

Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains

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Received 7 June 2012; accepted 17 July 2012
Available online 9 August 2012

In this work, the yeast ecology associated with the spontaneous fermentation of Grillo cultivar grapes from 10 vineyards was analyzed from grape harvest till complete consumption of must sugars. The microbiological investigation started with the plate count onto two culture media to distinguish total yeasts (TY) and presumptive *Saccharomyces* (PS). Yeasts were randomly isolated and identified by a combined genotypic approach consisting of restriction fragment length polymorphism (RFLP) of 5.8S rRNA gene and 26S rRNA and sequencing of D1/D2 domain of the 26S rRNA gene, which resulted in the recognition of 14 species belonging to 10 genera. The distribution of the yeasts within the vineyards showed some differences in species composition and concentration levels among 2008 and 2009 vintages. Due to the enological relevance, all *Saccharomyces cerevisiae* isolates were differentiated applying two genotypic tools (interdelta analysis and microsatellite multiplex PCR of polymorphic microsatellite loci) that recognized 51 strains. Based on the low production of H₂S, acetic acid and foam, ethanol resistance, growth in presence of high concentrations of potassium metabisulphite (KMBS) and CuSO₄ and at low temperatures, 14 strains were selected and used as starter to ferment grape must at 13 °C and 17 °C in presence of 100 mg/L of KMBS. Three strains (CS160, CS165 and CS182) showed optimal technological aptitudes.

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[Key words: Identification; Enological aptitudes; *Saccharomyces cerevisiae*; Spontaneous wine fermentation; Yeasts]

Yeasts responsible for the alcoholic fermentation of grape juice into wine are basically distinct in two groups: non-*Saccharomyces* (NS) species, growing during the first stages of fermentation, and *Saccharomyces* strains, which become dominant when the ethanol concentration increases.

Since the 80's, starter cultures belonging to the species *Saccharomyces cerevisiae* are commercially available in order to drive the alcoholic fermentation (1). However, despite the benefits due to the selected yeasts, in terms of effectiveness and ethanol yield, their employment in winemaking is quite controversial. One of the main reasons of objection for the routine use of commercial starter yeasts is due to their massive prevalence over the native microflora, with the consequent risk of loss of wine peculiarities (2). Furthermore, the recent growing interest for wines with definite "terroir" characteristics determined a re-discovery of wine fermentation by using indigenous yeasts occurring on grapes and/or in the winery environment (3).

Nowadays, starter cultures selected from autochthonous *S. cerevisiae* are commonly employed in winemaking to obtain wines with predictable quality and typicality. Although the inoculation of must with selected *S. cerevisiae* is expected to suppress the

indigenous NS strains, several studies have revealed that NS yeasts can indeed persist during the various stages of wine production driven by pure cultures of *S. cerevisiae* (4,5).

Regarding natural fermentations, *Saccharomyces* and NS yeasts do not coexist passively. Under these conditions, some enological traits of NS yeasts are not expressed, or may be modulated by *S. cerevisiae* cultures (6,7). During spontaneous fermentation, NS yeasts contribute to the aroma complexity of wines (8). Some authors reported that these yeasts provide typical aromatic notes that link the wines to the production region (9,10).

The modern trend of wine market is going toward products with given peculiarities. Among special wines, including fortified and non-fortified wines, Marsala produced in the homonymous area of western Sicily is historically known outside Italy since 1773, thanks to the English trader John Woodhouse. Marsala enjoys a "Denominazione di Origine Controllata" (DOC) status that is a recognition of quality (controlled designation of origin). This product requires a base wine for its production and the cultivar Grillo is one of the most cultivated grapevine in Sicily to this purpose.

Keeping in mind that wine production still remains a very traditional process, especially in areas where a long history and typicality of products is felt as an affection to the territory, the objectives of this study were to: examine the qualitative structure and the quantitative development of indigenous yeasts during the

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fermentation of Grillo cultivar (which represents the base wine for Marsala DOC product); to characterize *S. cerevisiae* isolates at strain level; and to investigate on the enological potential of *S. cerevisiae* strains.

MATERIALS AND METHODS

Sample collection Ten vineyards (Table 1) of the "Grillo" variety were sampled for grapes and berries within the Marsala wine production area (Sicily, Italy) during the harvesting of two consecutive vintages (2008 and 2009). The sampling was made in three 100 m² sub-areas (representing three replicates of the same vineyard) distant approximately 100–300 m from one another. In each vineyard, 15 grapes and 3.0 kg of grape berries (five grapes and 1 kg of berries from each sub-area) were randomly collected from undamaged grapes. All samples were then stored at 4°C during transport.

Grape samples (G) were placed into sterile plastic bags containing a washing isotonic peptone solution (10 g/L Bacto Soytone, 2 mL/L Tween 80) and incubated at 30°C for 3 h to collect the microorganisms hosted on peel surface (11).

Berries were crushed by stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 5 min at the highest speed to obtain must that was transferred into sterile flasks (5 L-volume) and maintained at 17°C until total sugar consumption. The samples collected for analysis were: grape must just pressed (M1), must at 1/5 (M2), 3/5 (M3) and 5/5 (M4) of sugar consumption.

Microbiological analysis Cell suspensions recovered from grapes and must samples were serially diluted in Ringer's solution (Sigma–Aldrich, Milan, Italy). Decimal dilutions were spread plated (0.1 mL) onto Wallerstein laboratory (WL) nutrient agar (Oxoid, Basingstoke, UK), incubated at 28°C for 48–72 h, for the counting of total yeasts (TY) and onto modified ethanol sulphite agar (MESA), prepared as reported by Francesca et al. (3), incubated at 28°C for 72 h, to detect presumptive *Saccharomyces* spp. (PS). Both media were supplemented with chloramphenicol (0.5 g/L) and biphenyl (1 g/L) to inhibit the growth of bacteria and molds, respectively. Analyses were carried out in duplicate.

Statistical analyses were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were analyzed using a generalized linear model (GLM) including the effects of vineyard (V = Guarrato, Lago Preola, Madonna Paradiso, Mazara del Vallo, Mothia, Musciuleo, Pietra Rinosca, Pispisia, Tre Fontane and Triglia Scaletta), year (Y = 2008, 2009) and sample type (S = G, M1–M4) and all their interactions (V*Y*S); the Student "t" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison. Significance level was $P < 0.05$.

Yeast isolation and identification Yeasts were isolated from both growth media used for counts. Three colonies per morphology were collected from the differential medium WL, while 10 colonies were randomly picked up from MESA. All

isolates were purified to homogeneity after several sub-culturing steps onto WL and at least two isolates (from each sample) sharing the same morphology were subjected to the genetic characterization.

The DNA extraction was performed using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

In order to perform a first differentiation of yeasts, all selected isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified and digested as described by Esteve-Zarzoso et al. (12). Gels were stained with SYBR® safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transilluminator and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA). Standard DNA ladders were 1 kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas). Five isolates representative of each group were subjected to an additional enzymatic restriction targeting the 26 rRNA gene following the methodology reported by Baleiras-Couto et al. (13). One isolate per group was further processed by sequencing the D1/D2 region of the 26S rRNA gene and/or 5.8S-ITS rRNA region to confirm the preliminary identification obtained by RFLP analysis (14). DNA sequencing reactions were performed at Primmbiotech S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at <http://www.ncbi.nlm.nih.gov>.

