainly because the patterns obtained by this technique have bands shared between all the isolates. The lowest genetic similitude was obtained with SSR analysis, where the first dendrogram branching was produced at a similitude coefficient value lower than 0.1. The evaluated SSR loci showed a high polymorphism between the yeast isolates analyzed, where only few of them remain closely associated (Fig. 4).

The molecular methods applied reflect the polymorphism at two levels—nuclear genomic and mitochondrial genomic level. Some strains exhibited differences in each genome, by clustering together with the nuclear markers (interdelta PCR and SSR analyses) and separately with the mitochondrial marker (RFLP mtDNA), such as isolates 12, 18, and 25 (Figs. 2, 3, and 4).

With a few exceptions, the yeast isolates were generally clustered in different ways, depending on the typing technique employed. Three clusters were conserved independently of the molecular method applied (cluster 7, 9, and 24; cluster 1, 2, and 6; and cluster 14, 15, and 21) (Figs. 2, 3, and 4). These clusters were mainly integrated by isolates from the winery equipment, and mostly constituted a part

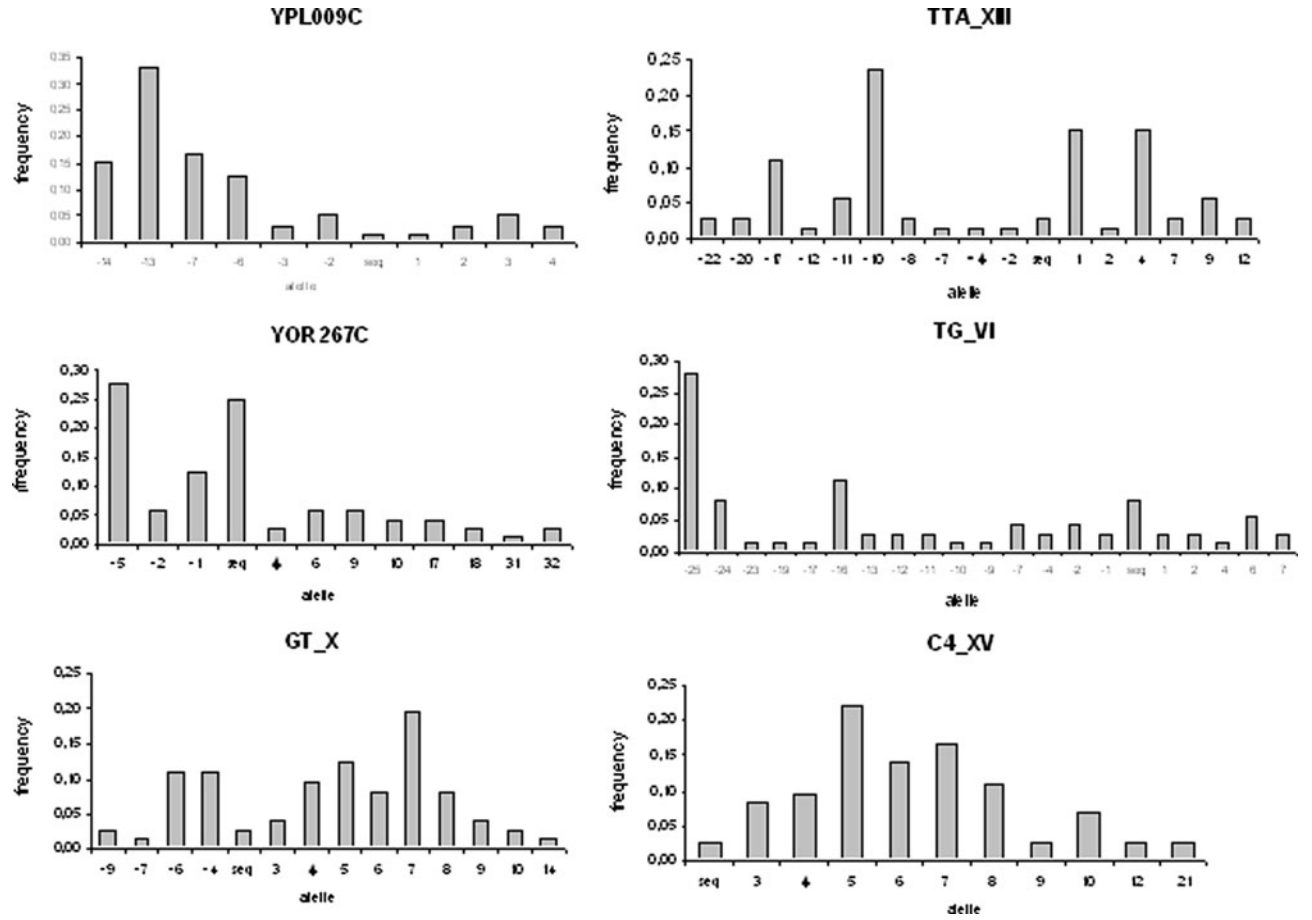

Fig. 1 Polymorphisms of alleles obtained for the six SSR loci on 28 native and 7 commercial *S. cerevisiae* wine strains. Alleles are expressed as + or − number of repeats relative to the sequenced (seq) strain

of the "perennial" population of the winery. Besides these strains, the clusters included some other strains that did not remain clustered with the three molecular markers. Furthermore, the dendrograms mostly showed clusters combining strains from winery and fermentation simultaneously (Figs. 2, 3, and 4).

