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Genetic and Molecular Diversity in *Saccharomyces cerevisiae* Natural Populations

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Summary

Two Saccharomyces cerevisiae populations isolated from grapes collected in two geographically distinct Italian regions, Tuscany and Sicily, were partially characterized by a multimethodological approach including genetic and molecular techniques. A total of 166 isolates, 61 from Sicily and 105 from Tuscany were characterized by random amplification of polymorphic DNA analysis. Preliminary results, obtained on 36 strains that were not differentiated with the above mentioned technique, showed that genetic analysis combined with restriction fragment length polymorphism using the DC4 probe are the most powerful combination of methods. Using this approach we were able to show that 34 of the 36 strains were unique and the remaining two fell into one class. This demonstrates a high level of biodiversity in these populations.

Keywords: Saccharomyces cerevisiae, biodiversity, genetics, wine yeast, DNA fingerprint

Introduction

In recent years the study of ecology and biodiversity of *Saccharomyces cerevisiae* strains isolated from natural environments has developed rapidly. This was mainly due to the application of genetic analysis *(1)* as well as to the improvement of the techniques addressed to the analysis of DNA polymorphisms.

Molecular methods used to characterise *Sacch. cerevisiae* strains include: a) pulsed field gel electrophoresis (PFGE) *(2,3)*; b) restriction fragment length polymorphism (RFLP) *(4)*; c) techniques exploiting polymerase chain reaction (PCR) such as random amplification of polymorphic DNA (RAPD) *(5,6)*, or amplification with primers targeting specific sequences, eventually associated with restriction analysis of the amplified fragments (PCR-RFLP) *(6–8)*; d) restriction enzyme analysis on mitochondrial *(9)* or genomic DNA *(10)*.

Although these techniques allowed the detection of diversity in *Sacch. cerevisiae* strains recovered from grapes and from natural fermentations *(1,3,11–13)*, an approach combining both genetical and molecular techniques is still lacking.

Here we describe preliminary results from the study of natural populations of *Sacch. cerevisiae* performed using the following strategy: 1) isolation of *Sacch. cerevisiae* strains from grapes and must fermentations; 2) typing of isolates by random amplified polymorphic DNA (RAPD). Strains not distinguished by RAPD analysis were analysed with the following methods: 3) Restriction Fragment Length Polymorphism (RFLP) and Pulsed Field Gel Electrophoresis (CHEF); 4) tetrad analysis examining: spore viability, homothallism/heterotallism, fermentation of sucrose, maltose and galactose, growth on a non fermentable carbon source (glycerol), copper resistance, hydrogen sulfide production; 5) sulfite resistance and killer phenotype.

Materials and Methods

Media and growth conditions. Media used were YPD medium (yeast extract 10 g/L, bacto-peptone 20 g/L, glucose 20 g/L), YPG (yeast extract 10 g/L, bacto-peptone 20 g/L, glycerol 30 g/L and 1% ethanol added

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after autoclaving), and minimal medium (MIN) (Difco yeast nitrogen base without aminoacids with $(NH_4)_2SO_4$). Media used for genetic analysis were prepared according to Mortimer *(1)*. Solid media included 2% agar (Oxoid). Growth temperature was 30 °C if not otherwise stated.

Grape collection and isolation of *Saccharomyces cerevisiae* strains. Grapes were collected in two Italian vineyards located in Tuscany (Montalcino) and Sicily (Castiglione di Sicilia), respectively. About 500 grams of grapes were placed in sterile jars, crushed with a sterile instrument and the resultant must was fermented at 20 °C. During fermentation must samples were diluted and plated on YPD. Single colonies were further isolated and *Sacch. cerevisiae* strains were selected by analyzing cell and colony morphology as well as the inability to grow on lysine agar *(14)*.

Genetic analysis. Strains were sporulated and subjected to tetrad analysis. Spores were scored for the following genetic traits: a) ability to ferment sucrose, maltose, galactose, and to grow on a non fermentable carbon source (glycerol); b) copper resistance; c) production of H_2S; d) spore viability was scored as: high>85% (H), medium between 85–50% (M), low<50% (L). Scoring was performed as described previously *(1)*.