Strain typing of *S. cerevisiae* isolates Intraspecific characterization of the isolates belonging to *S. cerevisiae* species was carried out through two techniques: interdelta analysis with primers delta 12 and delta 21 (15) and microsatellite multiplex PCR based on the analysis of polymorphic microsatellite loci named SC8132X, YOR267C and SCPTS7 (16). The PCR products were analyzed on agarose gel 2.0% (w/v) in 1 × TBE buffer and visualized as above reported.

Technological characterization of *S. cerevisiae* strains All strains belonging to the species *S. cerevisiae* were evaluated for their potential in winemaking. The ability to produce H₂S was tested using a qualitative method performed on Bismuth Sulphite Glucose Glycerin Yeast extract (BiGGY) agar (Oxoid) (17). H₂S was estimated by colony blackening after 3 days of incubation at 28°C. A five-level scale was used for color evaluation: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black. The resistance to various levels of ethanol (from 12 to 16 % v/v) and potassium metabisulphite (KMBS) (from 50 to 300 mg/L) were determined onto MESA. *S. cerevisiae* GR1 (3) and NF213, belonging to the culture collection of DEMETRA Department (University of Palermo, Italy), producing low amount of H₂S and resistant to high levels of KMBS and ethanol were used as control strains. Copper tolerance was evaluated as the ability of a strain to grow in presence of different concentration (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μmol/L) of CuSO₄ (18). The strains characterized by high production levels of acetic acid were indicated by the halo produced around colonies onto CaCO₃ agar plates after 7-day incubation at 25°C (19). *S. cerevisiae* GR1 was used as negative control, while *Hanseniaspora uvarum* TLM14 (DEMETRA culture collection) as positive control. The

TABLE 1. Microbial loads^a of samples collected from Grillo vineyards and micro fermentations.

Samples	Vineyards									
	Guarrato 37°56' N-12°32'E	Lago Preola 37°36' N-12°38'E	Madonna Paradiso 37°40'N-12°36'E	Mazara del Vallo 37°41' N-12°35'E	Mothia 37° 52'N-12° 28'E	Musciuleo 37°52' N-12°34'E	Pietra Rinosca 37°52' N-12°43'E	Pispisia 37°50' N-12°29'E	Tre Fontane 37°34'N-12° 42'E	Triglia Scaletta 37°43'N-12°31'E
TY (2008)										
G	6.0 ± 0.3	5.13 ± 0.3	3.54 ± 0.6	4.98 ± 0.7	6.92 ± 0.3	6.39 ± 0.2	5.12 ± 0.5	5.65 ± 0.2	6.41 ± 0.2	6.84 ± 0.5
M1	6.25 ± 0.3	5.60 ± 0.4	3.27 ± 0.3	5.98 ± 0.4	6.78 ± 0.4	6.64 ± 0.3	5.36 ± 0.4	6.67 ± 0.4	6.81 ± 0.3	6.99 ± 0.2
M2	7.38 ± 0.4	6.87 ± 0.8	7.15 ± 0.2	7.08 ± 0.2	8.28 ± 0.3	5.99 ± 0.5	5.77 ± 0.4	8.24 ± 0.4	7.17 ± 0.0	7.46 ± 0.2
M3	8.15 ± 0.1	8.05 ± 0.4	7.91 ± 0.7	7.96 ± 0.2	7.89 ± 0.4	4.93 ± 0.4	4.13 ± 0.2	7.84 ± 0.5	6.55 ± 0.5	8.01 ± 0.3
M4	8.09 ± 0.4	4.79 ± 0.4	4.42 ± 0.4	8.09 ± 0.5	7.98 ± 0.6	2.93 ± 0.1	1.39 ± 0.5	7.54 ± 0.6	4.16 ± 0.1	7.21 ± 0.5
PS (2008)										
G	2.47 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M1	3.06 ± 0.1	n.d.	n.d.	n.d.	3.92 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.
M2	6.20 ± 0.1	3.56 ± 0.2	3.12 ± 0.2	5.88 ± 0.7	7.14 ± 0.2	5.08 ± 0.1	3.29 ± 0.4	6.5 ± 0.8	5.23 ± 0.3	5.71 ± 0.6
M3	8.16 ± 0.8	4.14 ± 0.0	4.62 ± 0.5	6.46 ± 0.1	6.76 ± 0.3	2.24 ± 0.4	2.94 ± 0.3	7.16 ± 0.0	5.02 ± 0.1	7.50 ± 0.7
M4	7.36 ± 0.5	3.81 ± 0.2	3.44 ± 0.3	7.48 ± 0.3	7.02 ± 0.7	1.0 ± 0.0	n.d.	7.37 ± 0.5	2.02 ± 0.1	6.72 ± 0.5
TY (2009)										
G	5.56 ± 0.4	5.79 ± 0.2	5.93 ± 0.8	6.08 ± 0.2	4.07 ± 0.2	4.01 ± 0.3	5.77 ± 0.5	4.29 ± 0.3	4.36 ± 0.4	3.16 ± 0.6
M1	5.25 ± 0.8	6.30 ± 0.3	6.09 ± 0.6	6.6 ± 0.3	5.0 ± 0.3	5.54 ± 0.4	5.25 ± 0.4	5.03 ± 0.5	5.29 ± 0.4	3.98 ± 0.5
M2	7.39 ± 0.9	7.20 ± 0.3	8.25 ± 0.3	7.76 ± 0.2	7.97 ± 0.4	5.91 ± 0.7	7.20 ± 0.4	7.81 ± 0.3	8.09 ± 0.2	5.84 ± 0.2
M3	7.59 ± 0.4	7.27 ± 0.5	8.78 ± 0.7	7.38 ± 0.4	7.83 ± 0.6	4.26 ± 0.5	7.09 ± 0.2	7.55 ± 0.2	7.85 ± 0.6	6.77 ± 0.4
M4	7.27 ± 0.4	8.16 ± 0.6	8.17 ± 0.1	7.53 ± 0.1	7.97 ± 0.5	1.86 ± 0.4	5.95 ± 0.7	7.66 ± 0.3	7.54 ± 0.3	6.27 ± 0.7
PS (2009)										
G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.94 ± 0.5	n.d.
M1	n.d.	2.13 ± 0.7	1.84 ± 0.4	n.d.	2.66 ± 0.1	n.d.	n.d.	3.07 ± 0.1	3.44 ± 0.6	2.03 ± 0.1
M2	5.47 ± 0.3	5.47 ± 0.1	7.76 ± 0.6	2.87 ± 0.3	5.64 ± 0.5	3.85 ± 0.9	6.30 ± 0.4	5.22 ± 0.3	5.12 ± 0.2	4.15 ± 0.1
M3	7.4 ± 0.0	7.21 ± 0.5	8.77 ± 0.4	5.10 ± 0.1	6.60 ± 0.8	3.12 ± 0.2	5.85 ± 0.3	7.54 ± 0.7	7.22 ± 0.3	5.92 ± 0.6
M4	7.17 ± 0.3	7.04 ± 0.0	6.97 ± 0.2	6.90 ± 0.9	6.89 ± 0.6	n.d.	5.62 ± 0.9	7.07 ± 0.1	6.97 ± 0.1	6.16 ± 0.1

Abbreviation: G, grape berries; M1, grape must just pressed; M2, grape must at 1/5 sugar consumption; M3, grape must at 3/5 sugar consumption; M4, grape must at 5/5 sugar consumption; TY, total yeasts on WL nutrient agar; PS, presumptive *Saccharomyces* on MESA; n.d., not determined.