Most of the commercial strains included in this study were clustered separately from the other isolates analyzed, and just a few of them grouped with the strains mainly isolated from spontaneous fermentation. Only the commercial strain 35 was clustered repetitively with a non-commercial strain, grouping with strain 5 isolated from spontaneous fermentation in the three dendrograms, and showing different degrees of similarity according to the method applied (Figs. 2, 3, and 4).

Discussion

In this study, different molecular typing methods have been applied to evaluate the genetic relationships between 28 *Saccharomyces* strains isolated in a previous study [5]. The strains were obtained in the same cellar, and included isolates widely distributed on different winery equipment, those able to survive between vintages on the cellar surfaces, as well as those frequently found in spontaneous fermentation. Seven commercial strains widely used in this region, currently employed in this winery for the production of white wines, were also included in the analysis.

Recently, new genetic techniques have been developed to provide a differentiation of *Saccharomyces* strains based on single-nucleotide polymorphisms (SNPs), like multilocus sequence typing (MLS) [21, 22] or microarray techniques [23, 24]. Nevertheless, the molecular markers selected in this study still represent the simplest and most widely used techniques to study *Saccharomyces* biodiversity. They not only provide differentiation at the strain level but also give some information about the genetic relationship.

In the first approach, the discrimination power of the molecular techniques applied to differentiate closely related *Saccharomyces* strains was analyzed. Interdelta PCR with primers, delta 1 and delta 2, was the least discriminating method, as shown in previous studies [18], but the

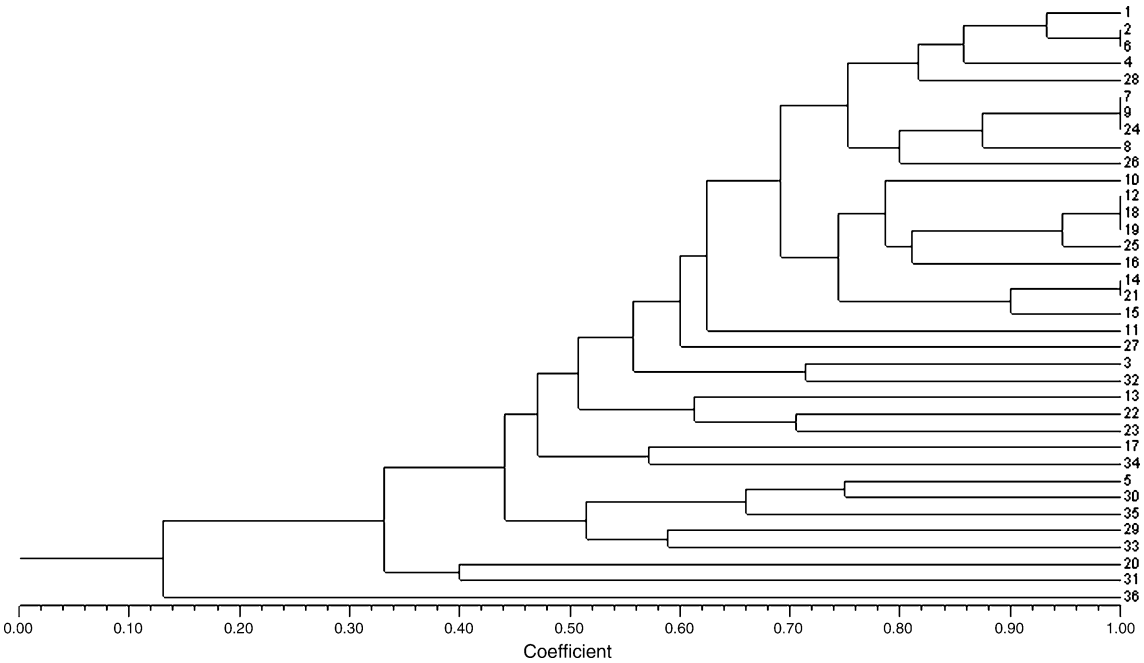

Fig. 2 Dendrogram showing molecular relationships based on improved PCR interdelta patterns of 28 wine *S. cerevisiae* isolates and 7 commercial selected strains. Cophenetic correlation $r = 0.88019$

