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Potenzialità dei microrganismi nella definizione e rafforzamento dei caratteri di tipicità delle varietà venete Tocai e Prosecco

Exploitation of microbial capability to enhance the characteristics of typical regional wines Prosecco and Tocai

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Summary

1. Introduction

1.1 Ecology of wine yeasts

Yeasts are widespread in nature and are found in soils, on the surface of vegetables and in the digestive tract of animals. Wind and insects disseminate them. They are distributed irregularly on the surface of the grape vine; found in small quantities on leaves, the stem and unripe grapes, they colonize the grape skin during maturation. Observations under the scanning electron microscope have identified the location of yeasts on the grape. They are rarely found on the bloom, but multiply preferentially on exudates released from microlesions in zones situated around the stomatal apparatus. *Botrytis cinerea* and lactic acid bacteria spores also develop on the proximity of these peristomatic fractures. The number of yeasts on the grape berry, just before harvest, is between 103 ad 105, depending on the geographical situation of the vineyard, climatic conditions during maturation, the sanitary state of the harvest, and pesticide treatments applied to the vine (Ribereau-Gayon P. *et al.* 2006).

Quantitative results available on this subject, anyway, are few. After the harvest, transport and crushing the crop, the number of cells capable of forming colonies on an agar medium generally attains 106 cells /ml of must. The number of yeast species significantly present on the grape is limited. Strictly oxidative metabolism yeasts, which belong to the genus *Rhodotorula* and a few alcohol sensitive species, are essentially found there. Among the latter, the apiculated species (*Kloekera apiculata* and his sporiferous form *Hanseniaspora uvarum*) are the most common. They comprise up to 99% of the yeasts isolated from certain grape samples. The following genera are associated with winemaking environment and they can be found but in lesser proportions: *Candida*, *Cryptococcus*, *Debaryomyces*; *Kluyveromyces*, *Metschnikowia Pichia*, *Saccharomycodes*, *Schizosaccharomyces* and *Brettanomyces* (and his sexual equivalent *Dekkera*) (Pretorius, 2000).

All the researches, that deal with this subject, confirm the extreme rarity of *S. cerevisiae* on grapes. Yet these yeasts are not totally absent. Their existence cannot be proven by spreading out diluted samples of must on a solid medium prepared in aseptic conditions but their presence on grapes can be proven by analyzing the spontaneous fermentative microflora of grape samples placed in sterile bags, then aseptically crushed and vinified in the laboratory in absence of contaminations.

1.1.1 Origin of wine yeasts

The fermentation of grape must is a complex ecological and biochemical process involving the sequential development of microbial species, as affected by particolar environment. The process includes the interaction of fungi, yeasts, lactic acid bacteria, acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms (Pretorius, 2000). Of all these, yeasts are the heart of biochemical interaction with the must derived from the varieties of *V. Vinifera* and other grape species.

Although the non-*Saccharomyces* yeasts are the predominant species as the grape, they grow well in the early stage of fermentation, but are subsequently replaced during the following steges by *Saccharomyces* yeasts, which are more tolerant to ethanol (Fleet and Heard, 1993). So, though many genera and species of yeasts are found in the musts, the genus *Saccharomyces* and mainly the specie *Saccharomyces cerevisiae* is the one responsible for alcoholic fermentation (Pretorius, 2000). The origins of non-*Saccharomyces* are grape skin and winery equipments (Fleet, 1993). However, the origin of *S. cerevisiae* is controversial; although the most significant finding was that it is practically absent from grapes and vineyard soils (Martini, 1993), some authors propose that this species is a "natural" organism present on plant fruits (Mortimer *et al.* 1999; Sniegowski *et al.* 2002). Others argue that there is an "artificial" origin and this species came from the hybridization of other *Saccharomyces* and then selected in a man-made environment (Martini, 1993), this model would be supported by the fact that *S. cerevisiae* has been found only in areas close to human civilization. Finally, some authors postulate that this species is a domesticated microorganism originating from its closest relative *Saccharomyces paradoxus*, a wild species found all around the world associated with insects, tree exudates and fermenting plant extracts. The occurrence of *S. cerevisiae* in the vineyard would be the consequence of back transportation from cellars by insects (Naumov, 1996).

Moreover, there is still a lack of agreement concerning the contribution to spontaneous fermentations of *S. cerevisiae* originating from the vineyard comparing to that originating from the winery. On one hand, spontaneous alcoholic fermentation is possible in sterilized vessels (Lopez *et al.* 2002) or in a newly built winery where *S. cerevisiae* has never been introduced (Beltran *et al.* 2002). On the other hand, as mentioned before, although it has

been found on damaged berries (Mortimer *et al.* 1999) wild *S. cerevisiae* is extremely rare on intact grapes (Sabate *et al.* 2002) whereas it can be found colonizing the winery equipment (Beltran *et al.* 2002; Sangorrin *et al.* 2002; Vaughan-Martini et al. 1995): some strains are even found in the winery over several years (Beltran *et al.* 2002; Frezier and Dubourdieu, 1992; Rosini, 1984).

1.1.2. Use of selected yeasts for oenological purposes

Originally, all wine was made by taking advantage of natural microflora for spontaneous fermentation; no deliberate inoculation was made to start the process. All the various yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural fermentations. A breakthrough was made in 1880 when Hansen, working at the Carlsberg winery in Denmark, isolated a pure culture derived from a single yeast cell and, in 1890, Muller-Thurgau from Geisenheim introduced the concept of inoculating wine fermentations with pure yeast starter cultures (Pretorius, 2000). In 1965, the first two commercial dried yeasts (ADWY) strains were produced for a large Californian winery (Fleet and Heard, 1993). These two strains, "Montrachet" and "Pasteur Champagne", were offered worldwide as allpurpose yeasts. The inoculation of selected pure yeast cultures into must is nowadays a common enological practice established since the 1970s, in order to produce wine with desirable organoleptic characteristics and to guarantee the homogeneity of successive vintages. Today, several yeast-manufacturing compagnie market a wide variety of dehydrated cultures of various *S. cerevisiae* strains, and most of worldwide wine production relies on the use of such commercial starter yeasts.
In the past 30 years, strains of *S. cerevisiae* have been selected for their enological properties and are used as starters in winemaking processes. Yet these strains involved in fermentation play an important rule in determining the characteristics of the final product, in terms of high alcohol percentage and absence of undesirable compounds (Perez-Coello *et al.* 1999) while the diversity of native *S. cerevisiae* strains present in spontaneous fermentations contribute to the chemical composition and sensory qualities of the resulting wine (Lurton *et al.* 1995). Moreover, several studies support the hypothesis that active dried yeasts reduce the variability of strains that appear in spontaneous fermentations

(Beltran *et al.* 2002; Fleet, 2003) and, possibly, the complexity of the resulting wine. For these reasons, winemakers looking for original flavours prefer spontaneous fermentation with indigenous yeasts. For the same reasons, lots of recent selection projects for new wine strains focus on ecotypical strains, trying to preserve biodiversity in selected areas and at the same time to guarantee optimal fermentation performances.

The recent discovery that an overabundance of living cells of *S. cerevisiae* is present in every kind winery is providing wine technologists with a large reservoir of strain diversity as a new source of locally selected starters for wine-making. Since *S. cerevisiae* populations, isolated from vineyard and wineries, endowed with enological properties wholly comparable to those of commercial starters, autochthonous starters may prevent excessive standardization engendered by the presence of only few active dry commercial starters in the international market (Martini, 2003).

1.1.3. Genomic characteristics of wine yeast

Industrial *S. cerevisiae* strains are highly specialized organisms, which have evolved to utilize their full potential in the different environments or ecological niches that have been provided by human activity. This selection process can be described as "domestication" and can be responsible of the special genetic characteristics of industrial strains (Querol *et al.* 2003). *S. cerevisiae* has a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns. Haploid strains contain approximately 12-13 megabases of nuclear DNA, distributed along 16 linear chromosomes whose size vary from 250 to 2000 kb (Barre *et al.* 1992). In contrast to most *S. cerevisiae* strains used in the laboratory, which are either haploid or diploid and have a constant chromosome electrophoretic profile, wine yeast strains are mainly diploid, aneuploid, or polyploid, homotallic and highly heterozygous, and show a high level of chromosome length polymorphism. Moreover, wine yeast strain seem not to remain genetically uniform (reviewed in Pretorius -Pretorius, 2000- and in Querol *et al. 2003*). Their exacerbated capacity to reorganize its genome by chromosomal rearrangements, such as Ty-promoted chromosomal translocations (Longo and Vézinhet 1993.; Rachidi *et al.* 1999), mitotic crossing over (Aguilera *et al.* 2000) and gene conversion (Puig *et al.* 2000) promotes a faster adaptation to environmental changes

than spontaneous mutations, which occur at comparatively very low rates. In particular, the ploidy of wine yeasts may confer advantages to adapt to variable external environments and increase the dosage of some genes important for fermentation (Bakalinsky et al. 1990; Salmon, 1997). The illegitimate recombination mediated by Ty elements and subtelomeric repeated sequences has several practical consequences: sporulation ability is very variable (between 0 and 75% ascus formation on a sporulation medium) and spore viability is also highly variable, ranging from 0 to 98% (Barre *et al*. 1993; Còdon *et al*. 1995). The meiotic segregants from wine strains diploidize with high frequency, indicating a high frequency of homotallism. Heterozygosity has been observed in both homothallic and heterothallic wine strains. In addition, the possibility of adaptive gross genomic changes occurring during laboratory growth conditions has been demonstrated by Hughes et al. (Hughes, Marton *et al.* 2000; Hughes, Roberts *et al.* 2000): those authors showed in multiple cases that the deletion of a single gene strongly favors the acquisition of a whole chromosome or a chromosome segment containing a compensatory copy of a close homolog of the deleted gene.

1.2 Saccharomyces wine yeasts taxonomy

The taxonomy of *Saccharomyces* wine yeasts has undergone multiple changes, due to successive reorganizations of the classification of this group (Pretorius 2000; Rainieri et al. 2003). *Saccharomyces* wine yeasts are all now considered to belong to the genus *Saccharomyces.* This taxon initially included four species - *S. cerevisiae, S. bayanus, S. paradoxus, S. pastorianus*- but has since been enriched by the addition of another five species: *S. kudriavzevii, S. tnikatae, S. cariocanus, S.arboricolus* and *S. eubayanus* (Vaughan-Martini and Martini 1998; Naumov et al. 2000; Rainieri et al. 2003; Shi-An Wang and Feng-Yan Bai, 2008; Libkind et al. 2011). Most wine yeasts used for alcoholic fermentation are now recognized as *Saccharomyces cerevisiae.* However, *Saccharomyces bayanus* may also mediate alcoholic fermentation, particularly in low-temperature conditions, as they are cryotolerant (Naumov et al. 2000). Strains of *S. paradoxus* have been isolated from vineyards, but their potential contribution to wine fermentation is unknown (Redzepovic et al. 2002).

Another level of complexity within the genus *Saccharomyces* group arises from the ability of strains of different species to mate and form hybrids (Barros Lopes *et al.* 2002). In recent years, the molecular characterization of *Saccharomyces* wine strains has revealed some of these strains to be hybrids (Masneuf et al. 1998). This situation extends the range of phenotypic diversity for wine yeasts and has potentially significant industrial implications.

These results underline some interesting features on the mechanism of *Saccharomyces* genus evolution. In fact the emergence of fermented beverages roughly matches the domestication of plants and animals, it is likely that some yeast lineages with favoured traits were also unwittingly domesticated. In contrast to extensive investigation into domestication of crops and livestock, studies of domestication of eukaryotic microbes have been limited, perhaps because of the inability to conduct direct field studies. Identifying the genetic basis of traits under selection during domestication may clarify the emergence of new traits and show the way toward further improvement. Because domesticated lineages derive from a subset of the original populations, a genetic bottleneck is likely to have caused the disappearance of some alleles, especially in microbes, which are often propagated clonally. In an age of accelerated habitat destruction and diminishing biodiversity, discovery of wild genetic stocks of domesticated microbes will facilitate preservation of their genetic resources for strain improvement. In this context, the new finding from sequencing the whole genome of *S eubayanus* becomes the starting point for understanding the evolution relationships among the environmental and technological *Saccharomyces* strains used for the production of fermented food and beverages (Libkind et al. 2011).

1.2.1 Genetic proprieties of S.cerevisiae wine strain

The genome structure of *S. cerevisiae* is intimately linked to its genetic properties, which reciprocally influence the life style and genome characteristics of this yeast. *S. cerevisiae* strains are mostly diploid in natural conditions and display vegetative reproduction through multi-polar budding.

One peculiarity of *S. cerevisiae* wine strains is that many are homothallic, and descendants of these haploid spores mate with their own progeny to form a diploid. Homothallism is

frequent in wine yeasts, with about 70% of strains known to be homothallic (Mortimer 2000), but the ecological significance of this property remains unclear. Mortimer et al. (1994) suggested that homothallism may provide required conditions for a process he called "genome renewal". According to this model, yeasts accumulate mutations during the vegetative stages of their life, rendering them heterozygous for various traits. Upon sporulation and the self-mating of homothallic spores, homozygous diploids are generated. This process makes it possible to eliminate recessive mutations deleterious for the strain or to ensure that recessive mutations increasing strain fitness are expressed. Genome renewal is therefore likely to play a role in adaptation of yeasts to stressful wine environment.
Mortimer (2000) proposed that about one third of wine S. cerevisiae cells were homozygous. Conversely, high levels of heterozygosity was deduced from various approaches and was shown to lead to massive differences in gene expression among segregants (Bradbury et al. 2005; Cavalieri et al. 2000).

The external crossing rate of S. cerevisiae was recently estimated indirectly, through an analysis of the whole genome sequences of three strains and the sister species *S. paradoxus* (Ruderfer et al. 2006). It was concluded that outcrossing was rare, occurring once every 50,000 divisions, corresponding to once every 17-137 years assuming that one to eight divisions occur per day (König et al. 2009).

1.2.3 The ploidy of wine yeasts

Early genetic studies on wine yeasts indicated that most strains were diploid though some were polyploid or aneuploid (Cummings and Fogel 1978; Thornton and Eschenbruch 1976; Takahashi 1978; Bakalinsky and Snow 1990). Various genetic data and DNA analyses have suggested that aneuploidy was common in flor yeasts (Martinez et al. 1995; Guijo et al. 1997). More accurate information about yeast aneuploidy was recently obtained through CGH analysis, with Infante et al. (2003) confirming that flor yeasts are aneuploid for several chromosomes. Unexpectedly, a similar karyotyping of commercial "fermentation" strains revealed no whole chromosome aneuploidy (Dunn et al. 2005).
Moreover, Legras et al. (2007) recently reported that 88% of the *S. cerevisiae* strains had allele patterns consistent with a diploid state. Unlike other industrial yeasts (baker's yeast

and brewing yeast strains), which have ploidy levels exceeding 2 n, most of the *Saccharomyces cerevisiae* strains used in wine-making seem to be diploid. Flor yeasts have features closer to other industrial yeasts, with more common aneuploidy and polyploidy certainly in relation to the occupancy of a specific ecological niche (König et al. 2009).

1.2.4 Chromosomal rearrangements

The existence of gross chromosomal rearrangements -translocations, deletions add amplifications of chromosomal regions was rapidly suspected based on the high level of chromosome polymorphism found in wine yeasts. Analysis of wine yeast chromosomes by pulsed-field gel electrophoresis (PFGE) demonstrates major chromosome length polymorphism between wine yeast strains (Vezinhet et al. 1990; Yamamoto et al. 1991). Such variations in chromosome size clearly resulted from gross chromosomal rearrangements (GCR).

Recombination between repeated Ty sequences interspersed throughout the genome is shown to be a major cause of chromosomal translocation (Rachidi et al. 1999; Codon et al. 1998). Other types of repeated sequences - tRNA and telomeric Y' sequences - may also serve as substrates for ectopic recombinations leading to chromosomal rearrangements (Carro et al. 2003).

One of the main conclusions of the CGH analysis is that wine yeasts are closely related. Indeed, "fermentation" strains do not contain the extensive amplifications of chromosomal regions observed in flor strains (Infante et al. 2003).

It has been suggested that flor yeasts must deal with high acetaldehyde, concentrations during wine aging, potentially inducing double strand breaks, the processing of which may favor GCR, (Infante et al. 2003). The differences between the environments of fermentation and flor yeasts may therefore support different evolutionary processes. CGH analysis is subject to certain limitations, which must be taken into account. This approach cannot detect reciprocal translocations or account for the existence of genes other than those already identified in the sequenced laboratory strain.

The effects on yeast fitness of most of these rearrangements remain unclear.

In flor yeasts, the amplified regions were shown to contain genes potentially useful for strain adaptation, such as *ADH*, which encodes alcohol dehydrogenase. This enzyme can detoxify the medium by removing acetaldehyde. In addition, a key gene for velum formation, *FLO1IIMUC1*, encoding a cell wall mucin, is found amplified and has an altered expression due to a promoter modification (Infante et al. 2003; Fidalgo et al. 2006). The best studied case of contribution to adaptation is that of a translocation between chromosome VIII and XVI which has a direct impact on sulfite resistance (Perez-Ortin et al. 2002).

The SSU1 gene encodes a plasma membrane protein that is thought to extrude sulfite anions and confers sulfite resistance (Park and Bakalinsky 2000). SSU1 is located on chromosome XVI in the laboratory strain, but an allele conferring higher levels of sulfite resistance is found associated with a translocation onto chromosome VIII (Goto et al. 1998). A survey of the translocation distribution shows that it is widespread in wine yeasts. This translocation is the only clear example identified o date in wine yeast in which a chromosomal rearrangement has been shown to be involved in adaptation to the Wine environment and to be selected in response to a technological practice-extensive sulfite use (König et al. 2009).

1.2.5 Yeast sequencing

In 1996, the budding yeast *S.cerevisiae* became the first eukaryotic organism to have its genome completely sequenced (Goffeau et al. 1996). The strain sequenced, S288C, is a commonly used laboratory strain that was obtained in the 1950s, by mating a strain isolated from a rotten fig (EM93) with a commercial strain (Mortimer and Johnston 1986). While experimental conditions may have left a significant footprint on the evolution of S288C (Gu et al. 2005), since 1996, the S288C genome sequence has been the only reference sequence available for *S. cerevisiae*. Today the genomes of several other yeast strains have been sequenced, including that of RM11-1a, a haploid derivative of a natural vineyard isolate, the clinical isolate YJM789 (Wei et al. 2007), and the diploid, heterozygous wine yeast strain EC1118 widely used as, starter in the

wine industry. The sequence divergence between these strains and the reference strain has been estimated at 0.5-1%, similar to that between humans and chimpanzees.

Recently, analysis of the EC1118 genome (Dequin et al., unpublished data) has revealed a sequence divergence relative to S288C or RM11 of 0.5 or 0.25% respectively. It has been estimated that there is less than 0.1% heterozygosity between EC1118 haplotypes, a value in the range of the variation observed between human beings (0.1-0.01%). About 50,000 SNIPs and only a moderate number of indels (5,000) with respect to the S288c genome have been identified in the genome of EC1118, suggesting that, as observed for YJM789, SNPs might be a primary cause of heritable phenotypic variation between strains. The genome of EC 1118 has in addition, remarkable peculiarities. Interestingly, the genome of this strain contained entirely new regions, carrying several genes involved in metabolic and transport functions. The study of the distribution of these fragments within the *S. cerevisiae* species, as well as their potential contribution to the adaptation of yeast to wine environment is under way (König et al. 2009).

1.3 Targets for selection and improvement of wine yeasts

Wine technologists gathered the basic properties required for the definition of a ‘‘selected *S. cerevisiae* strain for wine making’’ in two categories (Reed G and Chan SL. 1979): (1) primary or fitness traits, defined as those strictly associated with the formation of ethyl alcohol by fermentation, and (2) secondary or quality traits, defined as those related to the production of compounds that affect other parameters, such as the body of a wine, the higher alcohols complex (bouquet), and the appearance of undesirable off-flavors. Main primary and secondary traits are summarized in table 1.1, where some further traits, more specific and functional to the type of destre wine, are also listed (Pretorius 2000).