^a Log CFU/g for grape berries; Log CFU/mL for must samples.

growth at low temperatures was determined in Yeast Extract Peptone Dextrose (YPD) broth at 13 °C and 17 °C for 5 days. Growth patterns were examined through visual inspection of samples through a light microscope (Carl Zeiss Ltd.) (20). Foam production was examined according to Regodón et al. (21).

The strains selected for must fermentation were also evaluated for their enzymatic activities: β -glucosidase activity was tested in presence of arbutin, esculin, 4-methylumbelliferil β -D-glucopyranoside (MUG) and 4-nitrophenyl β -D-glucopyranoside (*p*-NPG) (22); proteolytic activity was assayed as reported by Bilinsky et al. (23).

All analyses were carried out in triplicate.

Micro fermentations The strains showing the best technological performances (low production of H₂S and acetic acid, resistance to ethanol, KMBS and CuSO₄, ability to grow at low temperatures, growth in suspended form and low foam production) were evaluated for their ability to ferment a grape must. Broth cultures in the stationary phase were washed twice in Ringer's solution and inoculated in 1 L of pasteurized Grillo grape must (pH 3.3, 21.6 °Brix, 151.6 mg/L yeast available nitrogen) added with KMBS (100 mg/L) at a final concentration of about 10⁶ CFU/mL. Micro fermentations were carried out at 13 °C and 17 °C. In order to allow CO₂ removal, the flasks were plugged with a Müller valve containing sulfuric acid (24) and the weight loss was monitored until the daily decrease was lower than 0.01 g (end of fermentation process). According to Ciani and Maccarelli (25), the fermentation power (FP) was evaluated as ethanol amount (% v/v) produced at the end of the process, the fermentation rate (FR) was calculated as CO₂ daily produced and the fermentation purity (FPu) was calculated as acetic acid (g/L) per ethanol (% v/v) produced at the end of micro fermentation. Two control micro fermentations were inoculated with *S. cerevisiae* GR1 and F1 (DEMETRA culture collection). At the end of fermentation, the wines were analyzed for residual sugar, acetic acid and glycerol content following the standard methods of the Organization of Vine and Wine (26).

RESULTS

Microbiological analysis The viable counts of TY and PS populations investigated in this study are reported in Table 1. TY counts on the grape surface were in the range 3.54–6.92 and 3.16–6.08 Log CFU/g in vintage 2008 and 2009, respectively. On average, higher levels of TY were observed on grapes collected in 2008 ($P < 0.05$), that were above 6 Log CFU/g for Mothia, Musciuleo, Tre Fontane and Triglia Scaletta vineyards. Data recovered from MESA showed that, except samples from Guarrato vineyard in the vintage 2008 and Tre Fontane vineyard in the vintage 2009, grapes did not host yeasts ascribable to PS group at detectable levels.

The yeast populations analyzed at different steps during sugar consumption were also monitored. TY load of M1 samples were higher than that detected on the corresponding grapes ($P < 0.05$). Regarding PS populations, the concentrations found for M1 samples from Guarrato 2008 and Tre Fontane 2009 were higher ($P < 0.05$) than those found in G samples and detectable levels were registered in six other M1 samples. During fermentation, both TY and PS counts increased significantly ($P < 0.05$); although often M3 samples showed higher levels than M4, not always the highest concentrations were displayed by M3 samples, since in some cases it was registered for M4 or M2 samples.

In general, the effect of vineyard, year and sample type was found to significantly ($P < 0.001$) affect count data of PS, while for TY the concentration levels were affected by vineyard ($P < 0.001$) and sample type ($P < 0.001$), but not by year. The combination of the three independent variables (V^*Y^*S) significantly affected both PS and TY counts.

Isolation and identification of yeasts A total of 1144 colonies from WL and 987 from MESA were isolated, purified to homogeneity and separated on the basis of appearance of colony morphology on WL. At least two cultures from each sample were morphologically selected obtaining 1021 isolates (614 from WL and 407 from MESA) which were subjected to molecular identification. After restriction analysis of 5.8S-ITS region and 26S rRNA gene, the isolates were clustered in 14 groups (Table 2): three of these groups (X, XI and XIII) were directly identified by comparison of restriction bands with those available in literature (12,27,28). These patterns corresponded to *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *S. cerevisiae* species. Eleven groups could not be identified by RFLP analysis, then the identification at species level

TABLE 2. Molecular identification of yeasts.

R.P. Isolate code	5.8S-ITS PCR	Size of restriction fragments			26S PCR	Size of restriction fragments			Species (% identity) ^a	Accession no.		
		CfoI	HaeIII	Hinfi		DdeI	Hinfi	MseI			Apal	
I	CS236	600	190 + 170 + 90	450 + 130	290 + 180 + 130	n.s.r.	1150	500 + 400 + 170	620 + 370 + 90 + 55	n.c.	Apal	JX129904
II	CS15	500	205 + 175	450	240 + 125	n.s.r.	1100	370 + 270 + 220	n.c.	n.c.	n.c.	JX129912
III	CS271	475	210 + 110	n.c.	235 + 235	n.s.r.	1100	340 + 210 + 75	750 + 130 + 90 + 65	n.c.	n.c.	JX129898
IV	CS244	540	260 + 210	n.c.	300 + 180 + 60	n.s.r.	1100	410 + 200 + 105 + 85	400 + 380 + 250 + 65	n.c.	n.c.	JX129901
V	CS206	650	345 + 275	570 + 80	260 + 240 + 140	n.s.r.	1100	265 + 200 + 185 + 160 + 140	410 + 390 + 280	n.c.	n.c.	JX129907
VI	CS231	750	335 + 115	n.c.	370 + 205 + 175 + 75	380 + 180 + 90 + 70 + 60	1190	n.c.	600 + 410 + 100 + 65	n.c.	n.c.	JX129905
VII	CS203	750	335 + 115	n.c.	370 + 205 + 175 + 75	400 + 175 + 90 + 60	1100	400 + 170 + 100	n.c.	n.c.	n.c.	JX129909
VIII	CS234	750	335 + 115	n.c.	370 + 205 + 175 + 75	310 + 160 + 90 + 70 + 60	1100	400 + 170 + 100	500 + 400 + 100 + 65	n.c.	n.c.	JX129914
IX	CS212	420	125 + 100 + 90 + 70	310 + 110	225	n.s.r.	1100	500 + 315 + 100 + 90 + 60	800 + 200 + 90	n.c.	n.c.	JX129906
X	CS240	720	315 + 290	340 + 220 + 85	315	n.s.r.	1100	500 + 400 + 170	600 + 400 + 60	n.c.	n.c.	JX129903
XI	CS51	400	200 + 90	300 + 100	200 + 180	n.s.r.	1100	n.c.	n.c.	n.c.	n.c.	JX129913
XII	CS280	500	215 + 190	400	230 + 160	n.s.r.	1150	500 + 400 + 125 + 100	1000 + 95	n.c.	n.c.	JX129897
XIII	CS325	880	380 + 360 + 140	340 + 255 + 175 + 140	375 + 130	n.s.r.	1100	500 + 210 + 190	1000 + 70	n.c.	n.c.	JX129896
XIV	CS27	650	650	700	310	n.s.r.	1130	500 + 250 + 190 + 170	1000 + 70	n.c.	n.c.	JX129911

All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp. Abbreviations: R.P., restriction profile; n.c., not cut; n.s.r., not subjected to restriction.
^a According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

TABLE 3. Geographical and annual distribution^a of yeast species during spontaneous fermentations.