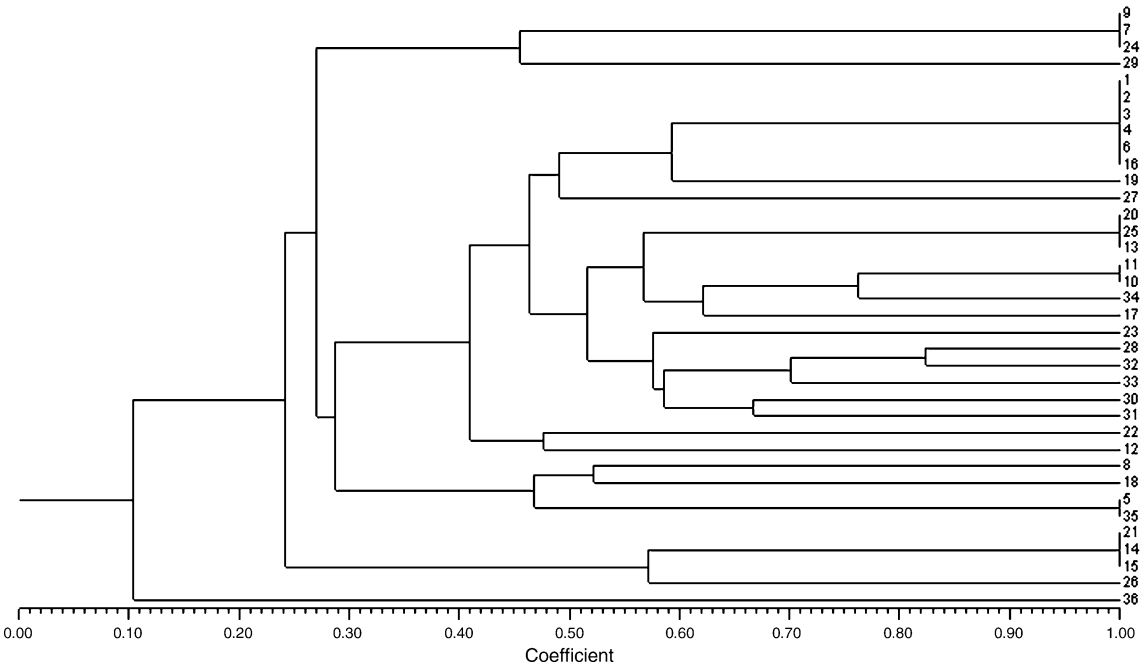

Fig. 3 Dendrogram showing molecular relationships based on RFLP mtDNA patterns of 28 wine *S. cerevisiae* isolates and 7 commercial selected strains. Cophenetic correlation $r = 0.85498$

differentiation efficiency was increased with the use of improved primers [10, 25].

The combination of the allele sizes from the six SSR loci showed a high degree of resolution, even though they were not adequate for the unequivocal characterization of the present population of 35 strains. According to the observed results, the SSR technique was not equivalent to the mtDNA RFLP, and showed higher discrimination power. Furthermore, the same/different SSR amplification profile did not always corresponded to the same/different