Table 1.1 Main desirable characteristics of wine yeast

Fitness traits	
Fermentation properties	**Technological properties**
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	High sulphite tolerance
High fermentation rate	Low sulphite binding activity
High ethanol tolerance	Low foam formation
High osmotolerance	Flocculation properties
Low temperature optimum	Copper resistance
Moderate biomass production	Resistance to desiccation
	Killer properties
	Proteolytic activity
	Low nitrogen demand
Quality traits	
Flavour characteristics	**Metabolic properties with health implications**
Low volatile acidity production	Low sulphite formation
Moderate higher alcohol production	Low biogenic amine formation
Low sulphite/DMS/thiol formation	Low ethyl carbamate (urea) potential
Liberation of glycosylated flavour precursors	
No phenolic off-flavours production	
High glycerol production	
Modified esterase activity	
Enhanced autolysis	
Hydrolytic activity	

Some of the requirements listed in Table 1.1 are complex and difficult to define genetically without a better understanding of the involved biochemistry and physiology. To date, no wine yeast present on the market has all the characteristics listed, and it is well established that wine yeasts have different behaviour concerning their winemaking abilities. Although this phenomenon can be ascribed to fermentation conditions that are hardly reproducible, the major source of variation can be attributed to the genetic constitution of the wine yeasts (Pretorius 2000).

1.3.1 Fitness traits

The technological traits influence the efficiency of the fermentation process. *S. cerevisiae* strains generally possess the technological characteristics required to perform an efficient fermentation. The determination of these traits is, however, necessary, since most of these characteristics are strain specifics.

1.3.1.1 Main fermentation properties

The rate of fermentation and the amount of alcohol produced per unit of sugar during the transformation of grape must into wine is of considerable commercial importance. The fermentation efficiency is intended as the uppermost concentration of ethanol obtainable by fermentation from an excess of sugar. The fermentation rate (vigour) is the measure of the ability of a starter to bring the fermentative process to a fast completion. It is normally represented as grams of CO_2 developed in 24 h, calculated as the average of a 3-day measurement period (Martini 2003). During wine yeast glycolysis, one molecule of glucose or fructose yields two molecules each of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapour (Boulton *et al.* 1996). The ethanol production and fermentation rate are closely linked to ethanol tolerance: in fact while ethyl alcohol is the major desired metabolic product of grape juice fermentation, it is also a potent chemical stress factor that is often the underlying cause of sluggish or stuck fermentations. Apart from the inhibitory effect of excessive sugar content on yeast growth and vinification fermentation, the production of excessive amounts of ethanol, coming from harvest of over-ripe grapes, is known to inhibit yeast growth rate, viability and fermentation capacity: cell growth stops at relatively low ethanol concentrations, and fermentation stops at relatively higher levels. Decreases in the rate of ethanol production are related to decreases in viable cell count. Cell growth inhibition by ethanol is noncompetitive and has been described as either a linear or an exponential function of ethanol concentration (Boulton *et al.* 1996; Benitez *et al.* 1996). Generally, sugar catabolism and fermentation proceed at a rate greater

than desired, and are usually controlled by lowering the fermentation temperature (Fleet, 1993). Occasionally, wine fermentation ceases prematurely or proceeds too slowly. The commercial implications of sluggish or incomplete wine fermentations are usually attributed to inefficient utilization of fermenter space and wine spoilage resulting from the low rate of protective carbon dioxide evolution and high residual sugar content. Conversely, financial losses through `runaway' wine fermentations arise from the fact that fermentor space is reduced because of foaming and volatile aroma compounds are lost by entrainment with the evolving carbon dioxide. Thus, yeast behaviours towards temperature are also very important in wine making control: a wide range of growth temperatures is suitable for wine strains, and fermentation efficiency should not swiftly decrease as small temperature changes happen. Optimal performance of wine yeasts in white wine fermentations, conducted at cooler temperatures (10±15°C) so as to minimize the loss of aromatic volatiles, and red wine fermentations, performed at higher temperatures (18±30°C) to enhance extraction of anthocyanin pigments, is therefore of critical importance to wine quality and costeffectiveness (Henschke, 1997).

1.3.1.2. Main technological properties

Several antimicrobial compounds, as well as ethanol, can interfere with yeast fermentation activity. Some of these compounds are usually added to fermentation tanks, as sulphite dioxide; other ones are found in grape must coming from agrochemical treatments as copper and pesticides; finally antimicrobial killer toxins are produced by some yeasts and are lethal to other sensitive ones. Sulphur dioxide is widely used in enology for its antioxidant activity and as antimicrobial agent towards yeast, acetic and lactic acid bacteria in general. Moreover, *Saccharomyces* is the most resistant yeast among wine-related species, so SO_2 addiction selects this microorganism inhibiting apiculated ethanol-sensitive species; thus tolerance to sulphite forms the basis of selective implantation of active dried wine yeast starter cultures into grape must. SO2 addiction, anyway, can affect differently fermentation kinetics and although *S. cerevisiae* tolerates higher levels of sulphite than most unwanted yeasts and bacteria, excessive SO_2 dosages may cause sluggish or stuck fermentations (Boulton *et al.* 1996) Wine yeasts strains vary widely in their resistance to

sulphite, and the underlying mechanism of tolerance as well as the genetic basis for resistance are still unclear. Within the *Saccharomyces* species, resistant strains are quite frequent (around 30%) and they can develop in presence of 150 ppm of SO_2, while more sensitive strains are inhibited at concentrations such as 100 ppm that mainly causes a prolongation of lag phase.

Wide application of copper-containing fungal pesticides (copper oxychloride) to control downy mildew (*Plasmopara viticola*) and, to a lesser extent, dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*) could lead to copper residues in musts that may cause lagging fermentation and affect wine quality detrimentally. This phenomenon recently increased due to the diffusion of the organic and integrated cultivations, where copper is widely used to reduce or eliminate the need of other chemical treatments. *S. cerevisiae* species exhibits a significant variability in copper resistance and the acquisition of this trait seems to be the result of an environmental adaptation (Romano, 2005).Several copper uptake, efflux and chelation strategies have been developed by yeasts to control copper ion homeostasis. In particular, copper sensitive strains do not change the metal concentration in wine, whereas resistant strains sensibly reduce this element accumulating copper inside the cell. Killer toxins are proteins produced by some yeasts that are lethal to sensitive wine yeast strains. The killers themselves, however, are immune to these mycovirus associated toxins. It remains controversial whether the growth and zymocidal activity of some wild killer yeasts have the potential to delay the onset of fermentation, cause sluggish or stuck fermentations and produce wines with increased levels of acetaldehyde, lactic acid, acetic acid and other undesirable sensory qualities. An unfortunate consequence of ignorance regarding the role of killer yeasts in wine fermentations is that some winemakers use co-cultures to inoculate fermentations, one strain being a killer and the other a sensitive strain. The advantage of using killer or neutral wine yeasts should therefore not be underestimated (Pretorius 2000).

1.3.2. Quality traits

The quality of wine is the outcome of complex chemosensory interactions that are difficult to predict because of the influences of many variables. The chemical composition of wine

is the foundation of both sensory response and wholesomeness, and it is determined by many factors. These include the grape variety, the geographical and viticultural conditions of grape cultivation, the microbial ecology of the grape and fermentation processes, and winemaking practices (Owens and Noble, 1997).

Microorganisms have a prominent role in determining the chemical composition of wine. They affect the quality of the grape prior to harvest and, during fermentation, they metabolise grape sugars and other components into ethanol, carbon dioxide and hundreds of secondary end-products that, collectively, contribute to the subtlety and individuality of wine character (Nykanen L. 1986).

1.3.2.1. Flavour characteristics

Alcoholic beverages contain mainly saturated, straight chain fatty acids. The volatile acid content of wine usually lies between 400 and 1000 mg/L, normally more than 90% of volatile acid consists of acetic acid. Although acetic and latic acid bacteria can be associated with high levels of short chain fatty acid, acetic, propanoic and butanoic acids are by-products of alcoholic fermentation (Ribereau-Gayon *et al.* 2000). Fermentation purity is expressed as of the ratio between volatile acidity (as g acetic acid/L) and ethanol (% volume) produced at the end of the fermentation process. High values of this ratio denote the ability to form few undesirable by-products in the course of fermentation. Wines cannot be commercialized if volatile acidity exceeds one tenth of the ethanol content.

Another fermentation by-product affecting wine quality is glycerol. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol. Due to its non-volatile nature, glycerol has no direct impact on the aromatic characteristics of wine. However, this triol imparts certain other sensory qualities; it has a slightly sweet taste, and owing to its viscous nature, also contributes to the smoothness, consistency and overall body of wine. Wine yeast strains producing a consistent amount of glycerol would therefore be of considerable value in improving the organoleptic quality of wine. Among other yeast metabolites, the formation of sulphite and sulphide by wine strains greatly affects the quality of wine. Sulphur is essential for yeast growth and *S. cerevisiae* can use sulphate, sulphite and

elemental sulphur as sole sources. Unlike sulphur dioxide (SO_2), which when properly used, has some beneficial effects, hydrogen sulphide (H_2S) is one of the most undesirable yeast metabolite, since it causes, above threshold levels of 50-80 g/L, an off-favour reminiscent of rotten eggs (Snow R. 1983). Even though the compositional variability of musts (i.e., the precursors of bouquet molecules variably distributed within grape varieties) is considered the main source of organoleptic specificity, today the wine technologists re-evaluate the role of yeast metabolism (strain-related by-products of fermentation) in the formation of bouquet and aroma. In fact, the growth, by means of alcoholic fermentation as energy source, is the best way for yeasts to make a contribution to wine flavour, as well (Henschke, 1997).This phenomenon is carried out by several mechanisms that involves the degrading of grape juice constituents and the production of a great amount of different compounds: mainly ethanol and other solvents that help to extract flavour components from grape solids, hundreds of secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds) that contribute considerably to wine aroma and the products of autolytic activity that characterizes the stationary phase of yeast growth. Moreover a great variety of exoenzymes are normally produced by these microorganisms that can transform neutral grape compounds into flavour active molecules (Nykanen L. 1986). These reactions, especially the production of secondary metabolites, vary with the species and strain of yeast. Tables comparing the diversity of metabolite production by different yeasts may be found in Fleet, Lema et al. 1996, Romano 1997, Heard 1999, and Lambrechts and Pretorius. Thus, the uniqueness and individuality of the flavour contribution by yeasts depends on the species and strains operating the fermentation.

1.3.2.2. Metabolic properties that influence wine safety

Today, it is generally accepted that moderate wine drinking can be socially beneficial, and that it can be effective in the management of stress and reducing the risk of coronary heart disease. In the selection and improvement projects concerning wine yeast strains, it is therefore of the utmost importance to focus on these health aspects and to obtain yeasts that may reduce the risks and enhance the benefits. Likewise, research in several laboratories

around the world is directed towards the elimination of suspected carcinogenic compounds in wine, such as ethyl carbamate, and asthmatic chemical preservatives, such as sulphites. It might even be possible to develop wine yeasts that could increase the levels of phenolic and antioxidative substances (e.g. resveratrol) associated with the so-called `French paradox', in which, despite the high dietary fat intake of the cheeseloving population of southern France, the death rate from coronary heart disease is significantly lower than the one found in industrialized countries (Pretorius, 2000)

1.4. Selection strategies for new strains of enological interest

Selection and genetic improvement of an organism is based on the ability to achieve a specific task or to do a precise function. In the case of wine yeasts, it is necessary that the selected strains have some basic traits combined with others more specific and functional to the type of wine desired (as summarised in Table 1.1). The primary selection criteria applied to most strain development programs relate to the overall objective of achieving a better than 98% conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate and without the development of offfavours. The growth and fermentation properties of wine yeasts have, however, yet to be genetically defined. What makes the genetic definition of these attributes even more complex is the fact that lag phase, rate and efficiency of sugar conversion, resistance to inhibitory substances and total time of fermentation are strongly affected by the physiological condition of the yeast, as well as by the physicochemical and nutrient properties of grape must.

1.4.1. Clonal selection

The starting point for the genetic improvement of wine yeasts is always the isolation from grapes, grape musts and wines of a high number of yeast strains, which are then submitted to the analysis of their oenological properties. The process, named ‘clonal selection’, produces pure strain clones which can either meet or not all the desired traits for winemaking, but it allows the constitution of a biodiversity background, which is very useful for successive selection steps or improvement programs. The selection is generally

carried out within the genus *Saccharomyces*. Yeast cultures are preferably isolated from grape juice or wine. *Saccharomyces* strains growing in these substrates are, in fact, well adapted to the oenological environment and can therefore ferment grape juice very efficiently. Nevertheless, *Saccharomyces* yeasts are scarcely present on grapes. Their isolation on solid media, may therefore not be suited, especially if a relevant number of cultures need to be collected. The use of an enrichment technique is generally preferred (Constantí *et al.* 1997; Versavaud et al. 1995). This method consists of creating the conditions that favour the growth of some microorganisms in a mixed population, and inhibit the growth of the rest. The high concentration of ethanol that accumulates in grape juice during fermentation is the main factor favouring the selection of *Saccharomyces* strains. Isolations are therefore carried out after the fermentation (or micro-fermentation) of grape juice. The resulting yeasts are then submitted to characterization steps.

1.4.1.1. Phenotypic characterization of yeasts

In the first step of a selection program a great number of isolates is subjected to different phenotypic tests with the aim of identifying and characterizing yeast strains and species. Taxonomists first delimitated the yeast species using morphological and physiological criteria. The first classifications were based on phenotypic differences between yeasts: cell shape and size, spore formation, cultural characters, fermentation and assimilation of different sugars, assimilation of nitrates, growthfactor needs, resistance to cyclo-heximide. Since then, many rapid, ready to use diagnostic kits have been also developed to determine yeast response to different physiological tests (Ribereau-Gayon *et al*., 2006)

Due to the relatively limited amount of yeast species significantly present on grapes and in wine, most of these phenotypic tests can easily identify oenological yeasts; some of them can be identified by simple observation of growing cells under the microscope. Small apiculated cells, having lemon-like shape, are typical of the species *Hanseniaspora uvarum* and its imperfect form *Kloekera apiculata*.

Saccharomycodes ludwigii is characterized by apiculated cells of a larger size (10- 20μm). Since most yeasts multiply by budding, the genus *Schizosaccharomyces* can be recognized because of its typical vegetative reproduction by binary fission. Finally, the budding of

Candida stellata produces star-shape cells. According to Barnett et al. 1990, the physiological characteristics can be used to distinguish between the principal grape and wine yeasts. These features can be studied individually setting up selective fermentation and growth tests, or in combined trials. On the basis of physiological tests the researchers Lafon-Lafourcade and Joyeux 1979 and, in the same period, Cuinier and Levau 1979, designed a ready to use kit (API 20 C system) for the identification of enological yeasts. It contains eight fermentation tests and ten concerning assimilation and resistance to cyclo-heximide. For a more complete identification, the API 50 CH system was developed, it contains 50 substrates for fermentation (under paraffin) and assimilation tests. Finally, Fleet and Heard in 1990 proposed a system that uses the different tests listed in Barnett's work. Appling this new method, it was found that some of these characteristics (for example sugars fermentation profiles) vary within the species and are even unstable for a given strain under vegetative multiplication. There is a considerable part of the current literature that uses the cell fatty-acyl composition as a means of yeast identification. This taxonomic tool has been applied especially to identify wine spoilage yeasts but also to characterize various species and strains (Kunkee RE, Bisson LF. 1993).

In general, during a selection program, the most used tests, among phenotype-based systems for distinguishing *Saccaromyces* species from other yeasts related to oenological environment, are based on selective growth media and phenotypic evaluation of colony colour and morphology (i.e. on WL nutrient agar). They have the great advantage to be easy to perform and very cheap but it was found that strains of *S.cerevisiae* can form colonies slightly different on these kind of media, and the morphological characteristics can be unstable under several multiplications. Thus this approach can not be considered decisive, since possible variations at strain level could lead to erroneous attributions. It is therefore currently accepted that phenotypic analyses are not sufficient to reach a trustworthy identification (Kurtzman and Robnett, 2003).

1.4.2. Selection of ecotypical yeast strains

The main critics of the practice of guided fermentations (using starter cultures) dislike the fact that the commercial wine strains, despite being numerous, possess very ordinary

characteristics. Commercial yeast strains produce wines with average qualities and do not enhance the aromatic traits that characterise many yeasts isolated from specific geographical areas. Studies on the improvement and the selection of wine yeasts to overcome this problem have recently been carried out.

In the last few years, there has been an increasing use of new local selected yeasts for controlled must fermentation in countries with a winemaking tradition. Though there are commercial yeasts to accomplish must fermentation, the use of local selected yeasts is believed to be much more effective. Local yeasts are presumed to be more competitive because they are better acclimated to the environmental conditions. Therefore, they would be better able to dominate the fermentation and become the most important biological agent responsible for the vinification. Selection of the appropriate local yeasts assures the maintenance of the typical sensory properties of the wines produced in any given region. Strains of *S. cerevisiae* can be isolated from vineyards and wine fermentations, and selected to be used as commercial starter cultures. It is now believed that strains of *S. cerevisiae* indigenous to vineyards and wineries tend to be homozygous for most of the genes by a process known as `genome renewal' (Mortimer *et al.* 1994). This process would eliminate the recessive lethal or deleterious genes that adversely affect yeast fitness (e.g. slower growth, lower fermentation rate, reduced spore viability, etc.). Genome renewal could also be responsible for the replacement of the parental heterozygous strains by the new homozygous diploids bearing new recessive alleles that increase fitness. The practical implications of genome renewal and yeast population dynamics in the vineyards and wineries (and even within yeast starter cultures) are farreaching, whether winemakers rely on spontaneous fermentation of grape juice or whether they inoculate grape must with selected wine yeast strains. Although dramatic improvements in most characteristics cannot be expected, intra-strain selection has been used for decades to obtain improved wine yeast strains and is still, up to date, one of the most utilized selection strategies (Pretorius, 1999)

The selection of wine yeasts for oenological use is traditionally carried out on the basis of their technological and quality-linked phenotypic characteristics. For this purpose different methodologies were designed.

1.4.2.1. Screening methods based on fitness traits

The technological characteristics required to wine strains may vary, depending on the musts and on the winemaking techniques used. However, some of these characteristics, like high fermentation vigour and ethanol production as well as low H_2S and acetic acid formation, are of particular interest for the selection of any kind of starter strain (Giudici and Zambonelli, 1992).

Recently a two-step procedure was proposed: a pre-selection based on resistance to SO_2, killer activity, growth at high temperature and low foam production, followed by a selection based on volatile acidity, ethanol production, and residual sugars. Another methodology based on phenotypic characteristics is carried on following four consecutive steps: (1) fermenting capacity of the strains (2) formation of volatile acidity, resistance to SO_2, production of H_2S, flocculation capacity and adherence to glass; (3) autolytic capacity of the yeast; (4) foaming properties of the autolysates obtained (Martinez et al. 2001).

The oenological traits can be evaluated by carrying out small-scale fermentations in synthetic media and eventually in grape juice.

To assess both fermentation efficiency and fermentation vigour, weight loss due to CO_2 formation during fermentation is usually followed: in particular Castelli in 1954 proposed microfermentations in grape must enriched in glucose to a final content of 30% (excess of sugar) in flasks stoppered with sulphuric acid-containing valves (in order to avoid water loss), performed at 25°C. Some years later, Ciani and Rosini (1990) proposed microfermentations performed on pastorized grape must where yeast cultures were pre-incubated in grape must for 48 h. Alternatively, microfermentations can be performed on synthetic must as described by Bely et al., (1990). In any case, fermentation efficiency (the uppermost concentration of ethanol obtainable) is calculated from weight loss at the end of fermentation (when no variations in weight are observed for two consecutive days). Fermentation rate is expressed as grams of CO_2 developed in 24 h, calculated as the average of a 3-day measurement period and followed during fermentation. Fermentation vigour is normally expressed as g of CO_2 produced in the first 48 hours following the inoculation of the must. The same of fermentation conditions (better if in untreated natural must) can be used to test sulphur dioxide resistance: after pasteurization, the must is split in

two: SO_2 as potassium metabisulphite is added (usually to a final concentration of 100 and/or 150 mg/L) to one aliquot. Both Flasks are inoculated and incubated at 25°C. After 2 and 7 days the weight loss caused by CO_2 production is determined, sulphite resistance is obtained by comparison with flasks where no SO_2 is added. SO_2 determination at the end of fermentation in un-sulphited must is also important: ability to produce SO_2 by sulphate reduction is widespread among *S. cerevisiae* natural isolate, and no strains completely unable to produce this anhydride have been ever described. Since production levels of some particular strains are astonishing (up to 200-300 mg/L and up to 500 mg/L if sulphite are previously added to must), this character should be considered during strain selection (SO_2 production lower than 25-30 mg/L is recommended. Zambonelli, 2003).