Species	Vineyards									
	Guarrato	Lago Preola	Madonna Paradiso	Mazara del Vallo	Mothia	Musciuleo	Pietra Rinosa	Pispisia	Tre Fontane	Triglia Scaletta
2008										
<i>A. pullulans</i>				G(4 ^b)			G(5 ^b) M1(5 ^b)			
<i>C. apicola</i>									G(6 ^b) M1(6 ^b)	
<i>C. zemplinina</i>				M1(5 ^b) M2(7 ^b)			M2(5 ^b)			
<i>Cr. flavescens</i>										
<i>Cr. magnus</i>										
<i>H. guilliermondii</i>										
<i>H. opuntiae</i>							M2(5 ^b) M3(4 ^b)			
<i>H. uvarum</i>	G(6 ^b ,2 ^c) M1(6 ^b ,4 ^c) M2(7 ^{b,c}) M3(8 ^b ,7 ^c)	M2(6 ^b) M3(8 ^b ,4 ^c) M4(8 ^b ,3 ^c)	M2(7 ^b ,3 ^c) M3(7 ^b ,4 ^c) M4(3 ^c)	M2(7 ^b ,5 ^c) M3(7 ^b)	G(6 ^b) M1(6 ^b ,3 ^c) M2(8 ^b ,7 ^c) M3(7 ^b ,6 ^c)	M1(6 ^b) M2(6 ^b ,5 ^c) M3(4 ^b ,2 ^c) M4(2 ^b ,1 ^c)		M2(8 ^b ,6 ^c) M3(7 ^b)	M1(6 ^b) M2(7 ^b ,5 ^c)	G(6 ^b) M1(6 ^b) M2(7 ^b ,5 ^c)
<i>I. terricola</i>										
<i>L. thermotolerans</i>	G(2 ^c) M1(6 ^b ,3 ^c) M2(7 ^b)						M2(5 ^b ,3 ^c) M3(4 ^b)			
<i>M. pulcherrima</i>	G(6 ^b) M1(6 ^b)		G(3 ^b) M1(3 ^b) M2(7 ^b)	M1(5 ^b)				G(5 ^b) M1(6 ^b) M2(8 ^b) M3(7 ^b)	G(6 ^b) M1(6 ^b)	
<i>P. kudriavzevii</i>							M2(3 ^c) M3(4 ^b ,2 ^c) M4(1 ^b)		M3(6 ^b ,5 ^c) M4(4 ^b ,2 ^c)	
<i>S. cerevisiae</i>	M2(6 ^c) M3(8 ^{b,c}) M4(8 ^b ,7 ^c)			M3(7 ^b ,6 ^c) M4(8 ^b ,7 ^c)	M1(6 ^b ,3 ^c) M3(6 ^c) M4(7 ^{b,c})	G(6 ^b)M1(6 ^b)		M3(7 ^{b,c}) M4(7 ^{b,c})		M2(7 ^b ,5 ^c) M3(8 ^b ,7 ^c) M4(7 ^b ,6 ^c)
<i>W. anomalus</i>										
2009										
<i>A. pullulans</i>		G(5 ^b) M1(6 ^b)		G(6 ^b) M1(6 ^b)		G(4 ^b)	G(5 ^b) M1(5 ^b)	G(4 ^b) M1(5 ^b)	G(4 ^b) M1(5 ^b)	
<i>C. apicola</i>										
<i>C. zemplinina</i>		M2(7 ^b ,5 ^c) M3(7 ^{b,c}) M4(8 ^b ,7 ^c)			M1(5 ^b ,2 ^c) M2(7 ^b ,5 ^c)		M2(7 ^b ,6 ^c) M3(5 ^{b,c})		M1(5 ^b)	G(3 ^b) M1(3 ^b ,2 ^c) M2(5 ^b ,4 ^c) M3(6 ^b ,5 ^c) M4(6 ^{b,c})
<i>Cr. flavescens</i>					G(4 ^b)					
<i>Cr. magnus</i>		G(5 ^b) M1(6 ^b)								
<i>H. guilliermondii</i>		M2(7 ^b)	G(5 ^b)						G(4 ^b) M1(5 ^b) M3(7 ^b ,6 ^c) M4(7 ^b ,6 ^c)	G(3 ^b) M1(3 ^b)
<i>H. opuntiae</i>	G(5 ^b) M1(5 ^b) M2(7 ^b) M3(7 ^b)	M1(6 ^b)	M1(6 ^b) M2(8 ^b)	M2(7 ^b) M3(7 ^b)			G(5 ^b) M1(5 ^b) M2(7 ^b)	M1(5 ^b ,3 ^M) M2(7 ^b) M3(7 ^b)	M2(8 ^b) M3(7 ^b)	
<i>H. uvarum</i>	M3(7 ^{b,c}) M4(7 ^{b,c})	G(5 ^b) M1(2 ^{b,c}) M2(7 ^b ,5 ^c) M3(7 ^{b,c}) M4(8 ^b ,7 ^c)	M2(8 ^b ,7 ^c) M3(8 ^b)	M2(7 ^b) M3(7 ^b)	M1(5 ^b) M2(7 ^b ,5 ^c) M3(7 ^b)	M2(3 ^c) M3(4 ^b ,3 ^c) M4(1 ^b)			M1(5 ^b ,3 ^c)	
<i>I. terricola</i>			G(5 ^b) M1(6 ^b)						G(5 ^b) M2(5 ^b)	
<i>L. thermotolerans</i>										
<i>M. pulcherrima</i>	M1(5 ^b) M2(7 ^b)	M1(5 ^b) M2(6 ^b)	M1(6 ^b)	M1(6 ^b)		M1(5 ^b) M2(5 ^b)		M2(7 ^b) M3(7 ^b)	M1(5 ^b)	
<i>P. kudriavzevii</i>	M4(7 ^{b,c})	M1(6 ^b) M2(5 ^c) M3(7 ^b)		M2(2 ^c) M3(7 ^b) M4(7 ^b ,6 ^c)			M3(7 ^b ,5 ^c) M4(5 ^{b,c})		G(1 ^c) M3(7 ^b)	
<i>S. cerevisiae</i>	M3(7 ^{b,c}) M4(7 ^{b,c})	M3(7 ^{b,c}) M4(8 ^{b,c})	M3(8 ^{b,c}) M4(8 ^{b,c})	M3(7 ^b ,5 ^c) M4(7 ^b ,6 ^c)	M3(7 ^b ,6 ^c) M4(7 ^b ,6 ^c)			M2(7 ^b ,5 ^c) M3(7 ^{b,c}) M4(7 ^{b,c})	M1(3 ^c) M2(5 ^c) M3(7 ^{b,c}) M4(7 ^b ,6 ^c)	
<i>W. anomalus</i>										

Abbreviations: *C.*, *Candida* spp.; *Cr.*, *Cryptococcus* spp.; *H.*, *Hanseniaspora* spp.; *I.*, *Issatchenkia* spp.; *L.*, *Lachancea* spp.; *M.*, *Metschnikowia* spp.; *P.*, *Pichia* spp.; *S.*, *Saccharomyces* spp.; *W.*, *Wickerhamomyces* spp.; G, grape berries; M1, grape must just pressed; M2, grape must at 1/5 sugar consumption; M3, grape must at 3/5 sugar consumption; M4, grape must at 5/5 sugar consumption.

