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Biodiversity study of wine yeasts belonging to the "*terroir*" of Montepulciano d'Abruzzo "Colline Teramane" revealed *Saccharomyces cerevisiae* strains exhibiting atypical and unique 5.8S-ITS restriction patterns

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ABSTRACT

The Montepulciano d'Abruzzo "Colline Teramane" premium wine DOCG is produced in the Teramo province (Abruzzo, Italy). This region has a great tradition in winemaking and the wine is produced by a spontaneous fermentation so it could represent a reservoir of wine natural yeasts with important oenological features. The aim of this study was to characterize the yeast community of this wine grape growing region in order to create a *Saccharomyces cerevisiae* bank, providing data on oenological properties for potential industrial applications. A total of 430 yeasts were isolated at the end of spontaneous fermentation. PCR-RFLP was applied for the identification at the species level and underlined that 14 strains exhibited unusual and characteristic restriction patterns different from those typical of the species *S. cerevisiae*. This difference was due to the insertion of base *C* at a position 138 in the ITS1 region that determined an additional cleavage site for the enzyme *Hae*III. This insertion could be associated to the fermentative performance and associated to the relationship existing between yeasts and a viticulture region or '*terroir*'.

1. Introduction

Saccharomyces cerevisiae, can be regarded as 'the wine yeast' because of its ability to transform sugar in ethanol, carbon dioxide and metabolites related to wine flavour (Jiménez-Martì et al., 2011). *S. cerevisiae* includes both wild and domesticated species, where the domestication event that resulted in grape wine yeasts likely occurred approximately 2700 years ago (Fay and Benavides, 2005; Mortimer and Polsinelli, 1999; Suzzi, 2011). The wine strains of *S. cerevisiae* are highly diverse; populations fermenting grapes are usually polyclonic, in fact over time, selection strategies have improved the performance of these strains, producing genetically distinct yeasts that are highly suited for specific industrial applications. The extent of genetic differences ranges from single-nucleotide substitutions to whole-genome duplication (Sipiczki, 2011; Suzzi, 2011). These genetic differences, translated into phenotypic differences, underscore the ability of yeast to adapt to varied environmental and stress conditions. In fact, strains of *S. cerevisiae* associated with wine producing area, often form a genetically differentiated group that is separate from natural strains isolated from other food fermentations (bread, beer, palm wine and sake) or from soil and oak tree habitats (Fay and Benavides, 2005; Legras et al., 2007; Liti et al., 2009; Schacherer et al., 2009). Damaged grape berries, but not undamaged, are an important source of yeast strains (Mortimer and Polsinelli, 1999). Moreover, some authors highlight that different viticultural region are characterized by a high diversity of yeast strains, suggesting the occurrence of natural specific strains associated with particular *terroir* (Carreto et al., 2008; Lopes et al., 2002; Renouf et al., 2006). Actually, most of European wine production relies on the use of starter cultures. Generally, these cultures consist of *S. cerevisiae* strains isolated from environments associated with wine producing regions, selected for their fermentative power, suitable fermentative kinetics at different temperatures, low acetic acid production, and resistance to sulphur dioxide.

Moreover, wine consumer demands wine of high quality and is well-disposed to reward not only originality and territoriality products, but also natural wine obtained from vineyards and

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winemaking practices that respect the environment and the typical oenological characteristics representative of those particular regions.

There is a currently growing demand for selection of yeasts able to improve the technological properties and sensorial features of wines. But because of the public attitude towards the use of GMOs (Genetically Modified Organism) in food products, novel wine strains will have to be generated using non GM approaches, such as the development of techniques for detecting strains that might improve wines in terms of their colour, aroma, structure and other technological properties (Suarez-Lepe and Morata, 2012). Moreover, consumer-oriented tendencies include the development of new fermentation technologies for optimizing wine quality and for producing new healthful wines (i.e. wines with higher antioxidant content and lower concentrations of toxic substances, such as ethyl carbamate or biogenic amines) with particular flavour profiles (Pizarro et al., 2007). For this reason during the last 15–20 years all –omic approaches (genomic, proteomic, metabolomic, phenomic) aimed the characterization of yeasts of oenological interest such as *Saccharomyces* e non-*Saccharomyces*, highlighting some unexpected features that can be useful to renew the fermentation processes and to obtain wine of great quality. For instance, a recent study underscores the anti-genotoxic and anti-mutagenicity action of some strains of *S. cerevisiae* used as starter cultures for Montepulciano d'Abruzzo fermentation (Trotta et al., 2012).

The aim of this study was to characterize the yeast community of this wine grape growing Montepulciano d'Abruzzo "Colline Teramane" area in order to create a *S. cerevisiae* bank, providing data on oenological properties for potential industrial applications.