1.4.2.2. Screening methods based on quality traits

Some of these characters can be studied using Petri dishes containing the suitable growing medium. Hydrogen sulphide production is evaluable on ABY or BiGGY agar at 25°C for 48 h [166]. The screening medium is inoculated with a small quantity of yeast biomass, and, after incubation, the colour of the growing colony (white, pale hazel, hazel, dark hazel, black) is observed: the darker the colony appears the higher is the H_2S quantity on BiGGY agar. Analogously, acetic acid production can be evaluated on calcium carbonate agar at 25°C during a period of 7 days incubation: the presence of an halo around the colony indicates strains producing high quantities of acetic acid, which causes dissolution of calcium carbonate salt on the plate. The acetic acid production is a stable character (Romano et al. 1998) but it is influenced by the must composition thus a quantification of acetic acid production during fermentation is also desirable. Alternatively, paper impregnated with PbAcO to saturation point can be used to carry out the qualitative control of H_2S production during fermentations. At the end of microfermntations (usually performed for fermentation efficiency or fermentation vigour determination), also some other endpoint products and by-products such as ethanol, acetic acid, succinic acid, glycerol, acetaldehyde, malic acid can be determined by standard chemical analysis, HPLC or enzymatic kits. Finally, the presence of several glycosidic enzymes and the quantification of their activity in oenological indigenous yeasts has been introduced as a

test, in order to select strains that contribute to enhance the primary aroma of the regional grapevine.

Yeast strains can be screened to determine the presence of β-glucosidase and glycosidase activities. The most popular screening test for β-glucosidase activity is carried out on agar plates with arbutin as substrate: yeast isolates that possess the proper enzyme are able to hydrolyse the substrate and a dark brown halo develops in the agar medium. Glycosidase activities can be determined by using the appropriate 4-methylumbelliferyl glycoside as substrate, as described by Manzanares et al.(1999). The presence of the enzymatic activity is then visualized as a fluorescent halo surrounding yeast growth after plate exposure to UV light. Alternatively, the same 4-MUG substrates can be used to perform the test in liquid growing media (Fia *et al*. 2005).

1.5 Aim of this work

The wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeast species. The main function is played by *Saccharomyces cerevisiae*, the species more tolerant to ethanol, that catalyses an efficient conversion of grape sugar into ethanol, carbon dioxide and other minor, but important, metabolites. In the past few years there has been a noticeable increase in the demand for autochthonous wine yeasts to be used as fermentation starters. The requirements for these yeasts are the ability to dominate during the fermentation process, and enhance, at the same time, the sensorial characteristics of the wines originating from different grapevine cultivars. In fact if commercial yeasts enable rapid and reliable fermentation reducing the risk of stuck and sluggish fermentations, they are ineffective in exalting the sensory properties of the regional wines losing the typical *terroir* character. On the contrary the autochthonous wine yeasts are indigenous strains isolated from natural grapevine environments, they are supposed to be the performers of spontaneous fermentations in the winemaking areas of origin, thus they can be selected for improving the *terroir* of local wines. Moreover they are often the starting point for a wine yeast selection programme as spontaneous fermentations in cellar are nowadays replaced by starter-guided vinification using selected strains in the form of active dried yeasts.

In this work results of the yeast selection program for the identification of autochthonous wine yeasts to be used for the vinification of "Lison-Pramaggiore bianco" wine are reported. From samplings in vineyard, performing single grape bunch fermentations, wine yeasts were isolated and characterised by means of molecular and physiological methods. This traditional wine was produced mainly in the North-East of Italy from a grapevine variety Tocai friulano. Till few years ago, its name was Tocai, one of the most popular wine in northern Italy. In recent years Hungary clamed the name Tokaj (and similar) to European Union to be used exclusively to define the wine produced in Hungary with the homologous grapevine. So the Italian wine had to change its denomination in "Lison-Pramaggiore bianco", the product public image suffered some serious damage and the wine lost its identity. In this context the yeast selection performed exclusively on the territory of

origin of the ancient Tocai (now included in the DOC area Lison-Pramaggiore) became a tool for reinforcing the identity of this local wine.

To investigate alternative ecological niches that can be a yeasts source contributing to understand the actual level of biodiversity in natural environment, samplings from vine bark were performed. The wine yeasts collected were genetically characterized and compared to the ones from grapes.

Moreover results about strains biodiversity in vineyard from DOC Lison-Pramaggiore and two other winemaking regions located in Northern Italy (DOC Prosecco di Conegliano – Valdobbiadene and Piave) are reported. By means of mitochondrial and microsatellites DNA analysis genetic differences and phylogenetic relationships were underlined.

Finally the characterization of yeast populations present in dried-grape musts and during the early stage of alcoholic fermentation that occur in manufacturing of Friularo Passito wine is reported. This traditional wine is produced in North–East of Italy using grapevine variety of Raboso piave. By means of conventional and molecular methods yeast species were identified and phenotypically characterized checking technical traits that influence the quality of the wine produced with high sugar grape must. The effect of added sulphites on yeast population was also evaluated.

2. Selection of autochthonous wine yeasts isolated from vineyard in Lison-Pramaggiore DOC area

2.1 Introduction

During the past few years there has been a noticeable increase in the demand for autochthonous wine yeasts to be used as fermentation starters. They are indigenous strains isolated from natural grapevine environments that are supposed to be the performers of spontaneous fermentations in the winemaking areas of origin, thus they can be selected for improving the *terroir* of local wines. The requirements for these yeasts are the ability to dominate during the fermentation process, and enhance, at the same time, the sensory characteristics of wines originating from different grapevine cultivars. In fact while commercial yeasts enable rapid and reliable fermentations reducing the risk of stuck and sluggish processes, they are ineffective in exalting the sensory properties of regional wines losing their typical *terroir* character.

In order to isolate new autochthonous yeast strains to enhance typical characteristics of the Lison (formerly Tocai) wine, sampling of single bunches of Tocai Italico grape variety was performed in the Lison-Pramaggiore DOC area.

2.1.2 The Tocai Italico grape variety

The Tocai Italico grape variety has great vegetative force and good production, its bunch of grapes is of medium size, with irregular shape, like a truncated pyramid; it is rather compact with two small wings. The grapes are round and their thin and fragile skin is green or yellow, according to the clone (figure 2.1).

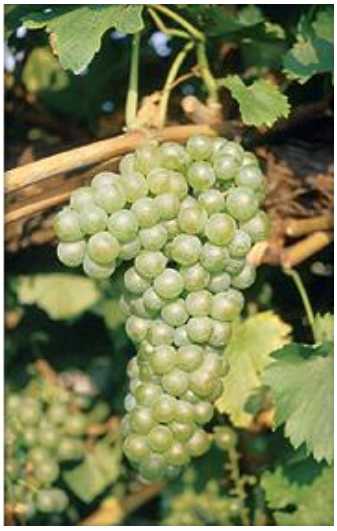


Figure 2.1 Tocai Italico grape bunch

The leaves are medium-large, three-lobed, with lyre-shaped petiolar sinus closed, lobes slightly overlapping; flap folded cup, with lower surface hairless.

The wine obtained from these grapes, named Lison, is straw-yellow with greenish hues. The scent is fine, delicate, moderately aromatic. The taste is pleasant, full and well rounded thanks to the low fixed acidity content with quite distinct bitter almonds aftertaste. The Tocai produced in some area of DOC Lison-Pramaggiore, with the oldest Tocai tradition, is decorated with the title "Lison Classico".

2.2 Materials and methods

2.2.1 Strains selection in Lison-Pramaggiore area

During the project were analyzed a total of 835 natural yeast isolates from grapes bunches and 249 bark portion of Tocai Italico variety.

To each sample was assigned an alphanumeric code: XYZ.

X is a letter (T = sample coming from bunches, TT = sample caming from bark portions);

Y corresponds to the sample sequence number;

Z corresponds to the colony sequence number isolated from the sample.

2.2.2 Culture media and growth condition

Media

Wallerstein Laboratory (WL medium) nutrient agar (Green & Gray, 1950).

Suspend 75 g WL nutrient agar (Oxoid) in a liter of distilled water.

Sterilize by autoclaving at 121 ° C for 15 minutes.

YM agar medium

- 3 g L^{-1} yeast extract (Oxoid);
- 3 g L^{-1} malt extract (Oxoid);
- 5 g L^{-1} vegetatone peptone (DIFCO);
- 10 g L^{-1} glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YM solid agar medium

- 3 g L^{-1} yeast extract (Oxoid);
- 3 g L^{-1} malt extract (Oxoid);
- 5 g L^{-1} vegetatone peptone (DIFCO);

- 10 g L^{-1} glucose (PROLABO)
- 16 g L^{-1} Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g L^{-1} yeast extract (OXOID)

20 g L^{-1} vegetatone peptone (DIFCO)

20 g L^{-1} glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

0,1 g L^{-1}	CaCl2
0,1 g L^{-1}	NaCl
1 g L^{-1}	KH2PO4
0,5 g L^{-1}	MgSO4•7H2O
3 g L^{-1}	tartaric acid

Micronutrients

0,2 mg L^{-1}	NaMoO4•2H2O
0,4 mg L^{-1}	ZnSO4•7H2O
0,5 g L^{-1}	H3BO3
0,04 mg L^{-1}	CuSO4•5H2O
0,1 mg L^{-1}	KJ
0,4 mg L^{-1}	FeCl3•6H2O
0,4 mg L^{-1}	MnSO4•H2O

Vitamins

400 µg L^{-1}	pyridoxine hydrochloride
400 µg L^{-1}	thiamine hydrochloride
2000 µg L^{-1}	Inosite

20 μg L^{-1}	Biotin
400 μg L^{-1}	Calcium pantothenate
400 μg L^{-1}	Nicotinic acid amide
200 μg L^{-1}	P-amino-benzoic acid

Variable components

0,3 g L^{-1}	(NH4)2SO4
0,3 g L^{-1}	(NH4)2HPO4
200 g L^{-1}	Glucose
0,2 g L^{-1}	Hydrolyzed Casein

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

Solution

Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937).
Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionised water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25 ° C, the liquid cultures, for fermentation inoculum, were subjected to agitation of 130 oscillations per minute.

2.2.3 Yeasts sampling and isolation from vineyards

Grape bunches isolation

Samplig:

The sampling was carried out by collecting the Tocai Italico grape bunches in the DOC Lison-Pramaggiore area, some days before harvest.
Within each vineyard, the vines were chosen farther away from roads and buildings (as potential commercial strains contamination sources) choosing bunches on the bottom, ripened as possible and not infested with visible moulds.
The collection has been made, at each stage, avoiding touching the grapes with hands and sterilizing scissors periodically in order to minimize contamination.
Stomaker sterile bags were used, filled with about 700-800 g of grapes (corresponding to one or two bunches, depending on size) and closed for the laboratory transport.
Samples collected in the vineyard were transferred to the laboratory where 8 g of sugars (4g of fructose and 4g of glucose corresponding to 2% of grapes weight) and 500µl of sulphur dioxide at 5% v/v, to facilitate the development of *Saccharomyces sensu stricto* yeasts, were added.
Bags was closed with a foam rubber cap previously sterilized in order to avoid the increase of pressure inside the bag, while maintaining the internal environment isolated from the outside. Each sample was then manually pressed and left to ferment spontaneously (at room temperature) for 2 to 3 weeks with skins, stalks and pips.
The fermentation process was monitored by measuring, for each bag, the daily weight loss.

Yeasts isolation

After fermentation, 5 mL of the must were took from each bag, and 6 serial dilutions (1:10) were performed on Ringer solution. 100 µl of the last three dilutions were plated on WL medium.
After 5 days at 25°C, colonies counting was performed and 16 colonies with *Saccharomyces*-like morphology were randomly stored.

2.2.4 Yeast isolates storage and purification

After the colonies determination of *Saccharomyces sensu stricto* group, by the multiplex PCR Sac18-Sac26, all the colonies confirmed belonging to the group were growth on liquid YPD medium for 24h at 25°C, then centrifuged and resuspended in 2 ml of a sterile solution composed of half YPD medium and 40% of glycerol. The vials were stored at -80°C.

2.2.3 DNA amplification

2.2.3.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 μL of sterile deionised water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

2.2.3.2 SAC26-SAC18 multiplex PCR

Saccharomyces sensu stricto colonies identification was performed by adopting the method developed by Nardi *et al*. 2006. The various components of the reaction mixture were used in the following final concentrations:

Table 2.2.1 PCR master mix composition

Primer SAC26F	0,2 μM
Primer SAC26R	0,2 μM
Primer SAC18SF	2 μM
Primer SAC18SR	2 μM
dNTPs (Amersham)	200 μM (each one)
Taq polimerasi (Promega)	0,02 U/μl
Buffer	1X
DNA	2 μl cellular suspension

Primers utilized are reported below (table 2.2.2).

Table 2.2.2 Primers for SAC26-SAC18 amplification

Name	Length	Sequence (5'-3')
SAC26F	22 nt	GAGAGGGCAACTTTGGGRCCGT
SAC26R	27 nt	ACCATTATGCCAGCATCCTTGACTTAC
SAC18F	23 nt	CTGCGAATGGCTCATTAAATCAG
SAC18R	25 nt	CCCTAACTTTCGTTCTTGATTAATG

The thermal protocol was the follows:

Initial incubation at 94°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 94°C for 15 s, annealing at 54°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gels with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.
Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.2.4 Mitochondrial DNA analysis

2.2.4.1 Yeasts total DNA extraction

Yeasts coat obtained on YM agar medium, growing yeasts for 48 h at 25 °C, was resuspended in 1 ml of sterile water and then centrifuged at 14000 rpm for 3 minutes in an Eppendorf microcentrifuge. After fluid discarding, the cells were resuspended in 500 μl of a solution containing 50 mM Tris-HCl, 20 mM EDTA, pH 7.4 and transferred to a 2 ml Eppendorf containing 0.3 g of glass beads of 425-600 μm (Sigma) and vortex for 3 minutes. 50 μl of 10% SDS were then added to the samples that were incubated in a thermostatic bath at 65°C for 30 minutes. At the end 200 μl of potassium acetate 5M were added and the samples were left on ice for 30 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes. 600 μl of supernatant was transferred to an Eppendorf tube and 600 μl of cold isopropanol were added. The samples were kept at room temperature for 5 minutes, stirring by inversion and then centrifuged at 14000 rpm for 10 minutes. The supernatant was removed and 500 μl of 70% ethanol were added. After centrifugation at 14000 rpm for 10 minutes and the supernatant discarding, the pellet was dried for 1 h at 37 ° C. The samples were resuspended in 50 μl of sterile water, to which 1.5 μl (10 mg / ml) of RNase (Amersham Bioscience E70194Z) were added. The samples were left at room temperature for 15-20 minutes and finally stored at -20 °C.

2.2.4.2 Total DNA enzyme restriction

The total DNA digestions were performed in 15 µl of volumes reaction containing 10 U of *HinfI* enzyme (Fermentas) and 10 µl of extracted DNA. The reactions were performed at 37 °C for 2 h.

2.2.5 Yeasts species identification by ITS1-5,8S-ITS2 region amplification and RFLP

2.2.5.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 µL of sterile deionised water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

2.4.2 ITS1-5,8S-ITS2 region amplification

The various components of the reaction mixture were used in the following final concentrations:

Table 2.4.1 PCR master mix composition

ITS1	2 µM
ITS4	2 µM
dNTPs (Amersham)	200 µM (each one)
Taq polimerasi (Promega)	0,02 U/µl
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 2.4.2).

Table 2.4.2 Primers for ITS1-ITS4 amplification

Name	Length	Sequence (5'-3')
ITS1	19 nt	**TCCGTAGGTGAACCTGCGG**
ITS4	20 nt	**TCCTCCGCTTATTGATATGC**

The thermal protocol was the follows:
Initial incubation at 95°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 53,5°C for 45 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gels with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.
Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.4.3 ITS1-4 RFLP analysis

The amplification products of the region ITS1-5,8S-ITS2 of rDNA were digested with enzymes HaeIII and MaeI (Amersham). The digestions were performed in 20 μl volumes reaction containing 10 U of enzymes and 10 μl of the amplified. The reactions were conducted at 37 ° C for 16 h.

2.5 Fermentation surveys on Synthetic Nutrient Medium (NSM) (Delfini, 1995)

2.5.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate 10 ml of YPD liquid medium. The tubes were left in incubation for 30 hours at 25 °C, moved to obtain a culture on stationary phase (approximately 10^7-10^8 cells/ml) measured by spectrophotometry (OD_{600} between 5 and 8).

2.5.2 Test preparation

Based on the OD of the respective pre-inoculation, for each strain the culture volumes to obtained a final OD_{600} of 0.5 (approximately 10^5 cells/ml) in 100 ml of medium at the beginning of fermentation, were calculated.

Each strain was inoculated in a 100 ml-Erlenmeyer flask sealed with silicon cap and supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use the synthetic must than the natural, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily growth monitoring operations.

The medium was sterilized by autoclaving at 100 ° C for 5 minutes.

Alcoholic fermentation development was controlled by measuring the weight loss daily from the beginning to the end of fermentation process. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

2.3 Results and discussion

2.3.1 Grape sampling of *Saccharomyces sensu stricto* in the Lison-Pramaggiore area

The sampling took place during the pre-harvest period in 2009 and2010, from 3 to 7 days before harvest. This period was chosen to avoid collection of contaminated grapes by selected yeasts in the vineyard reached by agricultural machinery during grapes harvest (Valero *et al*., 2005). In fact, the wineries in the area have been using commercial strains for a long time because of they rapid and reliable fermentation capabilities that reduce the risk of stuck and sluggish fermentations. Yeasts isolation starting from single fermented bunches, not only reduces possible contamination due to the presence of commercial yeasts in vineyard, but could also allow to isolate poorly competitive strains but with interesting quality characteristics (such as enhancement of primary aromas and production of secondary flavours).

Sampling was organized in order to cover the whole area of the DOC Lison-Pramaggiore evenly, but access to the vineyards requires a precise geographic knowledge of the territory. For this reason at this stage, the technical collaboration of the Lison-Pramaggiore Consortium, that contacted several wineries to ask for their collaboration and then also coordinated samplings in the vineyards, was needed.

Forty-six wineries contributed to the yeasts selection program. To evaluate the representativeness of the samples collected, their distribution on the territory was analyzed, evidencingthat the 193 samples collected evenly covered the entire DOC area, including the Lison-Classico macro-area that is considered the most valuable zone..

The samplings were performed at the following sites (table 2.3.1):

Table 2.3.1 Sampling distribution in the DOC Lison-Pramaggiore area.

SITES	N° OF GRAPE BUNCHES	WINERIES SAMPLED
Annone	9	2
Belfiore di Pramaggiore	22	7
Cinto	3	1
Fossalta di Portogruaro	25	6
Giai Annone Veneto	7	2
Lison	38	9
Loncon	45	8
Lorenzaga di Motta di Livenza	3	1
Motta di Livenza	11	3
Pramaggiore	10	3
Salvarolo	11	2
San Stino di Livenza	6	1
Sumaga	6	1
TOTAL	196	46

In 2010, twelve more bunches were collected from two wineries in Motta di Livenza and Lorenzaga sites, already sampled the year before. Hence a total 208 of bunches distributed in 13 different sites were considered for the present study. Based on these data we can say that sampling evenly covers the area considering both the geographic features that the localization of production areas.

2.3.2 Yeasts isolation

Bunches collected in the vineyard were transferred to the laboratory where 8 g of sugars (4g of fructose and 4g of glucose corresponding to 2% of grapes weight) and 500µl of sulphur dioxide at 5% v/v, to facilitate the development of *Saccharomyces sensu stricto* yeasts, were added into each plastic bags. This taxonomic group is the most important from a technological point of view because it includes all yeasts with the best technological and enological fermentation features that are the target of the selection program. In addition, this yeasts group is more resistant to SO_2 than contaminant apiculate yeasts that generally dominate the first stage of spontaneous fermentations.

Sugars were added as a nutrients source to support well the development of the microflora because grapes, having been collected earlier, had not yet reached their optimal sugar content .