^a The number reported between brackets refers to the highest concentration (Log cycle) of detection.

^b Yeast count onto WL nutrient agar.

^c Yeast count onto MESA.

was concluded by sequencing of D1/D2 domain of the 26S rRNA gene which was successful for all groups obtained by enzymatic digestions.

Yeast species distribution The distribution of yeast species among vineyards and vintages, as well as their concentration estimated for each sample, are reported in Table 3. *H. uvarum*, *M. pulcherrima* and *Aureobasidium pullulans* were the species most frequently encountered on grapes and musts soon after pressing. In general, the concentration levels detected on WL were higher than those found on MESA. *S. cerevisiae* was never detected on grapes and twice in M1 (Mothia 2008 and Tre Fontane 2009). The

concentration of *S. cerevisiae* was relevant (approximately 10⁶ CFU/mL) in M1 from vineyard Mothia in vintage 2008. The samples M2 and M3 were dominated by *H. uvarum*, *S. cerevisiae* and *Candida zemplinina* in both years reaching levels ranging between 6 and 8 orders of magnitude. *Hanseniaspora opuntiae* was also isolated in several M2 and M3 samples at high concentrations but only in the vintage 2009. At the end of the fermentation process, *S. cerevisiae*, *H. uvarum* and *Pichia kudriavzevii* were detected in several M4 samples of the two consecutive vintages and *C. zemplinina* only in 2008. Interestingly, in this technological step, the yeast levels found on MESA were comparable or even superimposable with

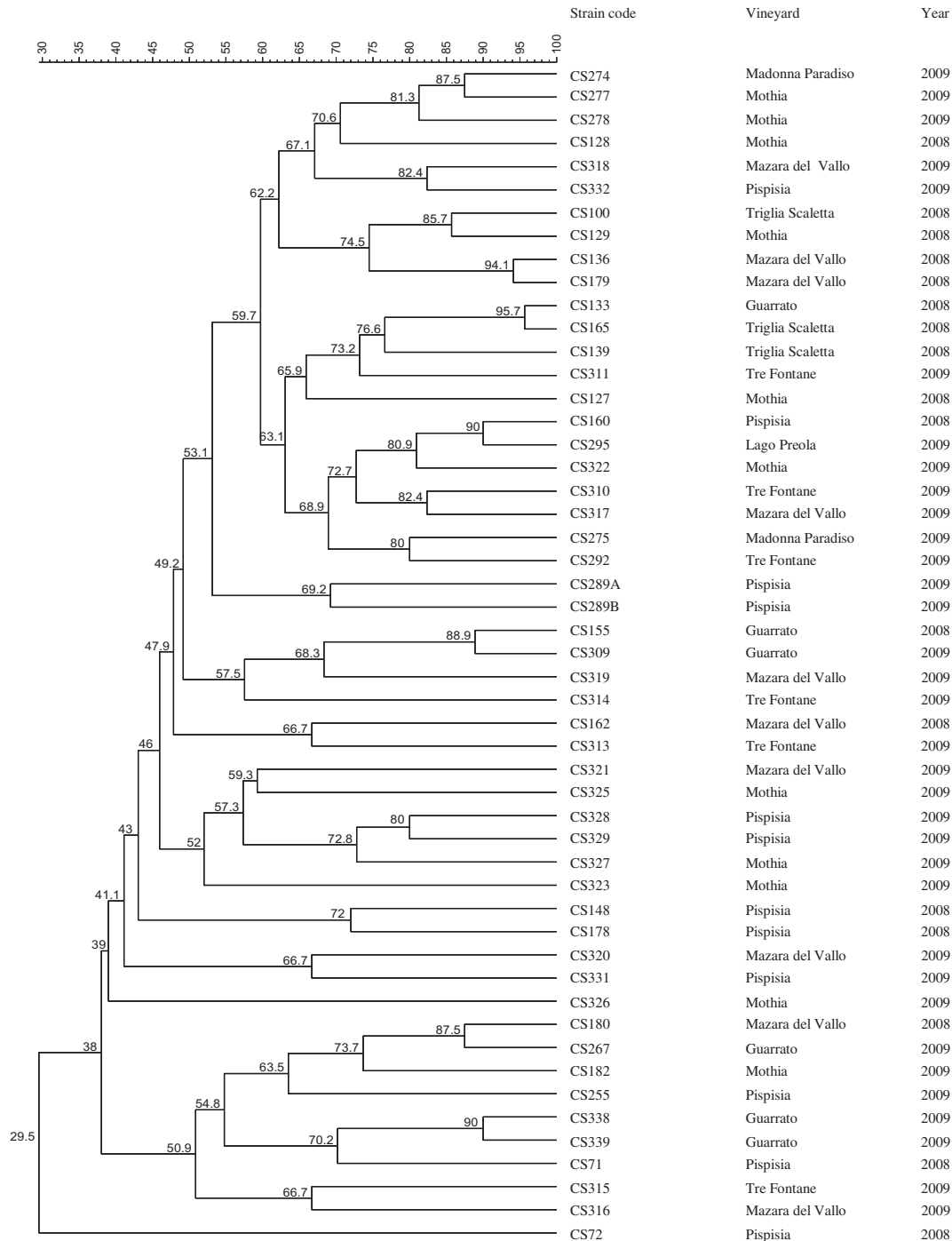


FIG. 1. Dendrogram resulting from interdelta analysis of *S. cerevisiae* strains.

those estimated on WL. Although in the samples obtained from Musciuleo and Pietra Rinosa vineyards *S. cerevisiae* was never isolated in both vintages object of analysis, it resulted dominant, alone (in the majority of the vineyards analyzed) or in combination with other species such as *H. uvarum*, *Hanseniaspora opuntiae* and *L. thermotolerans*, reaching concentrations within 6–8 Log CFU/mL. In general, when *S. cerevisiae* was not detected, the species dominating the fermentation process were *H. uvarum*, *P. kudriavzevii* or *C. zemplinina*.