Crude lysates from single colonies. Strains were grown 20 hours on YPD plates. Cells from colonies of about 1 mm in diameter were picked up, suspended in 50 μL of a 200 μg/mL zymolyase solution in sterile water, and incubated for 10 min at 37 °C. Cells were pelleted by centrifuging for 1 min at room temperature (RT), resuspended in 20 μL of sterile water, heated for 5 min at 95 °C and cooled on ice.

RAPD analysis. Two different oligonucleotides were used in RAPD experiments, 1283, 5'GCGATCCCCA3', RF2, 5'CGCGCCCCTGT3'. Primers, synthesized by standard phosphoramidite chemistry, were deprotected, dried, dissolved in TE (10 mM TRIS-HCl pH = 7.5, 1 mM EDTA) and used without further purification. Amplification reactions were carried out as described previously *(5)* using 5 μL of crude lysates and 0.25 U of Super Therm DNA polymerase (Advanced Biotechnologies Ltd). Electrophoresis conditions and the analysis of the amplification products were performed according to Paffetti *et al. (5)*.

Extraction and restriction of genomic DNA. DNA was extracted from frozen yeast cells as described by Barberio *et al. (10)*. DNA samples were tested spectrophotometrically for purity according to Sambrook *et al. (15)* and stored at 4 °C. Five micrograms of DNA were treated with 15 units of *Hind*III restriction enzyme (Boehringer, Mannheim) at 37 °C for 14 h. DNA fragments were separated by electrophoresis on 0.8% agarose gel in TEA buffer (0.04 M Tris-acetate, 0.001 M EDTA pH = 8) at 1.5 V/cm for 16 hours.

Probe preparation and Southern hybridization. The probe used for Southern hybridization (DC4) was an anonymous sequence of 2.4 Kb. It was obtained by *Hin*dIII restriction of a plasmid clone from a *Sacch. cerevisiae* genomic bank prepared in our laboratory. The probe was labelled with digoxigenin-11-dUTP and the hybridization signals were detected by using the chemiluminescence method following the instructions of the supplier (Boehringer, Mannheim). Southern blotting, hybridization conditions and analysis of the hybridization patterns were carried out as described previously *(5)*.

Pulsed field gel electrophoresis. Chromosomal polymorphisms were scored using CHEF gels, and the molecular weight marker was strain YPH80 (pulse marker 225–2200 Kb Sigma). Samples were prepared from each of the strains and run as described *(2,16)*.

Killer phenotype and sulfite resistance. Killer phenotype was checked according to Young *(17)* using *Sacch. cerevisiae* strain STX343, obtained by Yeast Genetic Stock Center as killer sensitive strain. Sulfite resistance was scored on YPD plates pH = 3.85 in the presence of 300 and 500 μg/mL sulphite *(18)*.

Results

Strain isolation. A total of 166 isolates, 61 from Sicily and 105 from Tuscany, were characterized as *Sacch. cerevisiae* strains.

Generation of random amplified polymorphic DNA. In order to screen large numbers of isolates we adopted the RAPD technique. This method is relatively fast, the limiting step being the DNA extraction. This step was shortened by setting up a method of amplification from crude lysates obtained from single colonies, as described in Materials and Methods.

Amplification by the 10-nt primers RF2 and 1283 was performed on all 166 strains. The reproducibility of the method was assessed repeating the same reactions using lysates obtained from three independent cultures of 14 randomly selected strains.

An example of the amplification profiles obtained with the two primers is shown in Fig. 1.