The samples were left to ferment spontaneously and during this period their weight loss was monitored. The fermentation was considered complete when the samples weight remained constant. At the end of fermentation, suitable dilutions of fermented juice were plated on selected WL media. Indigenous yeasts were isolated and, by means of plate count, yeasts concentration in fermented musts was determined to be on a range of 10^6-10^7 CFU/ml. The WL (Wallerstein Laboratory) proposed in the fifties by Green and Gray (1950) for the detection of yeast contaminants in beer production process, contains a dye, bromocresol green, which is differently absorbed by yeasts. Strains belonging to the *Saccharomyces sensu stricto* group scarcely absorb the dye and therefore their colonies show colours from cream to light green; they are opaque and have a smooth creamy texture. The use of this growth medium was also proposed for the wine industry (Cavazza et al., 1992). It was observed that the main vineyard yeasts have the ability to absorb the dye very well: the genus *Hanseniaspora*, the most commonly found on grapes, grown on WL medium, assumes a deep green colour distinguishable from the *Saccharomyces sensu stricto* group (Figure 2.3.1).

Therefore, on the basis of colony aspect, the *Saccharomyces*-like isolates were considered for further characterization.

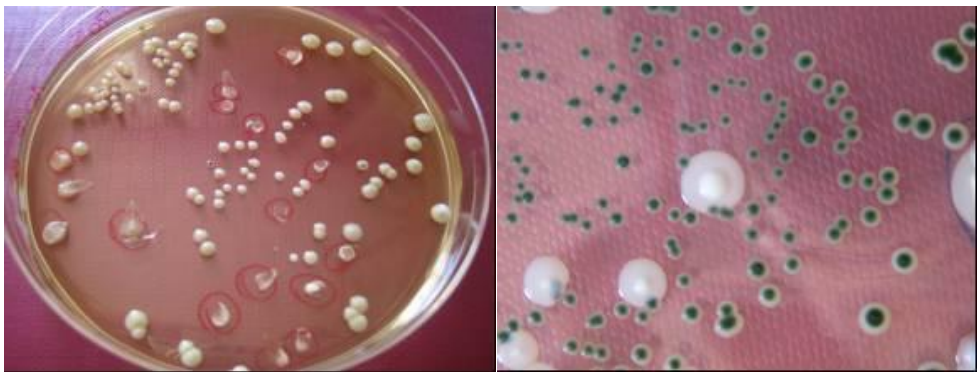


Figure 2.3.1 Different yeast colony morphologies on WL medium. A) Typical *Saccharomyces sensu stricto* colonies morphology; B) Apiculate yeasts (green colonies).

From every bunch fermented, a maximum of 16 colonies with *Saccharomyces sensu stricto*–like morphologies were collected. Finally a total of 835 colonies were isolated.

2.3.2.1 *Saccharomyces sensu stricto* abundance and distribution in the sampling

For unambiguous identification of the *Saccharomyces sensu stricto* yeasts isolates, all colonies collected from the two samplings were analyzed by molecular methods. For this purpose the group has developed a method for the *S. sensu stricto* group genetic identification based on a multiplex PCR (Nardi et al., 2006). The method provides the ability to discriminate this yeast group from other yeasts present in the enological environment on the basis of nucleotide differences within the DNA region coding for ribosomal RNA (rDNA). The D1/D2 region of 26S DNA is the stretch that carries more information about the differences between yeast species. In GenBank information on the sequence characteristics of the D1/D2 region of many yeasts species are present. It was therefore possible, according to a multiple sequence alignment (CLUSTALW), to identify the presence of two small highly conserved regions within the *sensu stricto* group that are sufficiently different from the other species known. The two stretches of DNA were used to construct the amplification primers (Sac26). A second pair of primers (Sac18) was designed to be used as an internal amplification control. The 18S rRNA sequence has a very high conservation level among all yeasts species (Figure 2.3.2).

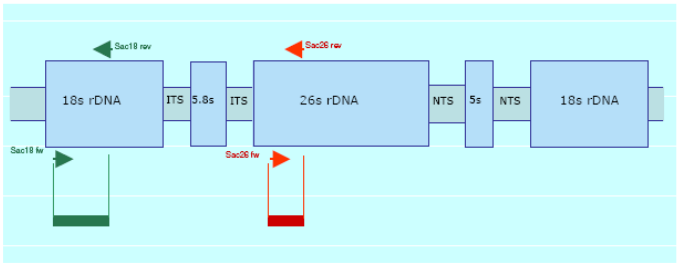


Figure 2.3.2 DNA region coding for yeasts ribosomal RNA. Primers position for *S.sensu stricto* identification are shown.

The method has been tested on all strains belonging to *Saccharomyces sensu stricto* group, *Saccharomyces sensu lato* and a selection of species (17) of enological interest. The results achieved confirmed that the method correctly discriminates *Saccharomyces sensu stricto* group from other wine yeasts. In particular the first produce two amplification DNA fragments (of 460 and 862bp length) and the other only the control 862bp fragment that can

be easily detected on an electrophoretic agarose gel (Figure 2.3.4). This protocol for the identification of *S. sensu stricto* is extremely fast because it allows the amplification of genomic sequences, with no DNA extraction and purification procedures, but simply from heat-lysed cells.

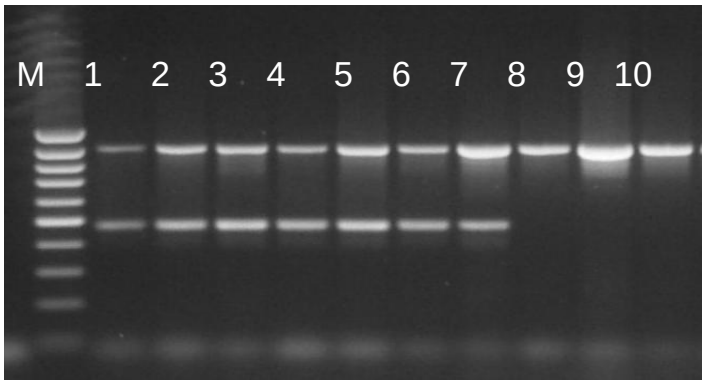


Figure 2.3.4 Multiplex PCR. M molecular weight standard (100bp, Amersham Bioscience) *S. sensu stricto:* 1) *S. bayanus*, 2) *S. cariocanus*, 3) *S. cerevisiae*, 4) *S. kudriavzevii*, 5) *S. paradoxus*, 6) *S. pastorianus*, 7) *S. mikatae*; *S.sensu lato* 8) *S.barnetti*, 9) *S.bulderi*, 10) *S. servatii.*

The multiplex PCR attested that 23% of colonies (195) of the 835 collected from WL medium are *Saccharomyces sensu stricto* . All positive colonies were recovered from 18 fermented bunches, which represent 8,6% of all bunches collected.

The yeasts selection program that involved the vineyards of Lison-Pramaggiore wine region was the third one conducted by the Microbiology group in the Veneto Region (north-east Italy) after yeast isolations from Prosecco (now called Glera grape variety) and Raboso Piave wine areas in past years (form 2004 to 2007). Comparing the results obtained on sampling conducted in the Lison-Pramaggiore area with those obtained in the two other areas, a clear difference between the red variety (Raboso Piave) and the two white ones was observed.

The results are reported in Table 2.3.2

Table 2.3.2 Yeast isolation in the three winemaking areas.

Areas	Grape bunches	Grape bunches	*Saccharomyces*	*Saccharomyces*

		containing *Saccharomyces*	*sensu stricto* (Multiplex PCR)	*sensu stricto* diffusion on bunches(%)
Prosecco	354	30	296	**8.5**
Raboso Piave	78	54	260	**69.2**
Lison	208	18	195	**8.6**

The two white wines, Lison (coming from Tocai Italico grape variety) and Prosecco (now called Glera grape variety), despite the large number of grape bunches sampled compares to Raboso, gave a much lower number of bunches that contained yeasts belonging to the *Saccharomyces sensu stricto* group.

2.3.2.2 Geographic distribution of samples with *Saccharomyces sensu stricto*

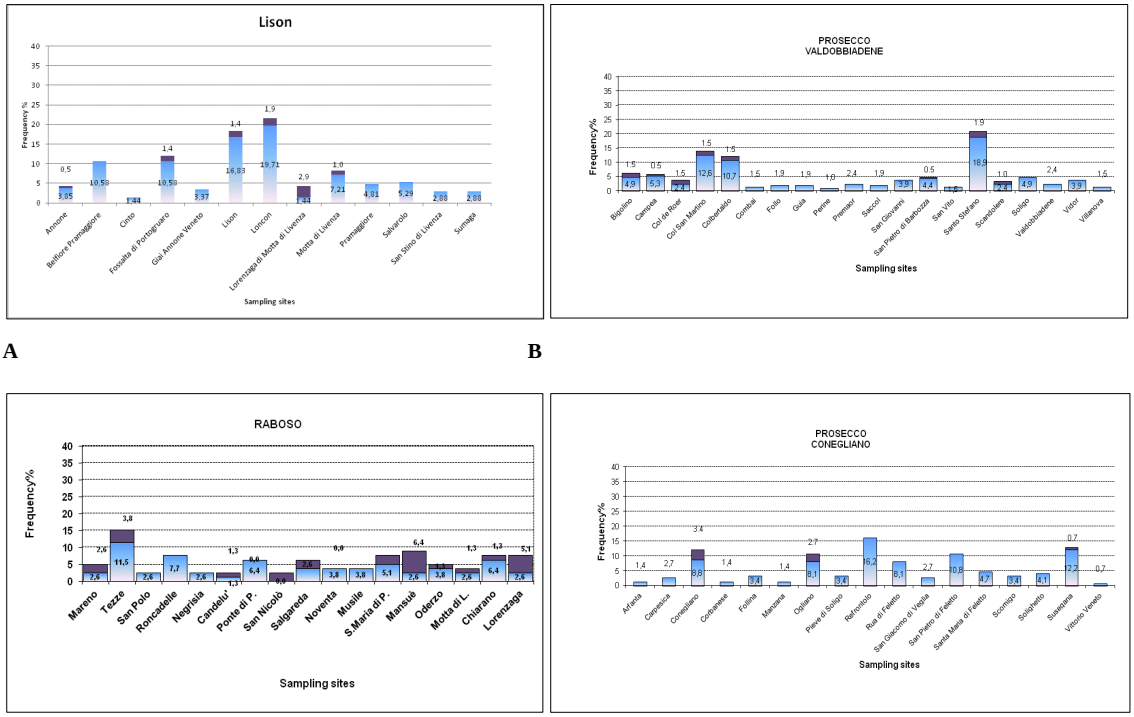
Observing the geographic distribution of grape bunches sampled, a higher concentration in the Lison Classico area (consisting principally by Annone Veneto, Pramaggiore, Lison, Loncon, Salvarolo, San Stino di Livenza and Sumaga sites) was observed, and indeed 119 of 205 samples collected were from that area, that is 58% of the total. This is due to the greater distribution of Tocai Italico grapes in the Lison Classico area than in all the others of the DOC Lison-Pramaggiore, because of historical and pedoclimatic factors.

All sampled areas had a low concentration of samples containing *S. sensu stricto*: in more than 50% of the sites, seven out of thirteen, no Saccharomyces strains were recovered (Figure 2.3.5 A).

Interesting data were found observing samples collected in the Lison Classico area, which include 9 sampled sites out of 13 in total (only Cinto, Fossalta di Portogruaro, Motta di Livenza and Lorenzaga sites are completely excluded). This area, although it was the most sampled, gave the lowest numbers of bunches containing Saccharomyces (see figure 2.3.5 A).

Observing the positive samples collected in the other two surveys, a low number of positive sites were observed also for the Prosecco area, where only 11 sites out of 37 sampled gave relevant results (figure 2.3.5 B). This area was subdivided into two subareas (Valdobbiadene and Conegliano) because of the differences in the pedo-climatic factors.

On the other hand, in the Raboso area, most of bunches contained yeasts belonging to the *S. sensu stricto* group, with 11 positive sites out of 17 (figure 2.3.5 C). Therefore this grape variety seems to better preserve yeasts presence.



A **B**

C **B**

Figure 2.3.5 Frequency of sites containing samples with yeasts belonging to *S. sensu stricto* group (violet) and without (light blue) in the Lison area (A), Prosecco (B) and Raboso (C).

2.3.2.3 mtDNA restriction fragment length polymorphisms analysis (RFLP)

To obtain a strain-specific characterization of the isolates identified as *S. sensu stricto*, a method proposed by several authors was chosen (Querol et al., 1996, Lopez 2001). This method uses mitochondrial DNA restriction fragment length polymorphisms analysis (mtDNA-RFLP) by enzymatic digestion of total DNA. The method is simple and yields results within 2 days. This technique has successfully been used by other authors to characterise strains of other yeast species (Martinez *et al*. 1995; Romano *et al*. 1996; Guillamon *et al*. 1997).

This is the most commonly genetic tool used for characterizing the *S. sensu stricto* group, in particular by usig the *Hin*fI restriction enzyme (Lopez et al., 2001, Schuller et al., 2004).

Restriction profiles obtained were compared by the GelComparII (Applied Maths) software that allows, by a matrix construction, to calculate the similarity level between strains and to draw it in a dendrogram.

The mtDNA RFLP analysis was conducted for all 195 isolates of the Lison area and it confirmed they all were Saccharomyces yeasts.

Dice similarity coefficient, which considers electrophoretic bands positions, but not their intensity, was used for the matrix construction. Moreover, for the dendrogram construction, determined by the UPMGA method, the "optimization" and "tolerance" values, which determine the variability level of the same profiles among replicates , were those chosen by the program. Under these conditions (optimization: 1.17%, tolerance: 1.5%), the analysis on the FR95 (Blastosel, Perdomini) commercial strain conducted repeatedly routinely gave a similarity degree of 100%,.

The mtDNA-RFLP analysis evidenced the presence of only 17 different profiles, which are considered as different strains, from the analysis of all the195 isolates, 10 coming from the survey conducted in 2009 and other 7 from the sampling done in 2010

Cluster analysis was performed by adding seventy commercial oenological strains and electrophoretic profiles comparison showed in one case 100% similarity with the profile of a commercial strain. This profile belongs to a enological yeast (the Mycoferm611) widely used in the Lison area. Furthermore, one profile was found in four fermented bunches coming from different vineyards sampled during the two harvest campaigns, so this profile can be considered the more present in the area.

Comparing the results obtained in the Lison area with those achieved in the two surveys on different grape varieties, the Lison has the lowest profiles rate (table 2.3.3).

Table 2.3.3 Yeast isolation in the three areas.

Areas	**Grape bunches**	**Grape bunches with *Saccharomyces***	***Sacchromyces* isolates (*Multiplex* PCR)**	**mtDNA profiles**
Prosecco	354	30	296	37
Raboso Piave	78	54	260	130
Lison-Pramaggiore	208	18	195	17

Saccharomyces strains biodiversity was notably higher on grape bunches of the red variety Raboso Piave. The 69% of bunches contained *Saccharomyces* strains. On the other hand only the 8,5% and 8,6%, of Prosecco and Lison bunches carried *Saccharomyces* yeasts. Moreover in the Lison area only in one sample two different yeast profiles were rescued, while in Raboso the 37% of positive samples gave four different profiles. The Prosecco situation is rather similar to Lison, with 73% of samples that contained only one single profile. To explain this result an interesting hypothesis focuses on specific features of the grape. In the case of Raboso, the grape peel is thick and hence more resistant to pest attack than Prosecco and Lison, therefore this vine variety needs less fungicidal treatments that could affect survival of yeasts in vineyard.

2.3.2.4 Species identification

Further genetic investigation was conducted to identify the species within the *Saccharomyces* genus, among the strains selected in the Lison area having different mtDNA profiles.

As for previous genetic investigations, also in this case the DNA region coding for ribosomal RNA was considered. In particular, the DNA trait between the two coding sequences for the 18S and 26S subunits, was studied. This trait encodes for the 5.8S rRNA subunit and contains two flanking areas called Internal Transcribed Spacer (ITS), particularly interesting for their high sequence polymorphism, higher than genes encoding for the 18S rRNA and 26S subunits (Cai *et al.*, 1996; James. *et al*., 1996). This variability is extremely high between organisms belonging to different species, but is very low within strains of the same species. This intra-specific polymorphisms can be highlighted by means of the ITS region amplification and subsequent restriction profile analysis by appropriate enzymes (Esteve-Zarzoso *et al*., 1999). Several authors have chosen the *Hae*III restriction enzyme to study enological yeasts (Baleiras Couto *et al*., 1996; Esteve-Zarzoso *et al*., 1999; Las Heras-Vazquez *et al*., 2003; Naumova *et al*., 2003). This enzyme was tested on several reference strains and allows to divide the species belonging to the *sensu stricto* group into two

subgroups in agreement with their genetic similarity (Kurtzman, 1998). The first group

includes *S. cerevisiae*, *S. paradoxus* and *S. cariocanus* and the second contains *S. bayanus*, *S. pastorianus*, *S. mikatae* and *S. kudriavzwevii*. (figure 2.3.6).

The enzyme *Mae*I was proposed for the first time by Mc Cullogh *et al.* (1998) to separate *S. cerevisiae* from *S. paradoxus*. Since the introduction of the three new non-European species in the *S. sensu stricto* group (*S.kudriazevii, S.mikatae, S. cariocanus*) was successive (Vaughan-Martini 1998, Naumov *et al*. 2000, Rainieri *et al.* 2000), the *S. cariocanus Mae*I restriction pattern was investigated. This enzyme discriminates between *S. cerevisiae* and *S. paradoxus*/ *S.cariocanus* species.

The analysis performed on all strains with different mtDNA profiles demonstrated that All belong to the *Saccharomyces cerevisiae* species.

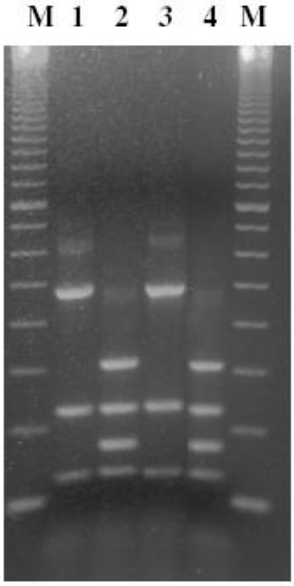


Figure 2.3.6 Separation of some *Saccharomyces* species by ITS analysis using *Hae*III enzymatic digestion. Lane: M, marker 100bp (Amersham Bioscience); 1, *S. mikatae*; 2, *S. paradoxus*; 3 *S. kudriavzwevii*; 4, *S. cerevisiae*.

2.3.3 Technological strains characterization in synthetic must

After isolation and genetic characterization, the second step of selection was the identification of strains with interesting technological characters. To evaluate the fermentative performance of the isolates, a representative for each electrophoretic profile obtained by the mtDNA-RFLP characterization, excluding the strain with a profile equal to

the commercial one, were inoculated in synthetic must (Delfini, 1995) under conditions that simulate enological setting. In addition to the 16 different Lison isolates, one commercial strain (EC1118) commonly used for winemaking, as internal control and comparison functions, was added.

Each strain was inoculated at a concentration of about $5*10^5$ CFU/ml in a 100 ml-Erlenmeyer flask closed with a silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use synthetic must with respect to natural juice for preliminar physiological assessments, is to standardize growth conditions and to facilitate significantly daily growth monitoring operations.

In particular the fermentative vigour, corresponding to the quickness of a strain to start and close the fermentative process, was evaluated. It was estimated by measuring flasks weight loss after 2 days from the start of the fermentative process. Another important character is the fermentative power, which is the maximum ethanol amount produced by yeasts during the fermentation of a must with an excess of sugar. This feature could be evaluated because the fermentation was performed in synthetic must with sugar concentration of 200g/l, that is the standard situation to evaluate the fermentative vigour, but too low to check the maximum alcohol production that requires 300 g/l of sugar (Delfini, 1995). In fact, literature data report that the majority of strains belonging to the *S. cerevisiae* species isolated in nature exhibits an excellent ability to produce ethanol that normally reaches 14-15% v/v (Vincenzini *et al*., 2005).

Observing fermentation kinetics, all isolates tested completed the alcoholic fermentation developing approximately 12% of alcohol and consuming all the sugar available. Only 2 strains (T317.2 and T411.10) were not able to use all sugar. Most of the strains tested had behaviours very similar to the commercial strain EC1118. In some cases the rate of sugars consumption seemed to be even higher than that shown by the commercial strain under the same experimental conditions. In general most of the yeasts tested (15 out of 17) revealed to have good fermentation characteristics (kinetics not showed).