Typing of *S. cerevisiae* strains and geographic distribution The 447 isolates belonging to the species *S. cerevisiae* were further genetically characterized. The interdelta analysis was able to separate the isolates in 51 groups, while microsatellite multiplex PCR recognized 44 different groups, showing a lower discriminatory power than the first technique. A dendrogram resulting from the cluster analysis of the 51 interdelta profiles is reported in Fig. 1. Except a few strains found in the same vineyard in a given year (CS136 and CS179; CS338 and CS339) which clustered at high levels (>90%), no particular similarities were found among strains isolated within the same vineyard. Furthermore, no strain was found in different vineyards or vintages.

The distribution of *S. cerevisiae* strains among the vineyards analyzed (Table 4) was found to be non-homogeneous. When *S. cerevisiae* was found, the number of strains recognized was between 1 and 12; the sampling from Lago Preola, Madonna Paradiso and Triglia Scaletta sites produced a very low number of strains, on the contrary Mothia, Pispisia and Mazara del Vallo were richer in *S. cerevisiae* biodiversity with 12, 11 and 10 different strains, respectively.

Technological screening of *S. cerevisiae* strains The 51 *S. cerevisiae* strains were screened for their enological characters (Table 5). Thirty-two strains were characterized by a low production of H₂S on Biggy agar plates (white–light brown colony) and resistance to high levels of ethanol (14–16% v/v). Moreover, 36 and 48 strains showed growth in presence of high concentrations of KMBS (150–300 mg/L) and CuSO₄ (400–500 mmol/L), respectively. Twenty-eight strains were found to produce low levels of acetic acid. The growth at low temperatures (13 °C and 17 °C) was positive for 22 strains, whereas all 51 developed in suspension. Only five strains were found to produce more than 2 mm of foam.

From the previous technological tests, 14 strains were selected and used as starters to ferment grape must at 13 °C and 17 °C in presence of 100 mg/L of KMBS. The results of the fermentation kinetics (Table 6) showed that, in terms of FP, FR and FPU, three strains (CS160, CS165 and CS182) showed better technological aptitudes than control strains.

After fermentation, enzymatic activities were determined as quality parameters (Table 6). The above three strains were characterized by optimal β-glucosidase activity, in particular onto agar plates containing esculin and MUG. However, no *S. cerevisiae* showed protease activity.

TABLE 4. Geographical and annual distribution of *S. cerevisiae* strains during spontaneous fermentations.

Vineyards	No. of <i>S. cerevisiae</i> isolates			No. of <i>S. cerevisiae</i> strains		
	2008	2009	Total	2008	2009	Total
Guarrato	28	43	71	2	4	6
Lago Preola	–	31	31	–	1	1
Madonna paradiso	–	33	33	–	2	2
Mazara del Vallo	26	38	64	4	6	10
Mothia	26	46	72	3	8	11
Musciuleo	–	–	–	–	–	–
Pietra Rinosa	–	–	–	–	–	–
Pispisia	34	47	81	5	7	12
Tre Fontane	–	48	48	–	6	6
Triglia Scaletta	47	–	47	3	–	3
Total	161	286	447	18	33	51

DISCUSSION

Microbial dynamics are important during long-term fermentation processes, such as wine productions, since the availability of the grapes occurs once a year and an anomalous evolution of the

TABLE 5. Technological screening of *S. cerevisiae* strains.

Strain code	H ₂ S ^a	Ethanol ^b	KMBS ^c	CuSO ₄ ^d	CaCO ₃ ^e	13°C ^f	17°C ^g	Growth pattern ^h	Foam ⁱ
CS71	2	4	6	10	–	+	+	S	F0
CS72	4	2	5	9	–	–	–	S	F0
CS100	3	3	4	8	+	–	–	S	F0
CS127	1	2	5	8	–	–	–	S	F1
CS128	0	4	6	10	–	+	+	S	F0
CS129	3	3	4	8	+	+	+	S	F0
CS133	0	4	6	10	–	+	+	S	F0
CS136	1	2	3	8	+	–	–	S	F0
CS139	4	3	5	8	–	–	–	S	F0
CS148	1	4	5	10	–	+	+	S	F0
CS155	1	4	6	10	–	+	+	S	F0
CS160	2	4	6	10	–	+	+	S	F0
CS162	1	4	6	10	–	+	+	S	F0
CS165	0	4	6	10	–	+	+	S	F0
CS178	2	1	3	8	+	–	–	S	F1
CS179	4	3	3	9	–	–	–	S	F0
CS180	1	4	6	10	–	+	+	S	F0
CS182	2	4	6	10	–	+	+	S	F0
CS255	4	4	4	9	–	+	+	S	F0
CS267	3	3	4	8	+	–	–	S	F0
CS274	2	2	3	9	+	–	–	S	F0
CS275	4	3	4	10	+	+	+	S	F0
CS277	3	1	4	9	+	–	–	S	F1
CS278	4	4	3	8	–	+	+	S	F1
CS289A	4	3	4	7	–	–	–	S	F0
CS289B	4	3	3	8	+	–	–	S	F0
CS292	2	3	4	8	+	–	–	S	F0
CS295	3	1	3	9	–	–	–	S	F0
CS309	4	3	4	8	–	–	–	S	F0
CS310	4	4	4	7	+	–	–	S	F0
CS311	3	2	5	8	–	–	–	S	F0
CS313	3	3	5	9	+	–	–	S	F0
CS314	4	2	5	9	+	+	+	S	F0
CS315	3	1	4	7	–	–	–	S	F0
CS316	4	2	4	10	–	+	+	S	F0
CS317	2	2	3	9	+	–	–	S	F0
CS318	2	1	3	8	+	–	–	S	F0
CS319	3	1	3	9	+	–	–	S	F0
CS320	4	2	5	10	–	+	+	S	F0
CS321	4	3	4	9	+	+	+	S	F0
CS322	3	1	3	8	–	–	–	S	F0
CS323	2	2	3	9	+	–	–	S	F0
CS325	1	3	4	9	+	–	–	S	F0
CS326	1	3	3	8	+	–	–	S	F0
CS327	1	2	4	8	+	–	–	S	F0
CS328	1	2	3	8	+	–	–	S	F0
CS329	1	3	5	10	–	+	+	S	F0
CS331	1	4	6	10	–	+	+	S	F0
CS332	1	4	3	8	+	–	–	S	F1
CS338	1	4	5	10	–	+	+	S	F0
CS339	1	4	5	10	–	+	+	S	F0

^a Color of colony on Biggy agar plates: 0, white; 1, beige; 2, light brown; 3, brown; 4, dark brown; 5, black.

^b 0, 0% (v/v); 1, 10% (v/v); 2, 12% (v/v); 3, 14% (v/v); 4, 16% (v/v) of ethanol contained in MESA plates at which strains showed growth.

^c 50 mg/L; 2, 100 mg/L; 3, 150 mg/L; 4, 200 mg/L; 5, 250 mg/L; 6, 300 mg/L of MBKS contained into MESA plates at which strains showed growth.

^d 0, 0 μM; 1, 50 μM; 2, 100 μM; 3, 150 μM; 4, 200 μM; 5, 250 μM; 6, 300 μM; 7, 350 μM; 8, 400 μM; 9, 450 μM; 10, 500 μM of CuSO₄ contained into YPD agar plates at which strains showed growth.

^e Result of analysis: +, precipitation halo; –, non-precipitation halo on CaCO₃ agar plates.

^f Result of analysis: +, growth; –, no growth at 13°C in YPD broth.