The analysis of the electrophoretic patterns showed that:

i) both primers gave rise to amplification profiles in all the strains examined;

ii) primer 1283 generated six amplification patterns, on Montalcino strains, characterized by a number of bands from 10 to 12 with size ranging between 280 and 1450 base pairs (bp). One profile was common to 90% of the strains analyzed. Three strain specific profiles were found. Three different amplification patterns were found for the Sicilian strains, characterized by a number of bands from 8 to 10 with size ranging between 305 and 1350 bp. The most common profile was shared by 68.8% of the strains.

iii) analysis of Montalcino population with primer RF2 generated five amplification profiles characterized by a number of bands from 12 to 14 with size ranging between 420 and 1480 bp. One pattern was common to 93% of the strains. Two amplification profiles, with 12 and 13 bands ranging from 290 to 1390 bp, differing in only one 326 bp fragment, were found in Sicilian strains. The 12-band pattern was common to 96.7% of the strains.

Combining the results obtained with both primers it was possible to distinguish 11 different pattern combinations in Montalcino strains, one common to 90% of the strains and 8 strain specific. The same analysis showed

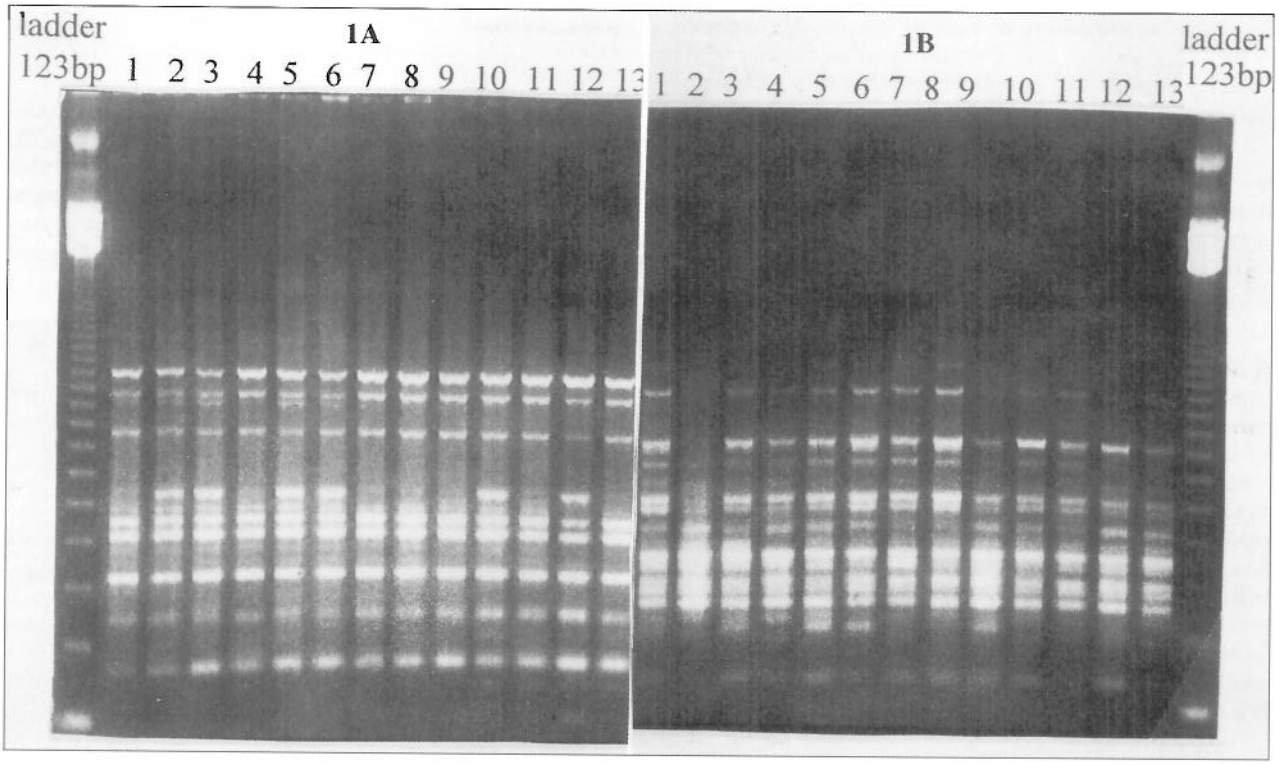

Fig. 1. An example of RAPD amplification patterns of 13 of the 105 Montalcino strains (lane 1–13). **1A** amplification with primer 1283, **1B** amplification with primer RF2. Molecular weight standard, is 123 bp DNA Ladder (the faster band is the 123 bp monomer). Amplifications patterns of lane 1 in both 1A and 1B represent the most common profile over the 105 Montalcino strains (90% of the strains with 1283 and 93% with RF2)