Considering the fermentative vigour, calculated as grams of glucose consumed after 2 days by the formula: sugar metabolized = weight losses *2,118 (Delfini, 1995), the relative frequencies observed, were plotted in the histogram in figure 3.5. Most of the isolates are in a class that includes intermediate values with glucose consumption on the range of 2-3

g/100ml. Six strains, including the commercial strain EC1118, have a glucose consumption over 3g/100ml, with one reaching more than 5g/100ml. In contrast only 2 among 17 strains tested (T411.10 and T317.2) showed very poor performance (glucose consumption less than 2g/100 ml). To better analyse the fermentative vigour of strains isolated in the Lison area, frequencies are compared with those obtained from Prosecco and Raboso surveys (figure 2.3.7).

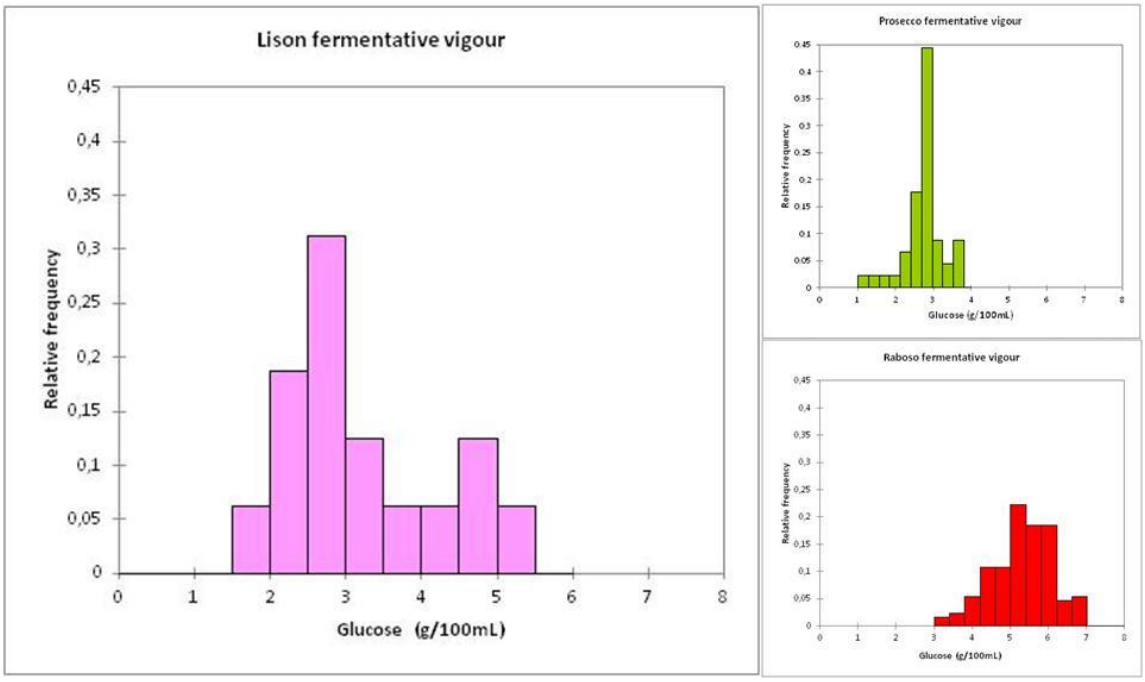


Figure 2.3.7 Fermentative vigour relative frequencies obtained on Lison (pink), Prosecco (green) and Raboso strains (red) fermented in synthetic must.

The strains coming from Lison have a behaviour similar to those selected in the Prosecco area, that have a glucose consumption between 2 and 3g/100ml. Therefore these strains in the first 2 days have a maximum fermentative vigour of less than 4g/100ml. Raboso strains behave very differently. In fact they have better fermentative kinetics with an high glucose consumption during the first 2 days of fermentation with an average over 5g/100ml. There are 13 out of 130 strains with a glucose consumption over 6g/100ml and only 2 strains in a range between 3-3,4g/100ml that is the lowest measured.

Moreover glucose consumption after 7 days of fermentation was analysed. The mean value registered on Lison strains was 12,8g/100ml with a standard deviation of about 2g/100ml.

Only strain T317.2 had a very low glucose consumption value (8g/100ml) confirming its low fermentative capability. Three strains exhibited high glucose consumption capability with more than 15g/100ml like the commercial EC1118 strain. The highest value was registered for T415.1 which confirmed to have the best fermentative power. Also in this case, glucose consumption after 7 days of strains from Prosecco and Raboso areas, is used for comparison (figure 2.3.8).

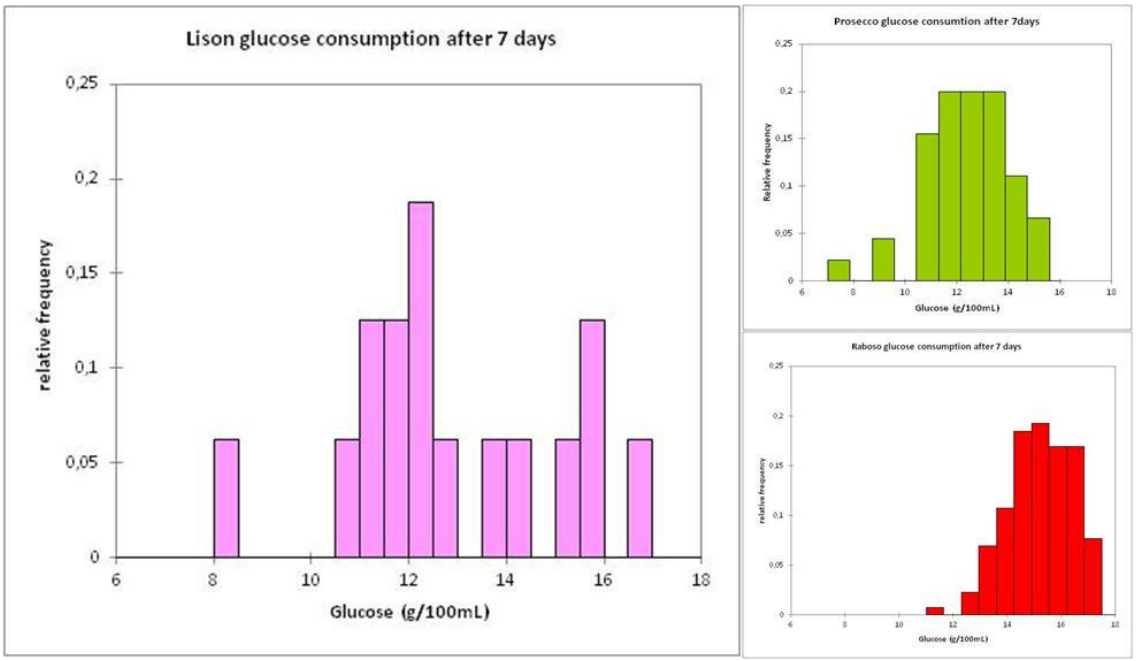


Figure 2.3.8 Relative frequency of glucose consumption after 7 days in Lison (pink), Prosecco (green) and Raboso (red) strains.

As for fermentative vigour, , similar behavior for Lison and Prosecco strains was observed even after 7 days. The mean value for Lison strains was 12,9g/100ml and the maximum was 16,7g/100ml, while for prosecco were 12,4 g/100mL and 15,5 g/100mL, respectively. Concerning strains selected from the Raboso area, the fermentation behavior was different. All the strains considered had high glucose consumption with an average of 15,2g/100ml and a maximum level of 17,4g/100ml with 10 strains out of 130 tested on a range of 16,8-17,4g/100mL. They confirm to have better fermentative power than the strains selected from white wine varieties.

Strains coming from the Lison area have intermediate fermentative vigour and glucose consumption after 7 days levels, i.e. on average better than strains selected from Prosecco but worse than those frome Raboso.

Furthermore, glucose consumed at the end of the fermentative process was evaluated. Strains from Lison completed the fermentative process having consumed about 18,5g/100m of glucose (range between 18,2 and 18,7g/100mL) that corresponds to 92,5% of the glucose added to the medium (figure 2.3.9).

The same feature was examined for comparison in strains coming from Prosecco and Raboso areas.

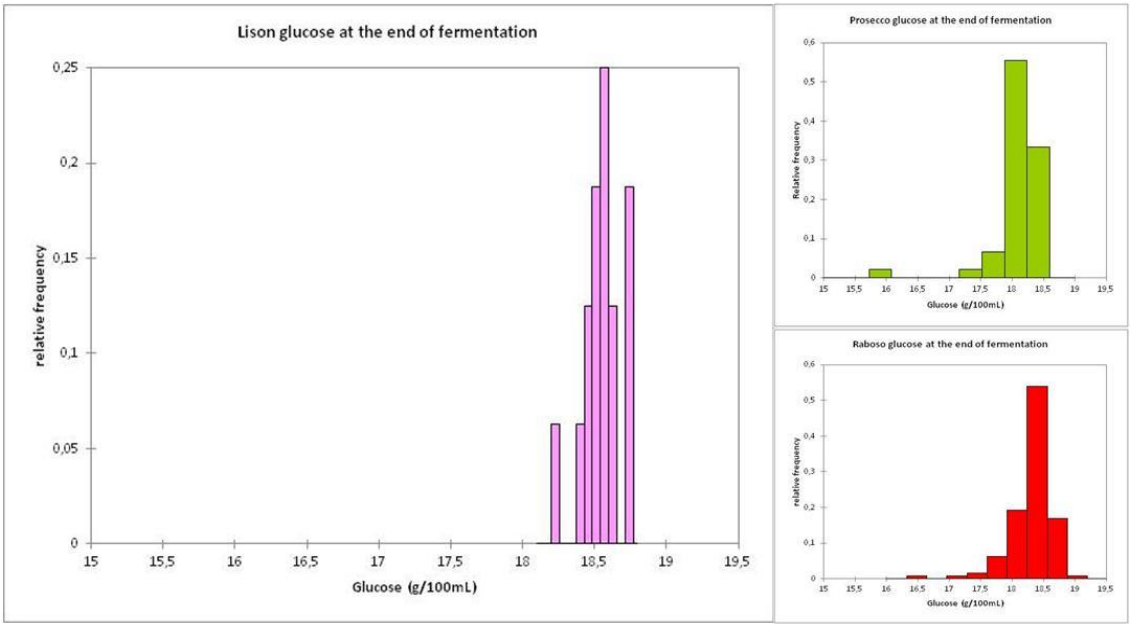


Figure 2.3.9 Strains relative frequency of glucose at the end of fermentation in Lison (pink), Prosecco (green) and Raboso (red) yeasts.

Considering the length of fermentation of the Lison strains, a mean value of 18,9 days was measured (figure 2.3.10). Only two strains spent 25 days to complete the fermentation process.

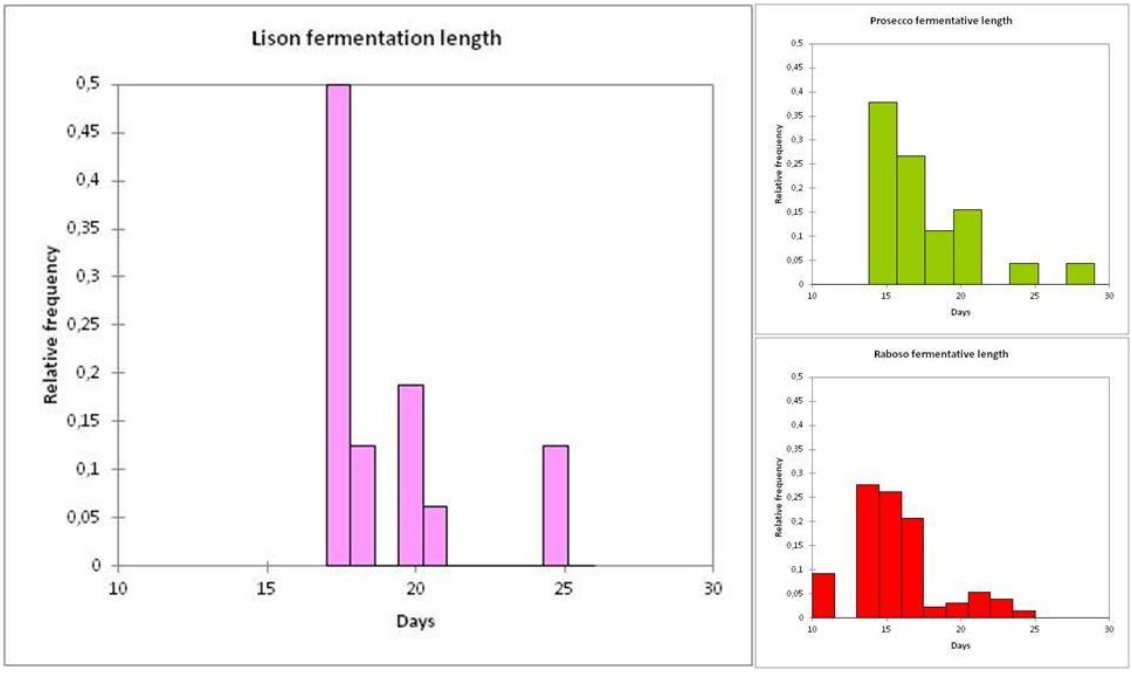


Figure 2.3.10 Strain relative frequencies of fermentation length in Lison (pink), Prosecco (green) and Raboso (red) strains.

Strains from Prosecco and Raboso concluded the fermentation in less time than the Lison ones, as indicated by mean values of 17,3 and 15,6 days for Prosecco and Raboso respectively. ForProsecco strains the minimum value was 14 and the maximum 28 days, reached by two strains. The Raboso strains confirmed to have the best fermentative performance with a minimum of 11 days reached by 12 yeasts and a maximum of 24 days spent by only two strains out of 130 tested (figure 2.3.10).

2.3.3.1 Sulphur dioxide and hydrogen sulphide production

Sulphite is widely used in winemaking for its antimicrobial and antioxidant properties, although its toxic effect on human health is proven. Wine yeasts usually produce low-to-medium SO_2 amounts, depending on their genetic characteristics and fermentation conditions. To better explore yeasts properties related to sulphites, sulphur dioxide (SO_2) and hydrogen sulphide (H_2S) productions were evaluated by growing yeasts on appropriate mediums.

The hydrogen sulphide production was evaluated by growing yeasts on Biggy agar plates (Oxoid), a specific medium that contains bismuth sulphite. This compound is converted into bismuth sulphur in the presence of hydrogen sulphide, so colonies turn brown with an intensity proportional to the amount of sulphur-containing substances produced.

Sulphur dioxide production was evaluated on Fucsine agar. The SO_2 produced by yeasts combining with the fucsine colorant (magenta colour) leads to the formation of a colourless compound, discolouring proportionally the growth medium.

The results obtained are reported in table 2.3.4.

Table 2.3.4 Sulphur dioxide and hydrogen sulphide production

Strain	SO_2 production	H_2S production
EC1118	2	4
T9.1	2	3
T21.1	2	3
T314.1	2	4
T317.2	3	3
T411.1	2	3
T411.10	2	2
T415.1	3	3
T424.1	2	4
T525.1	3	4
T602.3	3	3
T603.2	2	4
T605.3	2	4
T605.5	2	4
T605.7	2	4
T606.4	2	4
T606.8	2	3

For SO_2 production the range was set between 1 and 3 for high, medium and a low level of compound production. For H_2S production, values between 1 and 4 were chosen for low, medium-to-low, medium-to-high and high compound production.

The results show that most of the strains have a medium SO_2 and an high H_2S production. There are 4 strains that exibit a low level in SO_2 production (value 3 on the table) and a medium level in H_2S production. only T411.10 strain has a low level of H_2S production.

These results are in agreement with those obtained with strains from Prosecco and Raboso.

2.3.4 Physiologic characterisation in Lison natural must

The strains tested in synthetic must were then evaluated in must from Lison grapes to analyze their fermentation attitudes in an enological contest.

The strains were grown in a 200 ml-Erlenmeyer flask closed with silicon cap supplied with a bowed glass pipette and filled with 200 ml of natural must. The fermentation process was monitored by flask weight loss. Together with the 16 strains representative of the different profiles identified, the commercial strain Mycoferm61 used in the Lison-Pramaggiore area was also tested.

In this contest, fermentative vigour, glucose consumption after 7 days and fermentation length were evaluated. The results achieved were subdivided into relative frequency classes.

Concerning glucose consumption after 2 days of fermentation, the mean value was 5g/100ml, higher than that achieved in synthetic must (figure 2.3.11).

Only few strains had a glucose consumption as low as that obtained in synthetic must, within the range of 3-4g/100ml.

The 29,4% of the strains showed a very high fermentative vigour, including the commercial strain Mycoferm611 with a glucose consumption over 6g/100ml.

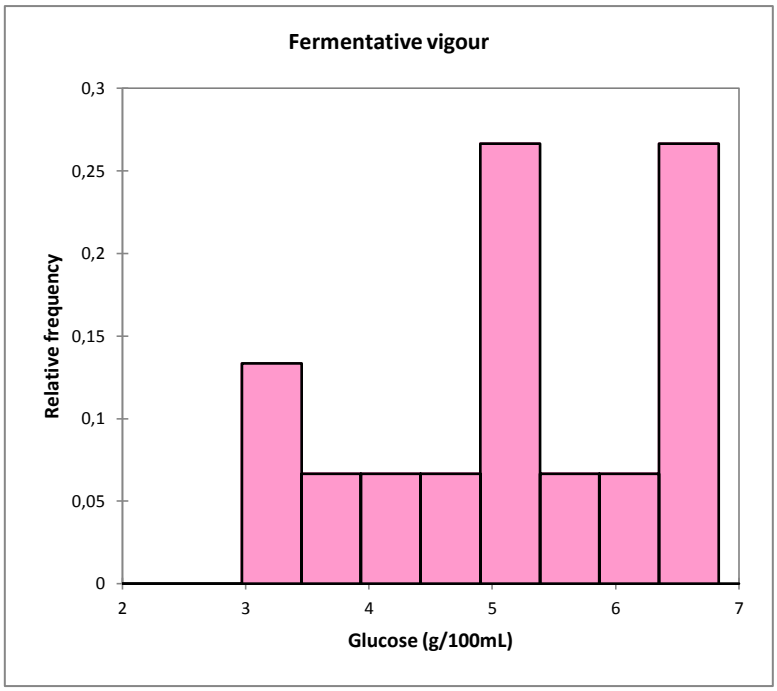


Figure 3.3.11 Strains relative frequencies of fermentative vigour

Observing glucose consumption after 7 days of fermentation, most of the strains had good fermentative performance (figure 2.3.12) with a mean value of 16,6g/100ml and 2 strains exceeding 19g/100ml.

Figure 2.3.12 Strains relative frequencies of glucose consumption after 7 days of fermentation.

Concerning fermentation length, most strains closed the process in about 20 days, 4 strains in 16 days and 4 yeasts in 27.

Finally, the fermentation products obtained were tasted by an expert panel chosen by the Lison-Pramaggiore Consortium.

Considering the low amount of must available (200mL), only 5 persons were engaged.

Only general positive and negative organoleptic notes were taken into account at this stage.

The evaluation was performed by expressing a ranking for strains expressing the best sensory characteristic. The first 8 strains of the list were considered for further discussion among the panelists. Finally one strain was chosens, namely T314.1. This yeast revealed good fermentative performance and exalted almond and apricot fruity notes considered typical for Lison wine .

3.3.5 Microvinification

A microvinification test was conducted on the strain selected by the panel after the fermentation in natural must, and on the commercial strain Mycoferm611 used as reference.

The strains were inoculated to a final concentration of about 10^6 cell/ml in 30 l of Lison must, whose
chemical characteristic are reported in table 2.3.5

Table 2.3.5 Chemical characteristics of Lison must used fo microvinification tests.

Sugars (g/L)	pH	Total acidity(g/l)	Malic acid (g/l)	Tartaric acid (g/l)
203	3,39	7,2	1,72	3,01

To follow the fermentation process, sampling was done every 3 to 5 days to determine the relevant chemical parameters (pH, sugar, total acidity, volatile acidity and alcohol degree). The process was stopped when sugar level dropped down 1g/l.
The fermentation kinetics, measured by sugars concentration, are described in figure 2.3.13.