^g Result of analysis: +, growth; –, no growth at 17°C in YPD broth.

^h S, suspended growth; F, flocculant growth in YPD broth.

ⁱ F0, foaming lower than 2 mm; F1, foaming among 2 and 4 mm; F2, foaming greater than 4 mm.

TABLE 6. Kinetics of alcoholic micro fermentations and enzymatic activities of *S. cerevisiae* strains.

Strain code	Fermentation power ^a		Fermentation rate ^b		Volatile acidity ^c		Fermentation purity ^d		Residual sugar ^e		Glycerol content ^f		Glucosidase activity ^g			Protease activity ^g			
	17°C		13°C		17°C		13°C		17°C		13°C		17°C		Esculin		Arbutin	MUG	p-NPG
	13°C	17°C	13°C	17°C	13°C	17°C	13°C	17°C	13°C	17°C	13°C	17°C	13°C	17°C					
CS71	11.32 ± 0.08	11.44 ± 0.05	1.39 ± 0.06	2.51 ± 0.04	0.57 ± 0.13	0.61 ± 0.08	0.05 ± 0.02	0.05 ± 0.01	2.46 ± 0.08	1.81 ± 0.08	7.40 ± 0.08	7.43 ± 0.08	+	-	+	-	-		
CS128	11.27 ± 0.21	12.73 ± 0.08	1.79 ± 0.12	3.20 ± 0.31	0.34 ± 0.05	0.35 ± 0.21	0.03 ± 0.01	0.03 ± 0.02	2.30 ± 0.02	1.30 ± 0.01	7.56 ± 0.02	7.61 ± 0.01	++	-	+++	-	-		
CS133	11.35 ± 0.04	12.71 ± 0.13	1.43 ± 0.05	3.29 ± 0.06	0.33 ± 0.21	0.36 ± 0.12	0.03 ± 0.04	0.03 ± 0.03	2.29 ± 0.01	1.31 ± 0.03	7.50 ± 0.13	7.62 ± 0.03	+++	-	+++	-	-		
CS148	11.03 ± 0.12	11.14 ± 0.07	1.44 ± 0.30	2.35 ± 0.01	0.41 ± 0.03	0.44 ± 0.13	0.04 ± 0.01	0.04 ± 0.04	2.77 ± 0.04	2.03 ± 0.03	7.51 ± 0.21	7.31 ± 0.02	-	-	-	-	-		
CS155	11.15 ± 0.34	12.70 ± 0.23	1.29 ± 0.07	2.26 ± 0.02	0.31 ± 0.08	0.32 ± 0.34	0.03 ± 0.03	0.03 ± 0.02	2.42 ± 0.11	1.31 ± 0.09	7.06 ± 0.02	7.64 ± 0.01	-	-	-	-	-		
CS160	12.63 ± 0.01	12.68 ± 0.02	1.76 ± 0.17	3.08 ± 0.23	0.28 ± 0.02	0.31 ± 0.07	0.02 ± 0.02	0.02 ± 0.01	1.33 ± 0.01	1.29 ± 0.02	7.55 ± 0.01	7.63 ± 0.31	+++	-	++	-	-		
CS162	10.12 ± 0.11	11.84 ± 0.11	1.28 ± 0.07	2.64 ± 0.04	0.51 ± 0.05	0.55 ± 0.03	0.05 ± 0.02	0.05 ± 0.01	3.24 ± 0.03	1.62 ± 0.12	6.97 ± 0.02	7.01 ± 0.12	-	-	-	-	-		
CS165	12.67 ± 0.14	12.50 ± 0.01	2.09 ± 0.02	2.64 ± 0.06	0.27 ± 0.11	0.30 ± 0.21	0.02 ± 0.02	0.02 ± 0.03	1.27 ± 0.01	1.42 ± 0.03	7.61 ± 0.04	7.59 ± 0.21	++	-	++	-	-		
CS180	12.49 ± 0.03	12.59 ± 0.31	1.08 ± 0.04	2.20 ± 0.17	0.36 ± 0.01	0.45 ± 0.21	0.03 ± 0.03	0.04 ± 0.04	1.41 ± 0.03	1.43 ± 0.06	7.03 ± 0.01	7.56 ± 0.05	+	-	+	-	-		
CS182	12.41 ± 0.23	12.84 ± 0.03	1.39 ± 0.03	3.25 ± 0.24	0.41 ± 0.06	0.47 ± 0.31	0.01 ± 0.01	0.04 ± 0.02	1.52 ± 0.09	1.26 ± 0.04	7.39 ± 0.11	7.63 ± 0.01	++	-	+	-	-		
CS329	11.29 ± 0.12	11.81 ± 0.06	1.07 ± 0.04	2.31 ± 0.04	0.57 ± 0.25	0.65 ± 0.28	0.05 ± 0.02	0.06 ± 0.02	2.09 ± 0.02	1.61 ± 0.21	7.52 ± 0.31	7.52 ± 0.03	+++	-	++	-	-		
CS331	11.31 ± 0.41	11.91 ± 0.41	1.02 ± 0.06	2.61 ± 0.07	0.49 ± 0.31	0.56 ± 0.37	0.04 ± 0.02	0.05 ± 0.01	2.33 ± 0.13	1.57 ± 0.07	7.21 ± 0.05	7.54 ± 0.02	+++	-	++	-	-		
CS338	11.25 ± 0.01	11.45 ± 0.09	1.07 ± 0.09	2.25 ± 0.21	0.59 ± 0.07	0.58 ± 0.18	0.05 ± 0.03	0.05 ± 0.01	1.98 ± 0.05	1.91 ± 0.09	7.20 ± 0.01	7.33 ± 0.11	+++	-	++	-	-		
CS339	11.13 ± 0.02	11.31 ± 0.07	1.09 ± 0.13	2.28 ± 0.17	0.48 ± 0.24	0.57 ± 0.02	0.04 ± 0.01	0.05 ± 0.03	2.68 ± 0.11	1.91 ± 0.09	6.77 ± 0.02	7.29 ± 0.01	++	-	+	-	-		
GR1	11.87 ± 0.11	12.01 ± 0.04	1.24 ± 0.19	2.77 ± 0.11	0.41 ± 0.20	0.48 ± 0.11	0.03 ± 0.01	0.04 ± 0.08	1.88 ± 0.12	1.67 ± 0.10	7.19 ± 0.09	7.33 ± 0.14	+++	-	++	-	-		
F1	12.03 ± 0.23	12.34 ± 0.06	1.27 ± 0.02	2.63 ± 0.04	0.44 ± 0.12	0.47 ± 0.01	0.04 ± 0.21	0.04 ± 0.03	1.61 ± 0.02	1.49 ± 0.03	7.41 ± 0.03	7.53 ± 0.12	++	-	+++	-	-		

^a Ethanol (% v/v) produced at the end of micro fermentation.

^b CO₂ produced after 3 days of fermentation (CO₂/day).

^c Acetic acid (g/L) produced at the end of micro fermentation.

^d Volatile acidity formed in relationship to ethanol produced at the end of micro fermentation.

^e Reducing sugars (g/L) at the end of micro fermentation.

^f Glycerol (g/L) produced at the end of micro fermentation.