Table 1. Results from genetic analysis

Strain	SUC	MAL	GAL	CUP	YPG	H_2S	% spore viability	CLASS
CS4, CS6, CS11, CS15	+/+	+/+	+/+	–/–	+/+	3/2	H	1
CS5, CS13, CS16	+/–	+/–	+/–	–/–	+/+	3/2	M	2
CS8, CS17	+/+	+/+	+/–	–/–	+/+	4/2	L	3
CS1	+/+	+/+	+/+	+/–	+/+	4/3	H	4
CS2	+/+	+/+	+/–	–/–	+/+	4/2	M	5
CS3	+/+	+/+	+/–	–/–	+/+	3/2	L	6
CS7	+/+	+/–	+/+	–/–	+/+	3/2	L	7
CS9	+/+	+/+	+/–	–/–	+/+	4/3	L	8
CS10	+/+	+/+	+/+	+/–	+/+	3/2	H	9
CS12	+/–	+/+	+/–	–/–	+/+	4/3	L	10
CS14	+/+	+/+	+/–	–/–	+/+	3/2	M	11
CS18	+/+	+/+	+/–	–/–	+/+	4/3	M	12
M1,M4	+/+	+/+	+/+	–/–	+/+	4/4	M	13
M6, M17	+/–	+/+	+/–	+/–	+/–	4/3	L	14
M13, M14	+/–	+/+	+/–	+/–	+/+	4/2	M	15
M2	+/–	+/+	+/–	+/–	+/+	4/3	H	16
M3	+/+	+/+	+/–	–/–	+/+	4/4	H	17
M5	+/–	+/–	+/–	–/–	+/+	4/3	L	18
M7	+/+	–/–	+/+	–/–	+/+	4/4	H	19
M8	+/–	+/+	+/+	–/–	+/+	4/4	L	20
M9	+/+	+/+	+/–	–/–	+/+	4/4	L	21
M10	+/–	+/+	+/+	+/–	+/+	4/4	M	22
M11	+/+	+/+	+/+	–/–	+/+	4/4	L	23
M12	+/+	+/+	+/+	+/+	+/+	4/4	H	24
M15	+/+	+/+	+/+	–/–	+/+	4/4	H	25
M16	+/–	–/–	+/+	+/+	+/+	4/4	M	26
M18	+/+	+/+	+/–	+/–	+/+	4/4	L	27

Six tetrads were dissected for each of the 36 strains, spores were analyzed for ability to ferment sucrose (SUC), maltose (MAL), galactose (GAL) and growth with glycerol (YPG) as a carbon source, copper resistance (CUP), production of H_2S (1–4). Spore viability was scored as: high>85% (H), medium between 85–50% (M), low<50% (L).

5 pattern combinations in Sicilian strains, one common to 67.2% of strains and 2 strain specific. Strains of the two populations did not show amplification patterns in common.

In order to assess if strains with the same RAPD profile were identical, 18 Montalcino and 18 Sicily strains sharing the most common RAPD profile were subjected to further investigation. In this preliminary work strains from Montalcino will be referred to as M1–M18 and those from Sicily as CS1–CS18.