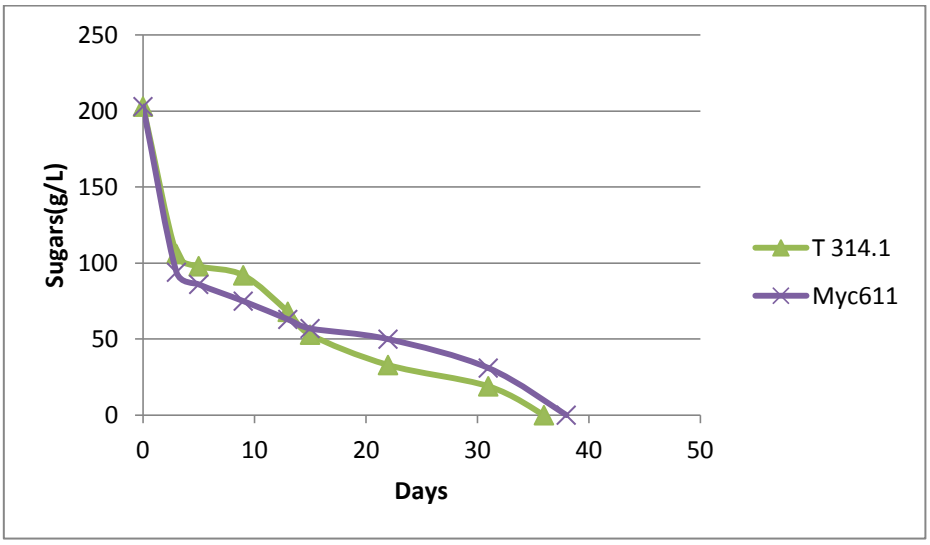


Figure 2.3.13 Fermentation kinetics of T314.1 and Myc611 strains in Lison must.

Considering sugars consumption during the fermentation process, T314.1 has a kinetic very similar to the commercial strain Mycoferm611. Both strains have a quite long fermentation length but consume all sugars present in the must.
The alcohol degree reached is about 12% (v/v) as reported in figure 2.3.14.

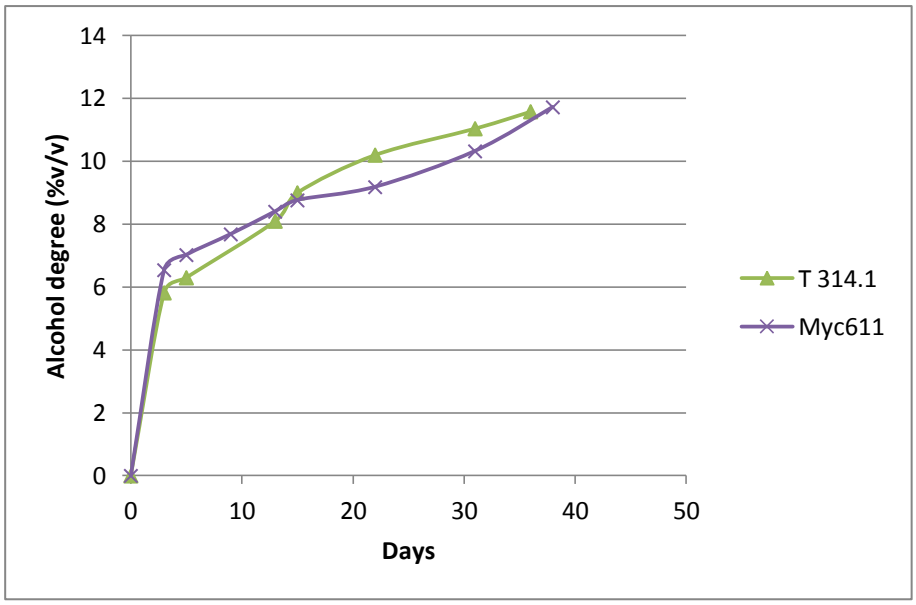


Figure 2.3.14 Alcohol production during fermentation.

The results achieved show that in natural environments there are autochthonous yeast strains with technological characteristics highly similar to those present in commercial yeasts.

2.3.6 Conclusions

A low number of samples containing *S. cerevisiae* were found in DOC Lison-Pramaggiore samplings. This situation is similar to what obtained in the Prosecco area during a survey conducted some years ago. The low yeast biodiversity level found could be linked to the territory climate characteristics as well as the Tocai Italico sensitivity towards fungal diseases, that, implying a greater use of pesticides in the vineyard, could negatively inluence yeast viability and development.

On the other hand, the higher yeast biodiversity found on the red vine variety Raboso Piave, could be linked to the thicker grape peel more resistant to pest attack than that of Prosecco and Lison grapes that requires fewer fungicidal treatments.

Concerning yeasts fermentative performances, strains coming from the Lison area showed fermentative vigour and glucose consumption very similar to those obtained by strains

coming from the Prosecco area, while the best performances were reached by strains from Raboso.

Nonetheless, the fermentative performance on natural Lison must, allowed to select one strain with good fermentative performances that at microvinification scale revealed kinetics similar to those of the commercial wine strain Mycoferm611, used in the Lison area for Tocai Italico wine making.

3. Genetic characterization and phylogenetic analysis of strains isolated from vineyards of NorthernItaly

3.1 Introduction

Genetic markers are observable traits which expression indicates the presence or absence of certain genes and they are classified into five broad groups: morphological, cytological, biochemical, protein and DNA. However protein and, more recently, DNA markers have revolutionized the availability of markers in ecological genetics studies. Genetic markers allow to characterize genetic diversity, so they are widely used for ecological genetic studies.

An ideal genetic marker for ecological genetic studies has six important characteristics (Weising *et al.* 1995):

- Detect qualitative or quantitative variation. The marker should be either present or absent, or the level of its expression should show discrete variation, that is, high versus low.
- Show no environmental or developmental influences. If an individual is translocated into three separate environments then it should display the same genotype irrespective of environment and if a marker is found in the juvenile it should also be present in the adult.
- Show simple codominant inheritance. In a diploid, both alleles at a locus should be visible in the heterozygote condition. In the dominant situation, one allele is present and it is impossible to distinguish between the dominant homozygote and the heterozygote condition.
- Detect silent nucleotide changes. The marker should be capable of detecting changes in the coding region of a genome that results in synonymous amino acid substitution, that is, mutations in codons that result in the incorporation of identical amino acid into a protein sequence.
- Detect changes in coding and non-coding portion of the genome. The markers should be randomly distributed across the genome, and no restricted to just one class of DNA.
- Detected evolutionary homologous changes. The markers used for genetic analysis should be homologous, that is, similar due to descent from a common ancestor. However, loci and alleles may be defined in genetic studies in manners other than by descent, for example, origin or state (Gillespie 1998).

None of the marker systems currently used in ecological genetics studies have all of these ideal characters. There are marker systems that are preferred for certain problems, for example, microsatellites will be preferred markers for detailed analysis of gene flow within populations, whilst other problems may be studied equally effectively using different marker systems, for example, PCR-RFLPs and allozyme analysis would be equally useful for estimating genetic diversity within a population. However, the choice of a marker system is a compromise between the properties of the marker and its availability.

The six most commonly used types of protein and DNA markers are allozymes, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites (or simple sequence repeats SSRs), and sequence analysis. However numerous other types of markers have been proposed, for example, single nucleotide polymorphism (SNPs; Curtler *et al.* 2001, Gibson 2002). Marker systems may be classified according to their modes of inheritance, that is, dominant (e.g., AFLPs) versus codominant (e.g., RFLPs), the numbers of putative loci that they detect at a locus, that is, few loci (e.g., allozymes) versus many loci (e.g., RAPDs), the numbers of alleles that they detect at a locus, that is, diallelic (e.g., RAPDs) versus multiallelic (e.g., SSRs) or their ease of use, that can be simple (e.g., RAPDs) or complex (e.g., AFLPs).

Allozymes were the firsts markers used. They are variant forms of an enzyme that are coded for by different alleles at the same locus. The majority of allozymes show codominant inheritance and the variants are attributed to nucleotide substitution causing charged amino acid replacement. They move at different speeds through a gel because they differ from each other in size and charge.

Allozymes are easily, safely and cheaply detected but they also have a low level of polymorphism. Gene variation is underestimated due to codon redundancy and synonymous nucleotide substitutions, although isoelectric focusing may identify additional polymorphism. The applications of allozyme markers include the estimation of gene diversity and population structure but they have limited phylogenetic power (Murphy *et al.* 1996; Mitton 1997).

Restriction fragment length polymorphism (RFLP) analysis measures DNA variation that affects the relative positions of restriction sites. Restriction enzymes are used to detect variation in primary DNA structure. The number of bases in the restriction site and the genome base composition determine the number of restriction sites identified in a genome.
RFLP markers are codominant and it is possible to detect nDNA and organelle DNA polymorphism in total DNA extracts. Their applications include estimation of gene diversity and population structure and may also be valuable as phylogenetic markers depending on the DNA sequence from which they are derived. On the other hand RFLPs are expensive, time consuming to detect and data from different laboratories are difficult to combine (Gardiner-Garden *et al.* 1992; Jansen *et al.* 1998).

Randomly amplified polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. Each band position on a gel is assumed to represent a diallelic locus (band present-absent). This locus definition means that RAPDs are dominant markers, that is, present-present homozygotes cannot be distinguished from present-absent heterozygotes at the phenotypic level.
The technique is cheap, simple, requires no sequence information, is PCR-based, and a large number of putative loci may be screened. It is useful at the initial stages of an investigation but it has been superseded by other technique because of its reproducibility, primer structure, marker dominance, product competition, product homology, allelic variation, genome sampling and non-independence of loci (Caetano-Anolles 1993; Weising *et al.* 1995; Harris 1999).

Amplified fragment length polymorphism (AFLP) analysis involves the selective amplification of an arbitrary subset of restriction fragments generated by double digestion of DNA with a frequently cutting and a rarely cutting restriction enzyme. Fragment ends are modified by the addition of double-stranded adapters, which provide the primer sites for subsequent PCR amplification. The number of bands generated in AFLP reaction is determined by the number of bases in the variable part of the selective primer and genome complexity. Most AFLP markers are scored as diallelic markers, where alleles are detected

as a band presence or absence, meaning that the markers are dominant. However, codominant AFLP markers may be detected because of small insertions or deletions in the restriction fragments.

These markers are highly polymorphic, either dominant or codominant and require no prior sequence knowledge. The majority of AFLP applications have been for genome mapping and breeding studies, although the application of AFLPs in ecological genetics is becoming widespread, especially for studies of gene diversity, population structure and clonality (Vos et al 1995). The disadvantages of these markers are that they requires a high degree of technical skill and relatively large amounts of high quality DNA (Rafalski *et al.* 1997; Robinson and Harris 2000).

In DNA sequence analysis the order of nucleotides in a piece of DNA is determined. Specific DNA regions are amplified by PCR and then subjected to cycle sequencing. Data are scored directly as the separate nucleotide bases. Direct DNA sequencing produces high-quality information, whilst automated technique and high powered computer facilities mean that large amounts of data can be generated. The data obtained can be used in applications that include estimation of gene diversity and population structure, and investigation of hybridization and gene flow. However, the approach has found its greatest value for phylogenetic analyses, where it is necessary to have ordered characters (Bishop and Rawlings 1997).

Microsatellites (SSRs) are short (10-50 copies) tandem repeats of mono- to tetra-nucleotide repeats which are assumed to be randomly distributed throughout the nDNA, cpDNA and mtDNA (Goldstein and Scholotter 1999, Jarne and Lagode 1996, Provan, Powell and Hollingsworth 2001). Primers are designed to conserved regions flanking the variable SSR. SSRs detect length variation that results from changes in the number of repeats units, to which stepwise mutation models are often applied. Consequently, regularly spaced bands (alleles) appear on gels. SSRs are relatively abundant and are thought to have a uniform coverage across the genome. Moreover they are codominant markers and it is possible to detect both nDNA and organelle DNA polymorphism in total DNA extracts. Mutation rates are high compared to other DNA markers, making them useful markers for intrapopulation

studies. The applications of SSR markers include estimation of gene diversity and population structure. Since SSR show a high number of alleles per locus they are ideally suited to the analysis of gene flow. The disadvantages of this kind of markers are that initial identification of SSRs is expensive and requires cloning and sequencing, whilst SSR primer pairs tent to be species-specific.

3.1.1Microsatellites and mtDNA analysis in *Saccharomyces cerevisiae* yeast

Autochthonous *Saccharomyces cerevisiae* strains isolated from natural environments associated with the wine production areas of interest, obtained from clonal selection, are now commercialized as active dry yeast. Such strains are able to efficiently ferment grape musts and produce desirable metabolites (e.g. glycerol, organic acids and higher alcohols), associated with reduced off flavours development (mainly H2S, acetic acid or phenolic compounds). Globally, they enhance the wine's sensorial characteristics and confer typical attributes to specific wine styles (Briones *et al.* 1995; Regodon *et al.* 1997). Commercially available yeast starters are now widely used in winemaking without any special containment and are annually released in large quantities, together with liquid and solid wine-making residues, in the environment around the winery. From an ecological point of view, these yeasts can be regarded as non-indigenous strains that are every year introduced in large quantities in the ecosystem surrounding a winery. In particular, it is not known if commercial strains are able to survive in nature and to become members of the vineyard microbiota.

In a recent study that was carried out in six vineyards of the Vinho Verde (Portugal) and the Languedoc (France) wine regions, it was shown that the dissemination of commercial yeast strains is limited to a very close proximity of the winery (10–200 m) where they have been used and that their permanent implantation in the vineyard did not seem to occur (Valero *et al.* 2005). Moreover, vine-associated autochthonous *Saccharomyces* biodiversity is not affected by long-term use of commercial yeasts.

Despite the great numbers of indigenous strains constantly selected from the environment, at the moment the description in terms of relative strains abundance in nature do not can be accurately estimated. Moreover, exploring the biodiversity of indigenous fermentative

strains can be an important contribution towards the understanding and selection of strains with specific phenotypes (Schuller, et al 2005). Comprehensive identification of polymorphisms among individuals within a species is essential both for studying the genetic basis of phenotypic differences and for elucidating the evolutionary history of the species.

Traditional morphological and biochemical tests are of limited value in revealing the genetic diversity of yeast strains of the same species. Molecular methods developed to study yeasts at both the species and subspecies level have several applications, including monitoring the dominance of the inoculated yeast strain, yeast population dynamics studies, studies of wine yeast strain origin and evolution, and protection of the industrial property on commercial yeast strains (Querol *et al.* 1992; Guillamon *et al.* 1998; Fernandez-Espinar *et al.* 2001; Torija *et al.* 2001). Available molecular typing techniques that have been applied to the genetic identification of wine yeast strains include separation of intact chromosomes by pulsed field agarose gel electrophoresis (Vezinhet *et al.* 1990; Guillamon *et al.* 1998); restriction analysis of the mitochondrial genome (Vezinhet *et al.* 1990; Querol *et al.* 1992); analysis of d sequences by PCR amplification (Ness *et al.* 1993; Lavallee *et al.* 1994; Legras and Karst, 2003); microsatellite markers (Balerias Couto *et al.* 1996; Techera *et al.* 2001; Gallego *et al.* 1998, Legras *et al.* 2005); PCR amplification of the mitochondrial, intron rich, COX1 gene (Lopez *et al.* 2003); Random Amplified Polymorphic DNA (RAPD-PCR) (Grando *et al.* 1994; Quesada and Cenis, 1995); single nucleotide polymorphisms (SNPs) (Jubany *et al.* 2008); or combination of several of these methodologies (Fernandez-Espinar *et al.* 2001). They all constitute powerful tools, not only for industrial and technological controls, but also for ecological investigations of the intraspecific diversity of the indigenous microflora of wines. Despite the availability of several molecular methods, data on *S. cerevisiae* typing are still limited. Moreover, some of methods, such as karyotyping or mitochondrial DNA polymorphism, are incompletely evaluated because of the low number of strains tested or because of the lack of studies on stability and reproducibility (Struelens M 1996). In addition, the discriminatory power of some methods appears insufficient when these methods are tested alone, moreover whole genome sequencing is a powerful approach for elucidating the population genetics of S. cerevisiae, but it is currently time consuming and expensive.

Mitochondrial DNA (mtDNA) of *S. cerevisiae* is a small molecule of 65-80 Kb which grade of variability can be shown with by restriction analysis. The high degree of polymorphism of mtDNA allows to analyse the variability of wine specific *S. cerevisiae* strains. Among all the molecular techniques described in literature, mitochondrial DNA restriction analysis appears as one of the most suitable methods to differentiate between strains. The Organization Internationale de la Vigne et du Vin (OIV OENO) in the resolution number 408 of 2011 regarding "Molecular tools for identification of *Saccharomyces cerevisiae* wine yeast and other yeast species related to winemaking" propose for the identification of wine yeast at strain level the mitochondrial DNA RFLP analysis. Querol *et al*. (1992) and then Lopez in 2001 developed a new mitochondrial restriction analysis method that clearly simplifies the characterization of *Saccharomyces cerevisiae* wine yeast strains. This mitochondrial analysis method consists of the standard miniprep isolation of total yeast DNA and the use of GqC-rich restriction endonucleases as *HinfI* or *RsaI*, which recognise a high number of sites in the yeast nuclear DNA, but few sites in the mitochondrial DNA. The method is simple and yields results within 3 days work. This technique has successfully been used by other authors to characterise strains of other yeast species (Martinez *et al*. 1995; Romano *et al*. 1996; Guillamon *et al*. 1997) because allows high throughput of strain identification in a short period of time. It can be used in wine industry because it's fast, secure and no PCR equipment is required. As showed by Shuller and Dequin (2004) mitochondrial DNA restriction analysis could be a good technique to differentiate yeast strains from the same ecosystem. This technique is also easy to use once the conditions have been carefully standardized and the reproducibility is better than other analysis.

Mitochondrial DNA has been the workhorse of research in phylogeography of higher eukaryotic organisms for almost two decades. However, concerns with basing evolutionary interpretations on mitochondrial DNA results alone have been voiced since the beginning of such studies (Munoz et al 2008). Recently, some authors have suggested that the potential problems with mtDNA are so great that inferences about population structure and species limits are unwarranted unless corroborated by other evidence, usually in the form of nuclear gene data (Zink and Barrowclough, 2008).

Microsatellites or simple sequence repeats (SSR) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp (Charlesworth *et al*. 1994). These repetitive sequences are a major component of higher organism DNAs. They are hypervariable in length (Tautz 1989) as a result of DNA replication errors, such as slipped-strand mispairing (Strand *et al*. 1993). Microsatellite alterations occur at a rate much higher than the mutation rate in nonrepetitive DNA (Wierld 1997). These alterations are likely to reflect DNA polymerase slippage (Sia 1997). During DNA replication, a transient dissociation of the DNA strands, followed by incorrect reassociation, results in one or more unpaired repeat units on either the template or the nascent strand. If these unpaired loops are not repaired, another round of replication will result in a tract that is shorter (if the unpaired repeats are on the template strand) or longer (if the unpaired repeats are on the nascent strand) than the original tract. The detection of microsatellite polymorphisms is a promising and powerful tool, providing accurate and unequivocal results expressed as base pair number (or as a number of repeats). Thus, microsatellites show a substantial level of polymorphism between individuals of the same species and are extensively used in humans for paternity exclusion tests (Helminen *et al*. 1988), forensic medicine (Hagelberg *et al*. 1991) and for molecular typing of different organisms (including cultivars of *Vitis vinifera*; Bowers *et al*. 1999). Microsatellites are particularly suitable for the detection of polyploids and have a higher discrimination power than nucleotide sequence-based methods such as multilocus sequence typing (MLST), particularly when closely related strains are compared (Ayoub *et al*., 2006).

The method has been successfully applied initially for typing clinical fungi such as *Candida albicans* (Lumen *et al*. 1998) and *Aspergillus fumigatus* (Bart-Delabesse *et al.* 1998).

After the entire *S. cerevisiae* genome was publicly available (Goffeau *et al.* 1996), different computer searches for short tandem repeats were conducted (Field and Wills, 1998; Katti *et al.* 2001; Aishwarya *et al.* 2007). Recently, several high throughput microsatellite polymorphism analyses have been performed (Legras *et al.* 2005, 2007; Schuller and Casal, 2007). This technique is the most appropriate for large-scale studies like determination of genetic proximity (phylogenetic studies) and biogeographical distribution of indigenous *Saccharomyces* strains and/or species by means of numerical analysis.

Nowadays, one of the greatest challenges for geneticists is the dissection of complex quantitative genetic variation into genes at the molecular level. Most traits of biotechnological interest in *S. cerevisiae* strains are complex traits that depend on multiple genes and their allelic variants. Codominant molecular markers like SSRs and SNPs are widely used for the molecular discrimination of individuals within eukaryotic species, for biodiversity studies, QTL mapping and linkage studies.