^g Result of analysis: +, high activity; ++, medium activity; +, low activity; -, activity not detected.

microorganisms in the fermenting musts may determine low quality products and conspicuous economic losses for producers.

In the present work, we pictured the structure of yeast communities present on the grapes of Grillo *cultivar*, in must and during its steps of spontaneous fermentations, focusing on the technological selection of *S. cerevisiae* strains. Ten vineyards, representing the principal sites of Marsala wine production area, were sampled during two consecutive years (2008 and 2009). Yeast counts reflected a non-homogeneous distribution among sampling sites and vintages, but, in general, the effect of vineyard, year and sample determined significant differences on the concentrations of TY and PS. The finding that the majority of yeasts occurring on grapes did not belong to the *Saccharomyces* genus is in agreement with previous reports (29).

The process of isolation resulted in the collection of 1144 yeasts. After restriction analysis of 5.8S-ITS rRNA region and 26S rRNA gene, 14 yeast groups were recognized. Only three of them were easily identified at species level, whereas for the other 11 groups, characterized by atypical restriction profiles of 5.8S-ITS, the sequencing of the D1/D2 domain of the 26S rRNA gene was necessary. Atypical polymorphism for this region is not surprising for yeasts, since many authors observed this behavior in several strains (30–32). At the end of the identification process, 14 species belonging to 10 genera (*Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Issatchenkia*, *Lachanceae*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Wickerhamomyces*) were found.

The yeast communities present on the samples resulted complex. As previously stated by other authors (29,33), NS yeasts were dominant on grapes and in must soon after pressing, while only a few species (*H. uvarum*, *S. cerevisiae*, *C. zemplinina* and *P. kudriavzevii*) represented the prevailing flora during the stages of fermentation. Although the frequency of the species is generally calculated on the total number of isolates collected from the different vineyards and in the entire period of observation, which may include consecutive vintages (20,34,35), we found this approach arbitrary. The species proportion is unavoidably altered by the isolation process, that is performed randomly. In this study we analyzed the yeast species distribution based on their effective concentrations (Table 3).

H. uvarum was the species mainly isolated during fermentation. In some cases it was found at levels of 10⁷–10⁸ CFU/mL in both vintages. Its high frequency of isolation at these stages confirms a general behavior observed for other grape varieties (34,36). The distribution of *H. uvarum* in different geographic regions might be linked to the low altitude and high temperature (37), climatic factors that characterize the area of production of Marsala wine. Within *Hanseniaspora* genus, *Hanseniaspora guilliermondii* is the species reported to be mainly present in warm climates (35), but in our study it was isolated in a few samples, not above 10⁷ CFU/mL, collected only during 2009 vintage. The species *H. opuntiae* was also isolated. Interestingly, this species was found when *H. uvarum* was absent and its presence was more frequent in the vintage 2009. *H. opuntiae* has been reported to be a member of the grape ecosystem (38) and to dominate the first stages of alcoholic fermentation (39), but no information is available in literature on its presence at the late phases of the process. In this work *H. opuntiae* was detected at approximately 10⁷ CFU/mL at 3/5 sugar consumption.

Another species isolated at high frequency on grapes and in must soon after pressing was *M. pulcherrima*. This result could be due to the capability of this species to prevail by inhibiting the growth of different yeasts, including *S. cerevisiae* (40). *A. pullulans* was also particularly present in these samples, but only in 2009 vintage. Generally, this species has been detected on unripe grape berries (11) and in grape musts (3,29) and Verginer et al. (41) reported its influence in the flavor development of red wines. In the

present study, strains of this species were isolated only from WL agar plates, even at 10^6 CFU/mL, showing their susceptibility to the selective conditions of MESA; hence, they do not represent potential wine contaminants. Among the yeast species isolated at low frequency, it is interestingly to note the presence of *Cryptococcus flavescens* isolated on grapes at 10^4 CFU/g in a single vineyard and reported to be isolated on this matrix only once before (34).

The spontaneous fermentations were then dominated by *H. uvarum*, *S. cerevisiae*, *C. zemplinina* and *P. kudriavzevii*. Despite the selective conditions of fermentation, NS populations reached levels of concentration comparable to the PS load until the end of fermentation. Several researchers have focussed on the positive influence of NS yeasts emphasizing their potential application as starters in wine productions (7,25). Furthermore, the use of *Hanseniaspora* spp. in combination with *S. cerevisiae* has been reported to contribute positively to the complexity and aroma of wine (6,42). This may be due to the capability of these yeasts, e.g., *H. uvarum* strains, to secrete several enzymes, such as β -glucosidase and proteases, that could contribute to the expression of varietal aroma of grapevine (37,43). *C. zemplinina* was also isolated in several samples at high concentrations (till 10^7 – 10^8 CFU/mL). These strains could represent an important source of starters to be employed for mixed fermentations with *S. cerevisiae*, since their interaction was demonstrated to increase the fermentation kinetics of grape must (44). Moreover, some *C. zemplinina* strains are osmotolerant, producers of low concentration of acetic acid and high amounts of glycerol from sugars (45) and may find application to reduce the ethanol content of wines produced by grape musts characterized by high sugar content, such as those produced in the Marsala area. Regarding *P. kudriavzevii*, it is usually detected on grapes (34) and in the early stages of alcoholic fermentation (46), thus, its finding at the latest stages of fermentation needs further investigation.

Yeast numbers and species recovered in this study are consistent with the presence of rotten berries hidden in undamaged clusters. The influence of rotten grapes on yeast species diversity is a well-known phenomenon (47). Hence, grape sampling plays a defining role in the structure of the yeast populations estimated (48).

S. cerevisiae strains selected from indigenous populations of a given area might drive the alcoholic fermentation better than commercial starters (49). Due to their enological importance, all *S. cerevisiae* cultures isolated in this work were investigated at strain level. Cluster analysis recognized 51 strains and showed that no common pattern was found among strains isolated from different vineyards or vintages. Many authors claimed that autochthonous yeasts are linked to a specific area (49,50) and stable in consecutive years (50), but for others, the occurrence of strains in the vineyards is only temporary (51).

Based on their technological properties, especially on their ethanol resistance, 14 *S. cerevisiae* strains were selected and tested as starters in Grillo grape must. Among them, only two couples of strains (CS133–CS165 and CS338–CS339) found in the same vineyard in the same year shared a certain genetic similarity, but no other strain was found in different vineyards or vintages. Three strains (CS160, CS165 and CS182) were characterized by a relevant FP, a capacity of paramount importance in this type of wine, since a high rate of sugar consumption is mandatory.

The main conclusions of this work are: yeast populations analyzed in 10 vineyards located in the area of Marsala DOC wine, which have never been explored before, showed some differences in species composition and concentration levels between the two consecutive years (2008 and 2009) object of study; *H. uvarum*, *C. zemplinina* and, interestingly, *P. kudriavzevii* were detected in place of or at comparable levels of *S. cerevisiae* in the stages of fermentation characterized by high ethanol concentration; 14 autochthonous *S. cerevisiae* strains displayed a technological potential to drive the fermentation of must into wine. The

technological investigation of NS isolates is being prepared in order to design mixed strain starters for the preservation of the typicality of the wines obtained with Grillo cultivar.

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