Genetic analysis. Six tetrads were dissected for each of the 36 strains and analyzed for ability to ferment sucrose (SUC), maltose (MAL), galactose (GAL) and growth with glycerol (YPG) as a carbon source (petite), for copper resistance (CUP), for production of H_2S and homotallism. In addition, we considered differences in other traits under genetic control such as spore viability, growth rate mutations and colony morphology mutations as criteria for distinguishing strains. The results obtained, considering homozygosity/heterozygosity for each trait, were pooled to obtain classes of combinations. Twenty-seven classes were found, 21 of which were unique and 6 common to at least two strains (Table 1). The overall level of heterozygosity was 80%.

All the strains analyzed were sporulation proficient, with spore viability ranging from 100% to 25%. Three Montalcino strains, M3, M7, and M15, which showed 100% spore viability, were completely homozygous with respect to the markers considered. Strains M17 and M6 were heterozygous for growth on glycerol indicating the segregation of a petite nuclear mutation. Higher heterozygosity was found with respect to SUC, GAL, CUP, and H_2S production. Strains M1 and M14 showed 2:2 segregation for a recessive gene controlling colony morphology, as two spores of the tetrad showed rough colonies with geometrical designs (Fig. 2) allowing to distinguish strain M1 from M4 (Class 12). Strains M3, M7, M8, M9, M10, M6, M17 and M16 showed segregation of slow growing colonies.

Restriction fragment length polymorphism. Genomic DNA from the same eighteen yeast strains was digested with the restriction enzyme *Hin*dIII and hybridized to probe DC4 (Table 2). This one is a *Sacch. cerevisiae* DNA fragment that revealed a good level of polymorphism, as already tested in our laboratory (unpublished

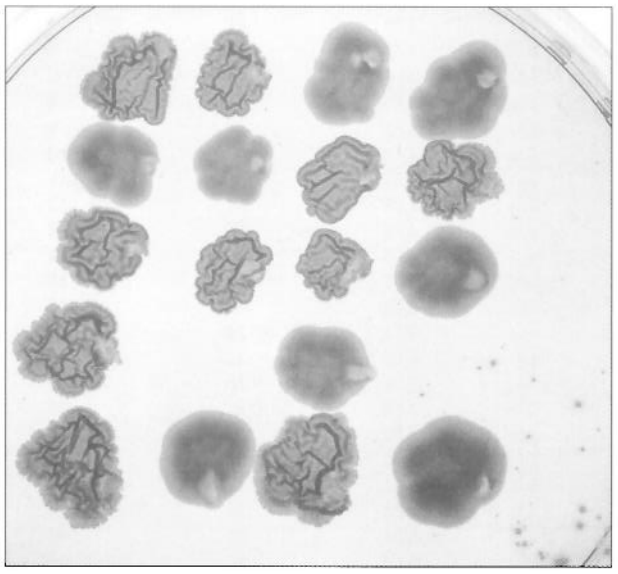

Fig. 2. Growth on YPD of yeast patches, derived from 4 spores (from left to right of every line) of five dissected tetrads of strain M1, shows 2:2 segregation for a recessive gene controlling colony morphology