In 2001, polymorphism analysis of selected microsatellite loci was proposed as a very powerful and unique method to discriminate *S. cerevisiae* at the strain level and that the discriminatory power of six microsatellite loci (Perez *et al*. 2001) is identical to the mtDNA RFLP (using enzyme *HinfI*) (Gonzalez Techera *et al*. 2001; Hennequin *et al*. 2001; Schuller *et al*. 2004, Valero *et al*. 2005). However, as several of the loci proposed by Hennequin *et al.* (2001) or Perez *et al.* (2001) present a very low allelic variation, it is clear that the maximum resolution of such technique is not attained (Legras *et al.* 2005).

In the last years, an increasing number of microsatellites have been described for *S. cerevisiae*, with the aim finding the most polymorphic loci with a high allelic diversity that can be applied for both strain delimitation and the description of relationships between strains that are related due to their common geographical or technological origin (Bradbury *et al.* 2006; Legras *et al*. 2007; Richards *et al.* 2009; Goddard *et al*. 2010).

The technique was improved from the six most polymorphic loci used by several authors in the first surveys to more than ten loci in the last studies (Legras *et al*. 2008).

Nowadays microsatellites typing is the favourite analysis conducted to understand the roles that ecology and geography play in shaping *S. cerevisiae*'s population structure and several authors have shown the importance to create a common database of microsatellite genotypes for *Saccharomyces cerevisiae*. There is an increasing need for standardization in the reporting of results from different laboratories as more *S. cerevisiae* strains and SSR markers are being tested. The discrimination power of the selected SSRs depends on the population of strains analyzed and, therefore, it would be very valuable information to be able to calculate allelic frequencies from strains coming from industrial, clinical or environmental settings. At present, it is not possible to extrapolate microsatellite data from different laboratories. Sizing with ladders, containing many or all of the observed alleles for a given SSR locus, is a common practice when analyzing human microsatellites and it

certainly allows comparison of data after careful validation procedures. The standard in humans is to report alleles as the absolute number of repeats. Only a small core set of loci have been selected and commercial kits providing premixed primers and allelic ladders are available. Because all users work with the same primers, these allelic ladders can be used to calibrate PCR product sizes to SSR repeat number for genotyping purposes (Butler, 2007). However, in some cases, there is still the need to reach a consensus on the definition of the core repeat structure to prevent confusion and allow a comparison of results between laboratories. Comparison with whole genome DNA sequence data shows that microsatellite profiling provides a simple and accurate method for identifying strains that are closely related genetically. SSR typing is a cheap and accessible method that has the following unique features compared with SNPs (or MLST):

(1) SSRs give clearcut information on ploidy levels. Many industrial strains are aneuploids or polyploids, and this has been associated with an adaptation mechanism (Querol *et al.* 2003).

(2) SSRs can be easily adapted to a simple method (using agarose gels) to monitor *S. cerevisiae* strains during alcoholic fermentation (Howell *et al.* 2004, Vaudano_and Garcia-Moruno, 2008) and to detect the presence of *S. cerevisiae–Saccharomyces bayanus* hybrids (Masneuf-Pomarede *et al.* 2007).

(3) The reason why some SSRs are highly polymorphic while others are invariable is still an open question. Variation in the efficiency of DNA mismatch repair at different sites in the yeast genome has been proposed as a possible explanation (Hawk *et al.* 2005). But one major factor of variability is the number of repeats.

(4) For closely related *S. cerevisiae* strains, MLST has proven to be less discriminatory than SSRs (Ayoub *et al.* 2006).

(5) Precise estimation and comparison of genetic variation among populations requires a large number of SNP relative to microsatellites because microsatellite loci typically have many alleles (more than 30 for *S. cerevisiae* for the most polymorphic loci), whereas two is the norm for SNP loci. Ascertainment bias in SNPs identification can also be a serious issue for studies of population structure since it has the potential to introduce systematic bias in estimates of variation within and among populations (Morin *et al.* 2004).

3.2 Materials and methods

3.2.1 Mitochondrial DNA analysis

3.2.1.1 Yeasts total DNA extraction

Yeasts coat obtained on YM agar medium, growing yeasts for 48 h at 25 °C, was resuspended in 1 ml of sterile water and then centrifuged at 14000 rpm for 3 minutes in an Eppendorf microcentrifuge.

After fluid discarding, the cells were resuspended in 500 µl of a solution containing 50 mM Tris-HCl, 20 mM EDTA, pH 7.4 and transferred to a 2 ml Eppendorf containing 0.3 g of glass beads of 425-600 µm (Sigma) and vortex for 3 minutes. 50 µl of 10% SDS were then added to the samples that were incubated in a thermostatic bath at 65°C for 30 minutes. At the end 200 µl of potassium acetate 5M were added and the samples were left on ice for 30 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes. 600 µl of supernatant was transferred to an Eppendorf tube and 600 µl of cold isopropanol were added. The samples were kept at room temperature for 5 minutes, stirring by inversion and then centrifuged at 14000 rpm for 10 minutes. The supernatant was removed and 500 µl of 70% ethanol were added. After centrifugation at 14000 rpm for 10 minutes and the supernatant discarding, the pellet was dried for 1 h at 37 ° C. The samples were resuspended in 50 µl of sterile water, to which 1.5 µl (10 mg / ml) of RNase (Amersham Bioscience E70194Z) were added. The samples were left at room temperature for 15-20 minutes and finally stored at -20 °C.

3.2.1.2 Total DNA enzyme restriction

The total DNA digestions were performed in 15 µl of volumes reaction containing 10 U of *HinfI* enzyme (Fermentas) and 10 µl of extracted DNA. The reactions were performed at 37 °C for 2 h.

3.2.1.3 Bands analysis

Restriction profiles obtained were compared by the GelCompareII (Applied Maths) software that allows, by a matrix construction, to calculate the similarity level between strains and to convert it into a dendrogram. For the matrix construction was used the Dice similarity coefficient which considers the electrophoretic bands position, but not their intensity. Moreover, for the dendrogram construction, determined by the UPMGA method, the "optimization" and "tolerance" values, which determine the minimum variability degree of a profile than other more similar, were those recommended by the program.

3.2.2 Microsatellites analysis

3.2.2.1 Strains

In this work 202 autochthonous strains of *Saccharomices cerevisiae* isolated from vineyards located in the DOCG Prosecco Conegliano-Valdobbiadene, DOC Piave and DOCG Lison-Pramaggiore areas were considered. The strains were obtained after single fermentation of bunches of Glera (ex Prosecco) variety, Raboso and Tocai Italico varieties.
The survey was also conducted on 37 commercial strains, coming from different substrates like wine, sake, ragi, beer, oak, bread, laboratory and clinical.
For each strains considered was also attributed a population name on the base of the isolation area.
All the strains considered are reported in table 3.2.1

Table 3.2.1 Strains tested for microsatellite polymorphism in this study

NAME	ORIGIN	POPULATION NAME
AWRI1631	Wine, AUSTRALIA (sequenced)	wine
AWRI796	Wine, AUSTRALIA (sequenced)	wine
BC187	Wine, USA (sequenced)	wine
BLA.GRCR	Wine, EUROPE	wine
CLB219w	Wine, EUROPE	wine
D47	Wine, EUROPE	wine
DBVPG1106	Wine, EUROPE (sequenced)	wine
DBVPG6040	Wine, EUROPE (sequenced)	wine
DV10	Wine, EUROPE	wine
EC1118	Wine, EUROPE (sequenced)	wine
F15	Wine, EUROPE	wine
FR95	Wine, EUROPE	wine

JAY270	Wine, EUROPE	wine
L1374	Wine, EUROPE (sequenced)	wine
L1414	Wine, EUROPE (sequenced)	wine
L1528	Wine, EUROPE (sequenced)	wine
LV10	Wine, EUROPE	wine
MYC611	Wine, EUROPE	wine
N.FERM	Wine, EUROPE	wine
P444	Wine, EUROPE	wine
QA23	Wine, EUROPE (sequenced)	wine
RM11a	Wine, EUROPE (sequenced)	wine
SIGMA1278	Wine, EUROPE (sequenced)	wine
V.PR.BL	Wine, EUROPE	wine
VIC17ES	Wine, EUROPE (sequenced)	wine
VIN13	Wine, EUROPE (sequenced)	wine
VL3	Wine, EUROPE (sequenced)	wine
VRB	Wine, EUROPE	wine
Y9	Ragi, ASIA	ragi
UC5	Sake, ASIA	sake
Clib382	Beer, EUROPE	beer
NCYC361	Beer, EUROPE	beer
6662	Bread, EUROPE	bread
YJM428	Clinical isolate, USA	Is.Cl
YJM653	Clinical isolate, USA	Is.Cl
S288C	Laboratory, USA	lab
NC02	oak tree exudates, North Caroline, USA	oak
B169.12	Autochthonous, Glera bunches	ProA
B173.16	Autochthonous, Glera bunches	ProA
B173.2	Autochthonous, Glera bunches	ProA
B173.4	Autochthonous, Glera bunches	ProA
B197.1	Autochthonous, Glera bunches	ProA
Big217.2	Autochthonous, Glera bunches	ProA
Big223.8	Autochthonous, Glera bunches	ProA
C261.4	Autochthonous, Glera bunches	ProA
P138.4	Autochthonous, Glera bunches	ProA
P148.1	Autochthonous, Glera bunches	ProA
P158.4	Autochthonous, Glera bunches	ProA
P173.3	Autochthonous, Glera bunches	ProA
P225.3	Autochthonous, Glera bunches	ProA
P227.11	Autochthonous, Glera bunches	ProA
P234.15	Autochthonous, Glera bunches	ProA
P234.5	Autochthonous, Glera bunches	ProA
P254.12	Autochthonous, Glera bunches	ProA
P254.2	Autochthonous, Glera bunches	ProA
P254.3	Autochthonous, Glera bunches	ProA
P283.4	Autochthonous, Glera bunches	ProA
P293.1	Autochthonous, Glera bunches	ProA
P301.16	Autochthonous, Glera bunches	ProA
P301.3	Autochthonous, Glera bunches	ProA
P301.4	Autochthonous, Glera bunches	ProA
P301.9	Autochthonous, Glera bunches	ProA
P303.6	Autochthonous, Glera bunches	ProA
P304.1	Autochthonous, Glera bunches	ProA
P304.11	Autochthonous, Glera bunches	ProA
P304.13	Autochthonous, Glera bunches	ProA
P304.2	Autochthonous, Glera bunches	ProA
P304.3	Autochthonous, Glera bunches	ProA
P304.4	Autochthonous, Glera bunches	ProA
P304.5	Autochthonous, Glera bunches	ProA
P304.6	Autochthonous, Glera bunches	ProA
P304.8	Autochthonous, Glera bunches	ProA
B125.2	Autochthonous, Glera bunches	ProB
S41	Autochthonous, Glera bunches	ProB
S43	Autochthonous, Glera bunches	ProB
S44	Autochthonous, Glera bunches	ProB
S45	Autochthonous, Glera bunches	ProB
S46	Autochthonous, Glera bunches	ProB

S47	Autochthonous, Glera bunches	ProB
X20.13	Autochthonous, Glera bunches	ProB
X22.4	Autochthonous, Glera bunches	ProB
X36.4	Autochthonous, Glera bunches	ProB
X39.14	Autochthonous, Glera bunches	ProB
R106.2	Autochthonous, Raboso bunches	RabA
R106.3	Autochthonous, Raboso bunches	RabA
R107.1	Autochthonous, Raboso bunches	RabA
R107.3	Autochthonous, Raboso bunches	RabA
R107.4	Autochthonous, Raboso bunches	RabA
R107.5	Autochthonous, Raboso bunches	RabA
R128.1	Autochthonous, Raboso bunches	RabA
R130.1	Autochthonous, Raboso bunches	RabA
R130.2	Autochthonous, Raboso bunches	RabA
R130.4	Autochthonous, Raboso bunches	RabA
R131.2	Autochthonous, Raboso bunches	RabA
R131.3	Autochthonous, Raboso bunches	RabA
R131.4	Autochthonous, Raboso bunches	RabA
R132.5	Autochthonous, Raboso bunches	RabA
R133.3	Autochthonous, Raboso bunches	RabA
R133.4	Autochthonous, Raboso bunches	RabA
R133.5	Autochthonous, Raboso bunches	RabA
R135.2	Autochthonous, Raboso bunches	RabA
R135.3	Autochthonous, Raboso bunches	RabA
R136.3	Autochthonous, Raboso bunches	RabA
R136.5	Autochthonous, Raboso bunches	RabA
R144.1	Autochthonous, Raboso bunches	RabA
R144.3	Autochthonous, Raboso bunches	RabA
R146.1	Autochthonous, Raboso bunches	RabA
R146.2	Autochthonous, Raboso bunches	RabA
R146.3	Autochthonous, Raboso bunches	RabA
R146.4	Autochthonous, Raboso bunches	RabA
R146.5	Autochthonous, Raboso bunches	RabA
R31.2	Autochthonous, Raboso bunches	RabA
R31.3	Autochthonous, Raboso bunches	RabA
R31.4	Autochthonous, Raboso bunches	RabA
R31.6	Autochthonous, Raboso bunches	RabA
R32.2	Autochthonous, Raboso bunches	RabA
R35.1	Autochthonous, Raboso bunches	RabA
R35.4	Autochthonous, Raboso bunches	RabA
R5.2	Autochthonous, Raboso bunches	RabA
R6.1	Autochthonous, Raboso bunches	RabA
R6.2	Autochthonous, Raboso bunches	RabA
R6.7	Autochthonous, Raboso bunches	RabA
R7.1	Autochthonous, Raboso bunches	RabA
R8.2	Autochthonous, Raboso bunches	RabA
R8.3	Autochthonous, Raboso bunches	RabA
R8.5	Autochthonous, Raboso bunches	RabA
R8.6	Autochthonous, Raboso bunches	RabA
R8.7	Autochthonous, Raboso bunches	RabA
R113.2	Autochthonous, Raboso bunches	RabB
R113.3	Autochthonous, Raboso bunches	RabB
R115.1	Autochthonous, Raboso bunches	RabB
R115.3	Autochthonous, Raboso bunches	RabB
R115.5	Autochthonous, Raboso bunches	RabB
R116.1	Autochthonous, Raboso bunches	RabB
R116.3	Autochthonous, Raboso bunches	RabB
R116.5	Autochthonous, Raboso bunches	RabB
R117.1	Autochthonous, Raboso bunches	RabB
R117.5	Autochthonous, Raboso bunches	RabB
R119.1	Autochthonous, Raboso bunches	RabB
R119.2	Autochthonous, Raboso bunches	RabB
R119.3	Autochthonous, Raboso bunches	RabB
R119.5	Autochthonous, Raboso bunches	RabB
R120.2	Autochthonous, Raboso bunches	RabB
R126.1	Autochthonous, Raboso bunches	RabB

R100.1	Autochthonous, Raboso bunches	RabC
R101.1	Autochthonous, Raboso bunches	RabC
R101.2	Autochthonous, Raboso bunches	RabC
R101.3	Autochthonous, Raboso bunches	RabC
R101.4	Autochthonous, Raboso bunches	RabC
R101.5	Autochthonous, Raboso bunches	RabC
R102.1	Autochthonous, Raboso bunches	RabC
R102.3	Autochthonous, Raboso bunches	RabC
R103.1	Autochthonous, Raboso bunches	RabC
R103.3	Autochthonous, Raboso bunches	RabC
R103.4	Autochthonous, Raboso bunches	RabC
R103.5	Autochthonous, Raboso bunches	RabC
R104.2	Autochthonous, Raboso bunches	RabC
R104.4	Autochthonous, Raboso bunches	RabC
R104.5	Autochthonous, Raboso bunches	RabC
R105.2	Autochthonous, Raboso bunches	RabC
R105.3	Autochthonous, Raboso bunches	RabC
R105.5	Autochthonous, Raboso bunches	RabC
R11.1	Autochthonous, Raboso bunches	RabC
R11.3	Autochthonous, Raboso bunches	RabC
R110.4	Autochthonous, Raboso bunches	RabC
R111.1	Autochthonous, Raboso bunches	RabC
R12.1	Autochthonous, Raboso bunches	RabC
R12.2	Autochthonous, Raboso bunches	RabC
R12.3	Autochthonous, Raboso bunches	RabC
R137.2	Autochthonous, Raboso bunches	RabC
R138.4	Autochthonous, Raboso bunches	RabC
R138.5	Autochthonous, Raboso bunches	RabC
R139.2	Autochthonous, Raboso bunches	RabC
R139.3	Autochthonous, Raboso bunches	RabC
R139.4	Autochthonous, Raboso bunches	RabC
R14.1	Autochthonous, Raboso bunches	RabC
R14.2	Autochthonous, Raboso bunches	RabC
R14.3	Autochthonous, Raboso bunches	RabC
R14.4	Autochthonous, Raboso bunches	RabC
R14.5	Autochthonous, Raboso bunches	RabC
R14.6	Autochthonous, Raboso bunches	RabC
R14.7	Autochthonous, Raboso bunches	RabC
R143.1	Autochthonous, Raboso bunches	RabC
R143.2	Autochthonous, Raboso bunches	RabC
R149.1	Autochthonous, Raboso bunches	RabC
R15.1	Autochthonous, Raboso bunches	RabC
R15.2	Autochthonous, Raboso bunches	RabC
R15.3	Autochthonous, Raboso bunches	RabC
R15.4	Autochthonous, Raboso bunches	RabC
R15.5	Autochthonous, Raboso bunches	RabC
R15.6	Autochthonous, Raboso bunches	RabC
R15.7	Autochthonous, Raboso bunches	RabC
R150.1	Autochthonous, Raboso bunches	RabC
R150.2	Autochthonous, Raboso bunches	RabC
R150.3	Autochthonous, Raboso bunches	RabC
R150.4	Autochthonous, Raboso bunches	RabC
R150.5	Autochthonous, Raboso bunches	RabC
R151.1	Autochthonous, Raboso bunches	RabC
R151.2	Autochthonous, Raboso bunches	RabC
R152.1	Autochthonous, Raboso bunches	RabC
R152.4	Autochthonous, Raboso bunches	RabC
R152.5	Autochthonous, Raboso bunches	RabC
R153.1	Autochthonous, Raboso bunches	RabC
R153.2	Autochthonous, Raboso bunches	RabC
R153.4	Autochthonous, Raboso bunches	RabC
R153.5	Autochthonous, Raboso bunches	RabC
R154.1	Autochthonous, Raboso bunches	RabC
R154.4	Autochthonous, Raboso bunches	RabC
R155.3	Autochthonous, Raboso bunches	RabC
R157.1	Autochthonous, Raboso bunches	RabC

R157.2	Autochthonous, Raboso bunches	RabC
R157.3	Autochthonous, Raboso bunches	RabC
R16.1	Autochthonous, Raboso bunches	RabC
R16.2	Autochthonous, Raboso bunches	RabC
R17.1	Autochthonous, Raboso bunches	RabC
R17.2	Autochthonous, Raboso bunches	RabC
T113B.1	Autochthonous, Tocai Italico bunches	TocA
T21.1	Autochthonous, Tocai Italico bunches	TocA
T23.1	Autochthonous, Tocai Italico bunches	TocA
T317.2	Autochthonous, Tocai Italico bunches	TocA
T415.1	Autochthonous, Tocai Italico bunches	TocA
T424.1	Autochthonous, Tocai Italico bunches	TocA
T522.13	Autochthonous, Tocai Italico bunches	TocA
T525.1	Autochthonous, Tocai Italico bunches	TocA
T9.1	Autochthonous, Tocai Italico bunches	TocA
T306.11	Autochthonous, Tocai Italico bunches	TocB
T314.1	Autochthonous, Tocai Italico bunches	TocB
T411.1	Autochthonous, Tocai Italico bunches	TocB
T411.10	Autochthonous, Tocai Italico bunches	TocB
T602.3	Autochthonous, Tocai Italico bunches	TocB
T603.2	Autochthonous, Tocai Italico bunches	TocB
T604.3	Autochthonous, Tocai Italico bunches	TocB
T605.3	Autochthonous, Tocai Italico bunches	TocB
T605.5	Autochthonous, Tocai Italico bunches	TocB
T605.7	Autochthonous, Tocai Italico bunches	TocB
T606.3	Autochthonous, Tocai Italico bunches	TocB
T606.4	Autochthonous, Tocai Italico bunches	TocB
T606.8	Autochthonous, Tocai Italico bunches	TocB
T611.4	Autochthonous, Tocai Italico bunches	TocB

3.2.2.2 DNA isolation

Yeast cells were cultivated in 10 ml YPD medium (36 h, 25° C, 150 rpm) and genomic DNA was
isolated by E.Z.N.A® yeast DNA kit (OMEGA Bio-Tech, USA).