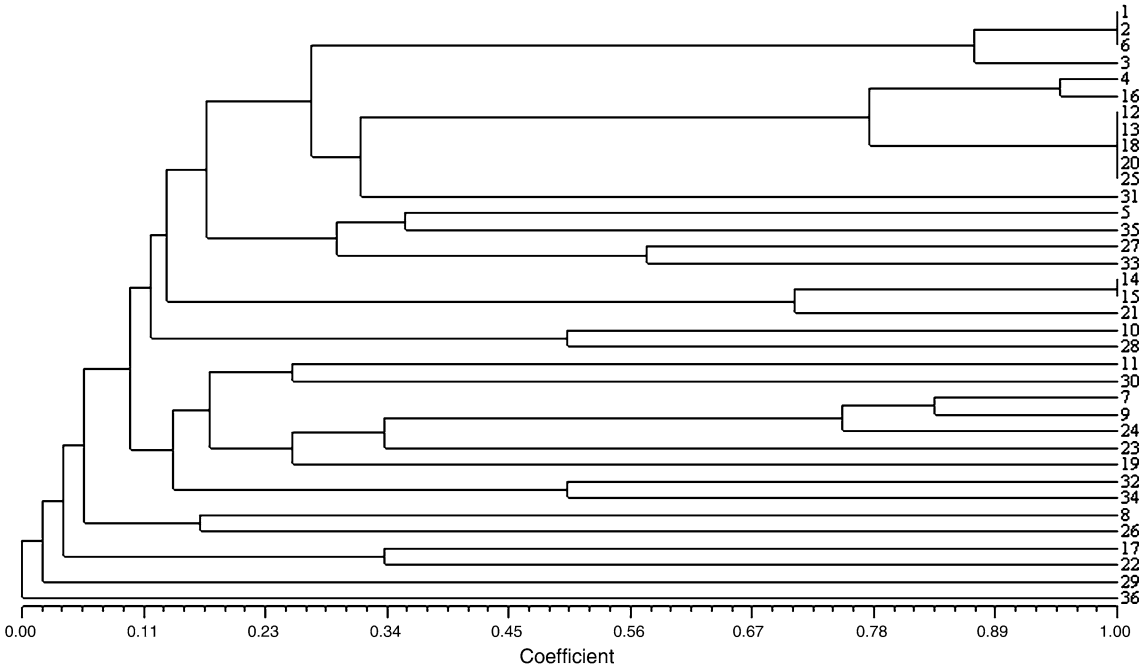

Fig. 4 Dendrogram showing molecular relationships based on SSR analysis patterns of 28 wine *S. cerevisiae* isolates and 7 commercial selected strains. Cophenetic correlation $r = 0.88078$

mtDNA RFLP patterns, as reported previously [18, 26]. In spite of this result, mtDNA RFLP analysis was adequate for the differentiation of yeast strains from the same ecosystem, because although some isolates were not discriminated by this technique, they remained closely related when the SSR method was used. Numerous authors have demonstrated that mtDNA RFLP analysis is an efficient technique to differentiate at the strain level [9, 18, 27, 28]. According to the results of this study, studies aiming to characterize the strains that are genetically closely related would need the inclusion of other SSR loci or the combination of SSR with other molecular typing techniques.

Molecular relationships among *Saccharomyces* strains from the same environment, represented by equipment, grapes, and musts spontaneously fermented from a winery located in the ZARM region, were evaluated, and complex relations were evidenced.

Only nine yeast isolates were repetitively grouped in three clusters. These groups comprised some of the "perennial" yeasts that were isolated from different winery equipment during the two vintages and were also found at the end of fermentation [5]. As shown in the dendrograms, these groups of yeasts always clustered together and had high degree of similarity. The isolates included in each of these clusters showed identical mitochondrial restriction patterns and the discrimination among them was achieved by molecular markers related to the nuclear repetitive sequences. It has been proposed that recombination processes could be favored by the presence of repeated sequences that may increase the probability of ectopic recombination events that generate different molecular patterns [29]. It is presumed that some kind of change at the nuclear level occur during the permanence of yeasts in the winery. Such changes could allow for yeast adaptation to the winery environment and survival from one vintage to the following by improving their competitive traits and tolerance to stress. Similar mechanism has been proposed by Bond to explain the dynamic genome of lager yeasts [30]. The monophyletic origin of each of these clusters and its rare relationship with the evaluated commercial strains suggest their American origin and the recent occurrence of microevolutionary events.

On the other hand, the rest of the isolates from the winery and fermentation showed a random clustering according to the molecular marker applied; this result suggests some kind of change at nuclear level, which could occur at a different rate in the nucleus with respect to the mitochondria, during the yeast life cycle. Recent studies have demonstrated a low stability of the genome in wine yeast [31], which may be due to the high reorganization capacity of its genome by Ty-promoted translocation, mitotic recombination, and gene conversion [16, 29, 32]. Alternatively, it has been proposed that ethanol and acetaldehyde introduce breaks in the DNA, with a much higher mutation rate on the mitochondrial genome. This may be due to a higher efficiency of the yeast nuclear DNA repair

system compared with the mitochondrial system that lacks proofreading activity [16, 33].

In this study, the commercial strains of European origin were not restricted to a sole cluster, but were generally clustered separately or appeared related to the fermentation isolates. The existence of some genetic relation between a few "winemaking-related" strains with some of the commercial strains could support the hypothesis that some American native strains may proceed from European strains as it was recently suggested [13]. Conversely, it has been suggested that the existence of "genomic resemblance" among the native *Saccharomyces* and commercial strains, caused by centuries of positive selection during wine production, could lead to the same characteristics explored during the process of selection for obtaining commercial cultures [23]. These authors suggest that resemblance in phenotype is reflected in genotypic characteristics.

Results of this study show the complex relationships found at the molecular level among the yeast isolates that share the same ecological environment. Moreover, this study has revealed that beyond the abundant diversity observed, the yeasts share many genetic characteristics. On the other hand, this study has demonstrated the importance of selecting an appropriate molecular method according to the main objectives of the research.

Acknowledgments This study was supported by Viticulture Regional Project MZASJ O7 from the *Instituto Nacional de Tecnología Agropecuaria* (*INTA*) (and Project PDT 32/06 from Dinacyt, Uruguay). L.M. is a fellow of *Consejo Nacional de Investigaciones Científicas y Técnicas* (*CONICET*) and *Programa de Doctorado en Ciencias Biológicas* (*PROBIOL*), *Universidad Nacional de Cuyo.*

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