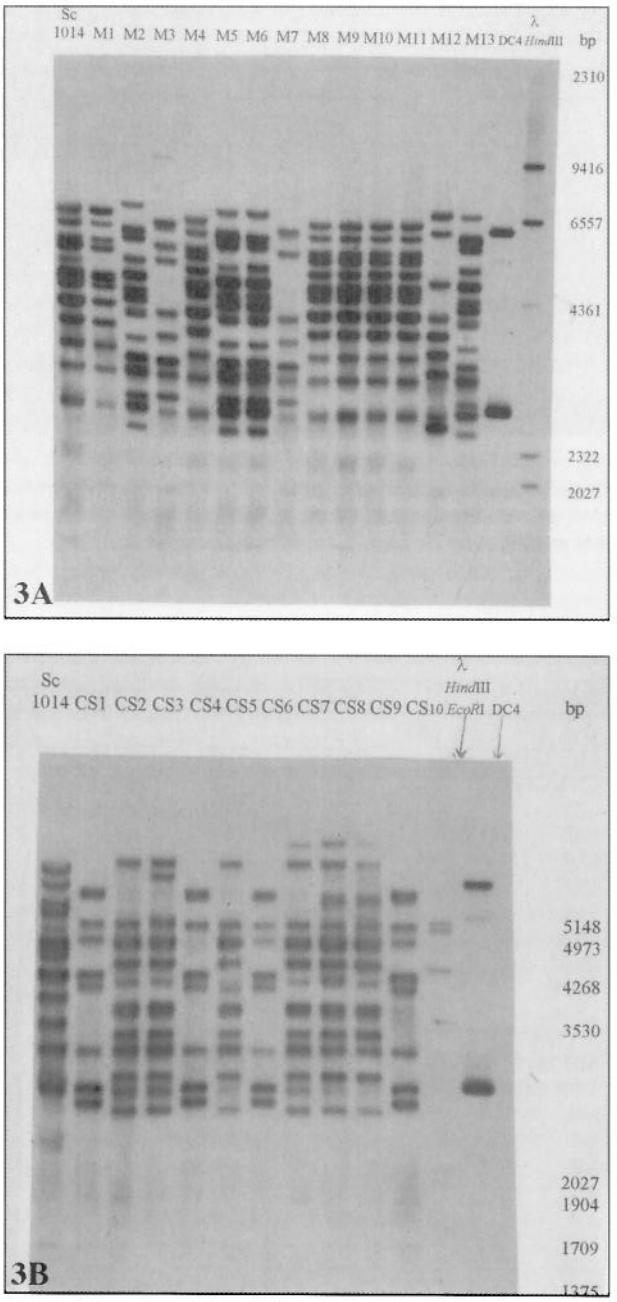

Fig. 3. Hybridization patterns of *Hin*dIII digested DNA from 13 Montalcino (M1–13, Fig. 3A) and 10 Sicily (CS1–CS10, Fig. 3B) strains, obtained with probe DC4. Sc1014 is *Saccharomyces cerevisiae* Type strain from DBVGP. Numbers at the right end indicate the size (Kb) of bands of the digoxigenin-labelled molecular weight marker. Lambda (λ) DNA/*Hin*dIII in 3A and Lambda (λ) DNA /*Hin*dIII/*Eco*RI in 3B

results). Preliminary sequence data (not shown) indicate that this encompasses part of a telomeric Y sequence. Hybridization profiles (Fig. 3) of M strains showed 10–17 bands ranging from 2500 to 6600 bp, with 8 hybridization profiles four of which were unique. Hybridization profiles of CS strains showed 7–13 bands ranging from 1740 to 7440 bp. Ten different profiles were present, eight of which were unique. This probe enabled us to differentiate strains not previously distinguished, namely CS4, CS15, CS6 (class 1), CS5, CS13, CS16 (class 2), CS8, CS17 (class 3), M6 and M17 (class 14).

Table 2. Results from RFLP analysis

Hybridization patterns	Strains
A	CS1, CS6, CS10, CS11, CS17
B	CS8, CS9, CS14, CS15, CS16,
C	CS2
D	CS3
E	CS4
F	CS5
G	CS7
H	CS12
I	CS13
L	CS18
M	M4, M8, M9, M10, M11
N	M13, M2, M5, M6, M14
O	M1, M15
P	M17, M18
Q	M3
R	M16
S	M7
T	M12

Table 3. Results from PFGE analysis

PFGE profiles	Strains
A	CS1, CS4, CS6, CS10, CS11, CS17
B	CS2, CS3, CS5, CS8, CS9, CS12, CS13, CS14, CS15, CS16, CS18
C	M2, M6, M13, M14
D	M8, M9, M11
E	M1, M12, M15
F	M17, M18
G	M4, M10
H	M3, M7
I	M5
L	M16