2. Materials and methods

2.1. Fermentation and yeast isolation

Grapes were harvested during 2007 in three vineyards located in Abruzzo region, a Montepulciano d'Abruzzo wine producing area in Central Italy where the wine is produced based on spontaneous fermentation and without any commercial enzyme preparations. The area of interest (AOI) was defined following the specifications of the Denomination of Guaranteed Origin (DOCG) production disciplinary (Ministerial Decree 20 February 2003, G.U. n. 54 of 6 March 2003). The territory extends from 13° 46 to 13°53 east longitudes, and from 42°25–42°54 north latitudes between the Apennines mountains and the Adriatic Sea (Herrera-Nuñez et al., 2011). The Montepulciano d'Abruzzo "Colline Teramane" (DOCG) premium wine is produced in the Teramo Province, Abruzzo, Italy. Nine samples, three for each vineyard were taken in October in order to ascertain which yeasts were present at harvesting. Five kg of undamaged and healthy grapes were harvested randomly in each of the three vineyards (named A, B and C) and were crushed in laboratory. The nine respective musts (2 L for each sample) were fermented at 25 °C and at the end fermentation aliquots were plated on Wallerstein Laboratory Nutrient Agar (WLN, Oxoid, Milan, Italy). For each sample, higher dilutions were used to isolate, randomly, 10-40 separated colonies from the petri dishes, in order to increase the probability to pick up strains belonging to the dominant species (Versavaud et al., 1995; Tofalo et al., 2009). Four-hundred and thirty isolates that presumptively belonged to the species *S. cerevisiae* were purified by repetitive streaking on YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar, all Oxoid). The isolates, belonging to the Culture Collection of the Faculty of BioScience and Technology for Food, Agriculture and Environment (University of Teramo), were stored at −80 °C in YPD broth supplemented with glycerol (20% v/v final concentration). Standard procedures for physiological identification of yeasts, as well as instructions for the preparation of materials and testing conditions were followed according to Kurtzman et al. (2011).

2.2. Yeast identification

Yeast cells were grown aerobically in YPD at 28 °C. DNA was isolated according to Querol et al. (1992). The 5.8 internal transcribed spacer (ITS) rRNA region was amplified in a Bio-Rad thermocycler (MyCycler, Bio-Rad Laboratories, Milan, Italy) using primers ITS1 and ITS4 as described previously (Esteve-Zarzoso et al., 1999; Tofalo et al., 2009).

Species identification was confirmed by sequencing of the D1/D2 region of the 26S rRNA was carried out according to Kurtzman and Robnett (1998), using primers NL-1 and NL-4. PCR products of the 5.8S-ITS and D1/D2 domain of isolates per distint RFLP gel were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences AB, Uppsala, Sweden) following the manufacturer's instructions, and delivered to B.M.R. (Padua University, Italy) for sequencing. The sequences obtained were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschult et al., 1997). ClustalX sofware was used to construct multiple sequence alignments. The ITS sequences analysed in this study were deposited in the NCBI (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbankunder) under the accession numbers KF486908 (KML18 strain), KF486909 (KML4 strain); KF486910 (KML2 strain), KF486911 (KML87 strain), KF486912 (KML123 strain).

2.3. Saccharomyces cerevisiae typing

Two oligonucleotides M13 (5′-GAGGGTGGCGGTTCT-3′) and LA1 (5′-GCGACGGTGTACTAAC-3′) with arbitrarily chosen sequences, were used for isolates biotyping. The reaction mixture and PCR conditions were performed according to Tofalo et al. (2007, 2011). RAPD-PCR patterns were acquired using the Gel Doc 2000 (Bio-Rad, Milan, Italy). Conversion, normalization, and further analysis of the patterns were carried out with the *Fingerprinting* II Informatix software (Bio-Rad). Band similarities between RAPD-PCR patterns were analysed using Pearson coefficient, and correlation coefficients were calculated by the unweighted pair group method with arithmetic averages (UPGMA). The reproducibility of the RAPD fingerprints was assessed by comparing the PCR products obtained with M13 and LA1 primers and DNA prepared from three separate cultures of the same strain.

Repeated interspersed delta sequences (with primer δ12: 5′-TCAACAATGGAATCCCAAC-3′ and δ21 5′-CATCTTAACACCGTATATGA-3′) were used to characterize the 14 atypical *S. cerevisiae* strains. PCR amplifications were carried out according to Legras and Karst (2003). PCR products were separated by electrophoresis in 2% (w/v) agarose gels containing 1 μg/μl ethidium bromide, at 80 V for 1 h in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). Electrophoresis gels were acquired using the Gel Doc 2000 (Bio-Rad).

2.4. Fermentative performance

The fermentative vigour, is the speed for which yeast starts the fermentation whereas the fermentation power is the maximum quantity of sugar that a wine yeast is capable of fermenting in must (OIV, 2012).

For this reason, to evaluate fermentative performances the *S. cerevisiae* strains were tested in microvinification trials using must without grape skin from Montepulciano d'Abruzzo cultivar (280 g/l fermentable sugars, 7.4 g/l titratable acidity and a pH 3.2). The must samples (95 ml), after treatment at 70 °C for 30 min, were