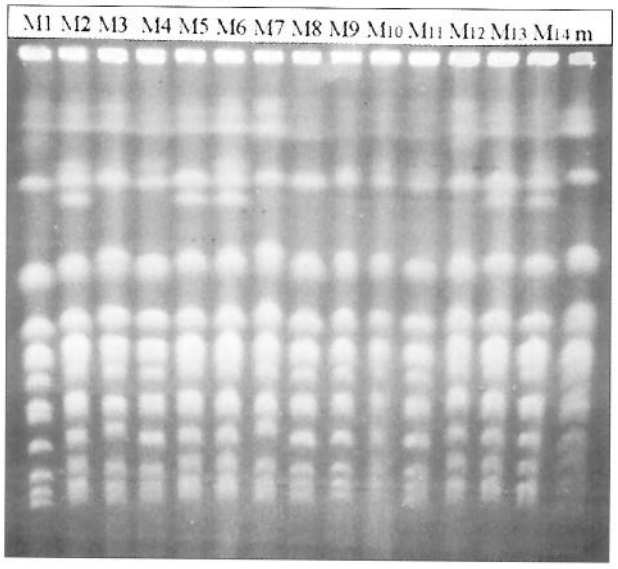

Fig. 4. CHEF gel analyses of 14 Montalcino strains, »m« lane contains *Sacch. cerevisiae* YPH80

PFGE. Results of PFGE, analyzed according to the previously used criteria, showed 8 electrophoretic profiles in M strains (Fig. 4), two of which unique, and two profiles in CS strains (Table 3).

Killer phenotype and sulfite resistance. All CS strains were able to produce killer toxins, while only one strain (M16) from Montalcino was a killer. With respect to resistance to sulfite, 75% of the strains, regardless of their origin, were resistant to 300 μg/mL sulfite and 50% were resistant up to 500 μg/mL.

Discussion

RAPD analysis with primer 1283 and RF2 enabled us to recognize 16 different patterns, 10 of which were strain specific. Although this percentage (6%) is low the method is fast and its differentiation power could be improved using more or other primers.

Both genetic analysis and RFLP with DC4 probe showed to be powerful techniques, allowing the distinction of 21 and 12 strains with unique features, respectively. The level of heterozygosity (2/3) shown by genetic analysis at one or more loci is in agreement with data previously obtained by Mortimer *et al. (1)*; moreover the fact that in Montalcino strains 100% viability is associated with complete homozygosity could be explained by genome renewal *(1)*.

PFGE analysis showed a lower power of discrimination compared to RFLP, as only 2 strains showed unique profiles and both were already distinguished.

The analysis of killer phenotype and sulphite resistance were not useful for strain discrimination despite their ecological significance.

In conclusion, the combined use of genetic analysis with DC4 probe hybridization allowed the distinction of 34 strains out of 36 tested, indicating the presence of a high level of diversity in *Sacch. cerevisiae* natural populations.

If we consider that the strains are part of a larger population of natural wine yeast we can estimate the size of this population using statistical considerations. With 34 unique strains and two strains representing a 35th class the estimated size of this population is 2448 distinct individuals. This estimate assumes that these individuals are equally frequent and that selection of the strains is random. This diversity can represent a valuable resource to develop new strains of interest.

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Genetička i molekularna raznolikost u prirodnoj populaciji *Sacch. cerevisiae*

Sažetak

Dvije populacije Sacch. cerevisiae, izolirane iz grožđa podrijetlom iz dviju talijanskih pokrajina – Toskane i Sicilije, djelomično su okarakterizirane multimetodološkim pristupom uključujući genetičke i molekularne postupke. Ukupno 166 izolata, 61 iz Sicilije i 105 iz Toskane, okarakterizirani su analizom amplifikacije polimorfne DNA. Preliminarni rezultati, dobiveni na 36 sojeva koji nisu bili diferencirani prema spomenutom postupku, pokazuju da je genetička analiza povezana s polimorfizmom duljine restrikcijskih fragmenata, koristeći DC4 sondu, najsupješnija kombinacija. Koristeći taj postupak utvrđeno je da je jedinstveno 34 od 36 sojeva, a preostala dva pripala su u jedan razred (klasu). Rezultati upućuju na visoki stupanj bioraznolikosti tih populacija.