3.2.2.3 Microsatellites amplification

To achieve this analysis, 18 microsatellite loci (Legras *et al.* 2007, Richards *et al.* 2009) were combined in two sets of nine loci (Table 2.6.2) and amplified using the Type-it Microsatellite PCR kit (Qiagen, Milan, Italy). PCR reactions were run in a final volume of 12,5 µl containing 20–80 ng of yeast DNA. Amplification was performed using a Gene Amp 9700 (Applied Biosystems) thermal cycler under a three-stage temperature programme: stage one: 95°C – 15 min, stage two (34 cycles): 95°C – 30 s, 57°C – 2 min, 72°C – 1 min, stage three: 60°C – 30 min

Table 3.2.2 Characteristics of the 12 loci and primers used in the study

Locus name	Motif	ORF or coordinates	Primers	Dye
MIX 1				
C3	CAA	YGL139w	FW-CTTTTTATTTACGAGCGGGCCAT RV-AAATCTCATGCCTGTGAGGGGTAT	NED
C5	GT	VI-210250/ 210414	FW-TGACACAATAGCAATGGCCTTCA RV-GCAAGCGACTAGAACAACAATCACA	VIC
C8	TAA	YGL014w	FW-CAGGTCGTTCTAACGTTGGTAAAATG RV- GCTGTTGCTGTTGGTAGCATTACTGT	FAM
C11	GT	X-518870/ 519072	FW-TTCCATCATAACCGTCTGGGATT RV-TGCCTTTTTCTTAGATGGGCTTTC	FAM
YKR072c	GAC	YKR072c	FW-AGATACAGAAGATAAGAACGAAAA RV-TTATTGATGCTTATCTATTATACC	PET
SCYOR267c	TGT	YOR267c	FW-TACTAACGTCAACACTGCTGCCAA RV-GGATCTACTTGCAGTATACGGG	VIC
SCAAT2	TAA	YBL084c	FW-CAGTCTTATTGCCTTGAACGA RV-GTCTCCATCCTCCAAACAGCC	PET
SCAAT3	TAA	YDR160w	FW-TGGGAGGAGGGAAATGGACAG RV-TTCAGTTACCCGCACAATCTA	NED
SCAAT6	TAA	IX-105711/ 105883	FW-TTACCCCTCTGAATGAAAACG RV-AGGTAGTTTAGGAAGTGAGGC	PET
MIX 2				
SCAAT5	TAA	XVI-897051/ 8970210	FW-AGCATAATTGGAGGCAGTAAAGCA RV-TCTCCGTCTTTTTTGTACTGCGTG	NED
C4	TAA +TAG	(i) XV-110701/110935	FW-AGGAGAAAAATGCTGTTTATTCTGACC RV- TTTTCCTCCGGGACGTGAAATA	NED
C6	CA	XVI-485898/ 485996	FW-GTGGCATCATATCTGTCAATTTTATCAC RV-VIC-CAATCAAGCAAAAGATCGGCCT	VIC
YPL009c	CTT	YPL009c	FW-AACCCATTGACCTCGTTACTATCGT RV-TTCGATGGCTCTGATAACTCCATTC	FAM
C9	TAA	YOR156c	FW-AAGGGTTCGTAAACATATAACTGGCA RV-TATAAGGGAAAAGAGCACGATGGC	NED
SCAAT1	TTA	XIII-86902/ 87140	FW-AAAGCGTAAGCAATGGTGTAGATACTT RV-CAAGCCTCTTCAAGCATGACCTTT	VIC

YKL172w	GAA	YKL172w	FW-CAGGACGCTACCGAAGCTCAAAAG RV-ACTTTTGGCCAATTTCTCAAGAT	FAM
YLR	TC	XII-823393/ 823562	FW-CTGGAATGAAATTAAACAAAAGC RV-TCTTCCTTTTCTACTATCTTCTC	PET
YLL049	TA	XII	FW-GCAACATAATGATTTTGAGGT RV-GTGTCTTGTGTGAGCATAGTGGAGAA	PET

3.2.2.4 PCR product analysis

PCR products were sized for 18 microsatellite loci on a capillary DNA sequencer (ABI 3130 XL, Applied Biosystems) with the DS-33 Matrix Standard Kit (Dye Set G5, Applied Biosystems) using the polyacrylamide Pop7 and the size standard GeneScan500LIZ®. Before the analysis, the PCR amplicons were first diluted 100 fold and then 0,5µl of the dilution was added to 9,35µl of formamide (Applied Biosystem) and 0,15µl of GeneScan500LIZ® size marker, and the mixture was denaturated at 95°C for 5 min. Allele distribution into classes was carried out using GeneMapper software version 4.1 (Applied Biosystems).

3.2.2.5 Population analysis

The chord distance Dc (Cavalli-Sforza & Edwards 1967) was calculated between each strain with a laboratory-made program (Legras *et al.* 2007). All trees were obtained from distance matrices derived with neighbour of the Phylip 3.69 package, using Mega 5.05 (Kumar et al . 2004) for tree-drawing. All trees were rooted by the midpoint method. The reliability of the tree topologies was assayed through a jackknife procedure. The validity of nodes was obtained with the consens program (Phylip 3.69 package). Population genetic tests (PCA and FstP) were also conducted by the GenAlEx 6.41 program.

3.3 Results and discussion

3.3.1 Mitochondrial DNA analysis

With the aim to understand the genetic relationships among the strains isolated in the in three different wine producing areas (DOCG Prosecco of Conegliano-Valdobbiadene, DOC Piave and DOCG Lison-Pramaggiore) located in Veneto (north-east Italy), two approaches were performed. The first one considered the profiles obtained with the mtDNA-RFLP analysis, while the second one the polymorphism obtained by the microsatellites analysis. For this purpose 202 strains of the autochthonous yeasts collection were considered. Samplings were performed in a total of 162 wineries collecting 97 grapes of Glera variety in the Prosecco area, 20 of Raboso Piave variety in the Piave DOC and 45 of Tocai Italico variety in the Lison-Pramaggiore area during the 2004–2010 pre-harvest period. All the isolates were analyzed by their mtDNA RFLP (with the restriction enzyme *Hinf*I) and a restriction profile was attributed to each isolate. Each profile was analyzed by GelCompareII (Applied Maths, Belgium) software that allows, by a matrix construction, to calculate the similarity level among strains and to convert it into a dendrogram. For the matrix construction the Dice similarity coefficient, which considers the electrophoretic bands position, but not their intensity, was used. Moreover, the dendrogram was determined by the UPMGA method and values of the "optimization" and the "tolerance" parameters, which calculated the minimum variability degree of a single profile, were chosen to optimize profile reproducibility. Concerning this aspect, using the optimization and the tolerance values of 1.17% and 1.5% respectively), the analysis of three replicates of the profile that identified the commercial strain conducted Blastosel Fr 95 (Perdomini, Italy) gave a similarity degree of 100%. When the same profile was found in more than one sample, one strain from each sample was taken into account. The analysis point out the presence of 184 different profiles. In order to better understand yeasts evolution, 28 commercial wine strains, including 10 strains which genome sequence is available, 2 beer strains (NCYC361 and Clib382), 2 clinical isolates (YJM428 and YJM653), 1 bread (6662), 1 laboratory (S288c), 1 oak (NC02), 1 sake (UC5) and 1 ragi (Y9) strains, were analyzed (see materials and methods for details). For each profile, obtained from

autochthonous yeasts, a specific code was given. In table 3.2.1 the mtDNA profile code, the number of samples with the specific profile and the name of the strains with the mtDNA profiles, were reported for each sampled area.

Table 3.3.1 Different mtDNA profiles found in samples collected in the wine producing area Lison,-Pramaggiore, Conegliano-Valdobbiadene and Piave

LISON- PRAMAGGIORE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
A	1	T9.1
B	4	T21.1; T23.1; T113b.1; T306.11
D	1	T411.1
E	1	T411.10
F	1	T415.1
G	1	T424.1
H	1	T522.13
R	1	T314.1
S	1	T317.2
U	1	T525.1
AA	2	T602.3 T606.3
AB	2	T603.2; T604.3;
AC	1	T605.3
AD	1	T611.4
AE	1	T605.5
AF	1	T605.7
AG	1	T606.4
AF	1	T606.8

CONEGLIANO-VALDOBBIADENE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
P1	1	P301.4
P2	1	P301.16
P3	1	P293.1
P4	1	S47
P5	1	B125.1
P6	8	P227.11; X39.14; P304.8; P303.6; X20.13; X22b.4; S43;S44
P7	1	P148.1
P8	1	B125.6
P9	1	B217.2
P10	1	B125.5
P11	5	P173.3; B173.1; C261.1; P225.3; X36.4
P12	1	B169.12
P13	2	S41; S45
P14	1	P304.13
P15	1	P158.4
P16	1	P304.6
P17	1	P304.5
P18	1	P283.4
P19	1	P234.15
P20	1	P304.3
P21	1	P301.3
P22	1	P138.4
P23	1	S43
P24	1	P254.10
P25	1	P254.16
P26	1	P304.2
P27	1	P254.1
P28	1	P304.1
P29	1	B223.8
P30	1	P304.4
P32	1	B125.3
P33	1	P234.5
P34	1	P304.11
P35	1	B173.4
P36	1	B197.1
P37	1	B173.2
P38	1	P219.1

PIAVE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
R101	1	R8.3
R102	1	R6.1
R103	1	R5.2
R104	1	R8.2
R105	1	R7.1
R106	1	R8.7
R107	1	R12.2
R108	1	R11.1
R109	1	R12.1
R110	1	R12.3
R111	1	R14.4
R112	1	R14.5
R113	1	R14.7
R114	1	R15.1
R115	1	R15.2
R116	1	R15.5
R117	1	R16.1
R118	1	R16.2
R119	1	R31.6
R120	1	R17.2
R121	1	R15.7
R122	1	R14.1
R123	1	R14.2
R124	1	R14.3
R125	1	R17.1
R126	1	R32.2
R127	1	R11.3
R128	1	R15.3
R129	1	R35.1
R130	1	R31.4
R131	1	R8.6
R132	1	R14.6
R133	1	R6.7
R134	1	R31.3
R135	1	R15.6

PIAVE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
R136	1	R35.4
R137	1	R31.2
R139	1	R104.5
R140	1	R107.3
R141	1	R107.4
R142	1	R104.2
R143	1	R130.2
R144	1	R130.1
R145	1	R130.4
R146	1	R157.1
R148	1	R146.2
R150	1	R153.4
R151	1	R150.2
R152	1	R153.2
R153	1	R150.3
R154	1	R153.1
R155	1	R150.1
R156	1	R101.1
R157	1	R100.1
R158	1	R117.1
R159	1	R105.2
R160	1	R105.5
R161	1	R151.2
R162	1	R104.4
R163	1	R111.1
R164	1	R139.2
R165	1	R139.3
R166	1	R139.4
R167	1	R137.2
R168	1	R128.1
R169	1	R143.2
R170	1	R143.1
R171	1	R154.1
R172	1	R154.4
R173	1	R105.3

PIAVE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
R174	1	R119.5
R175	1	R146.4
R176	1	R146.5
R177	1	R115.3
R178	1	R115.5
R179	1	R116.1
R180	1	R116.3
R181	1	R133.4
R182	1	R146.1
R183	1	R116.5
R184	1	R119.2
R185	1	R133.5
R186	1	R150.4
R187	1	R150.5
R188	1	R152.1
R189	1	R103.1
R190	1	R138.5
R191	1	R107.5
R192	1	R138.4
R193	1	R152.4
R194	1	R152.5
R195	1	R136.3
R196	1	R136.5
R197	1	R131.4
R198	1	R131.3
R199	1	R131.2
R200	1	R106.3
R201	1	R106.2
R202	1	R153.5
R203	1	R101.3
R204	1	R101.4
R205	1	R101.2
R206	1	R101.5
R207	1	R144.3
R208	1	R144.1

PIAVE		
mtDNA profiles code	Samples containing the profile	Isolate name for each sample
R209	1	R107.1
R210	1	R103.3
R211	1	R103.5
R212	1	R126.1
R213	1	R113.3
R214	1	R113.2
R215	1	R103.4
R216	1	R157.2
R217	1	R146.3
R218	1	R135.3
R219	1	R135.2
R220	1	R102.2
R221	1	R102.1
R222	1	R119.1
R223	1	R117.5
R224	1	R133.3
R225	1	R157.3
R226	1	R119.3
R227	1	R149.1
R228	1	R102.3
R229	1	R110.1
R230	1	R151.1
R231	1	R120.2
R232 (P6)	1	R155.3
R233 (P36)	3	R110.4; R132.5; R104.4

Indeed 132 different profiles were obtained analyzing strains selected in Piave (Raboso wine) area, while 37 and 17 were found in Conegliano-Valdobbiadene (Prosecco wine) and Lison-Pramaggiore (Lison wine) sites.

Moreover 3 profiles for Lison (B, AA and AB profiles) and Prosecco (P6, P11 and P13) were found in samples coming from different vineyards, while mtDNA profiles isolated of Raboso strains are collected each one from only one sample.

Two Prosecco profiles, the P6 and P36, were found also in Raboso area in 4 different samples, R155.3 (P6 profile), R110.4, R132.5 and R104.4 (P36 profiles).

Strains profile comparison is reported in the dendrogram in Figure 3.2.1. On the basis of mtDNA band similarities, the different profiles were divided into 16 principal clusters that are represented with a cone-shaped form in the Figure 3.2.19. For each cluster, strain names are reported on the right side. Commercial strains are in bold font underlined, while whole-genome sequenced commercial strains are in red bold font.

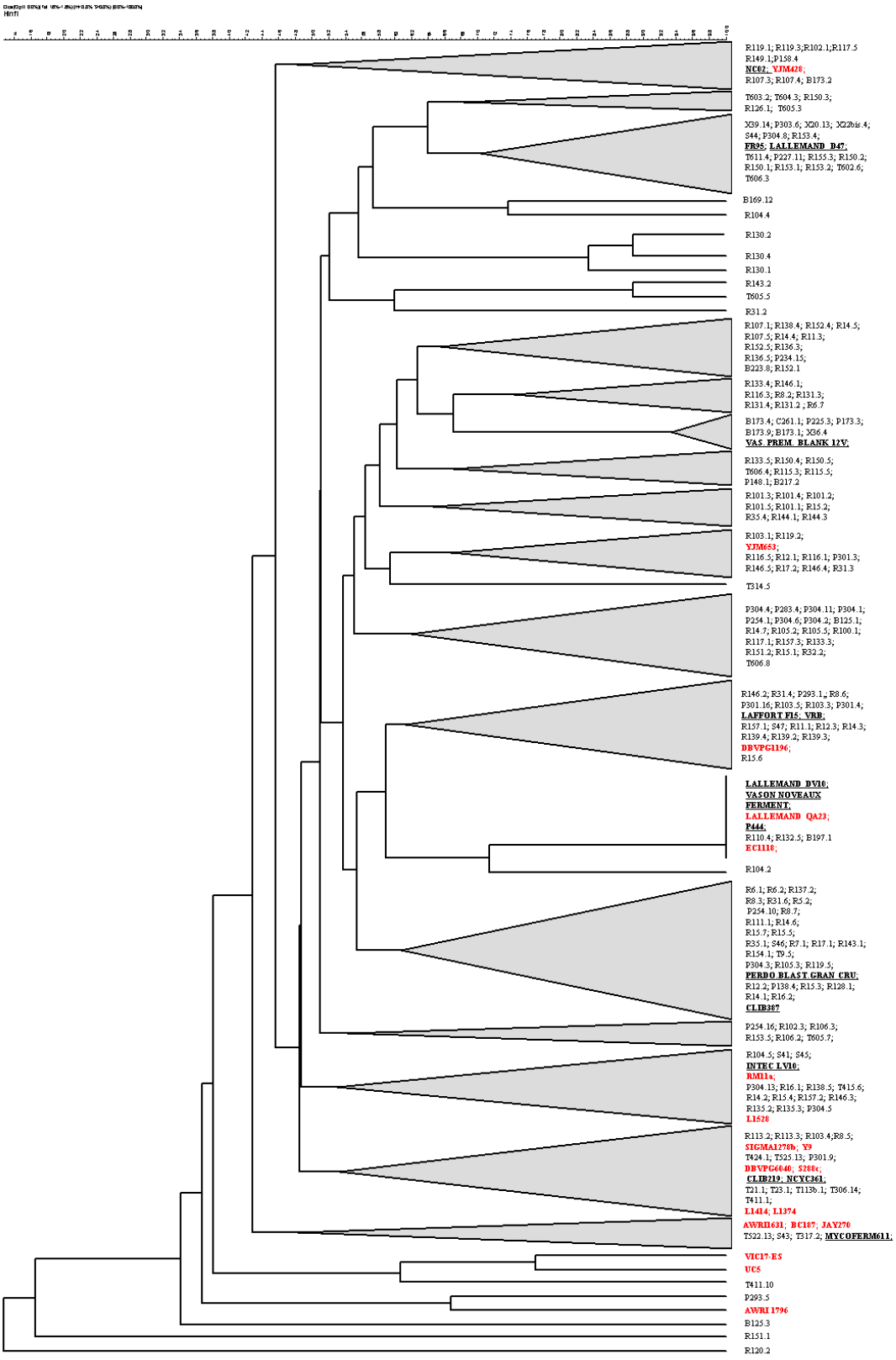


Figure 3.3.1 Similarity relationships among the strains isolated from the three winemaking on the basis of mtDNA analysis.

No cluster contained all the strains coming from the same Winemaking area, but the profile are mixed all over the dendrogram. Moreover there is not a clear separation between strains with technological relevance form those isolated from different environment without important technological traits.

The first cluster, starting from the upper side, grouped 9 different strains, 7 detected in Raboso area, corresponding to the 78% of the total, and 2 from Prosecc (the 22%). There are also a commercial strain, NC02, selected on oak exudates, and the sequenced clinical strain YJM428.

In the second cluster there are 4 strains, 2 detected in Lison area and 2 coming from Raboso. The analysis point out the 100% of similarity between the profile R212 detected in Raboso strains, and the AC profile found in Lison sites.

The third cluster grouped 10 strains, 6 isolated in Raboso, corresponding to the 60% of the total, 2 in Prosecco (20%) and 2 in Lison (20%) areas. The P6 profile is identical to the profile obtained from the commercial strain Laffort F15, while the R150 and the AD profiles selected on Raboso and Lison grape bunches respectively have 100% of similarity with the commercial strain Lallemand D47. Concerning the fourth cluster, 13 strains are present of which 11 selected in Raboso area, the 85% of the total, and 2 isolated from Prosecco (the 15%). Moreover, the profiles P29 and R188 are identical. In the fifth cluster there are 8 different strains all selected in the Raboso area. In the following cluster (the sixth), 2 strains, profiles P35 and P11, selected in the Prosecco area, was found. In particular the profile P11 has 100% of similarity with the commercial wine strain Vason Premium Blank 12V. In the seventh cluster there are 8 strains, 5 coming from Raboso, the 63%, 2 from Prosecco (25%) and 1 from Lison area (12%). The eighth cluster point out the presence of 9 different strains, all selected in the Raboso area. In the ninth cluster there are 10 strains selected in Raboso area that have an high profile similarity with the sequenced clinical strain YJM653.

The following cluster grouped 19 different strains, 11 detected in Raboso area, that correspond to the 58% on the total, 7 from Prosecco (37%) and only 1 Lison strain (5%). In the eleventh cluster 17 strains are present, 13 selected in Raboso area, corresponding to the (76,5%), and 4 different strains collected from Prosecco. One of these strains (profile P28)

have the 100% of similarity with the commercial wine strain VRB, that is the most common commercial starter used in winemaking in the Raboso area.

Concerning the strains that are not grouped in the 16 main clusters, there are 3 commercial strains (Lallemand DV10, P444 and Vason Noveaux Ferments) and 2 sequenced ones (Lallemand QA23 and EC1118) that have the 100% of similarity. Therefore the 2 strains selected in Raboso area and one selected from Prosecco (P36 profile) correspond to commercial wine strain.

The cluster 12 is the largest, with 28 total strains detected. The 82% of the strains were detected in Raboso area, while 11% and 7% were achieved from Prosecco and Lison respectively. In particular the profile R107 has the 100% of similarity with the commercial wine strain Perdomini Blastosel GranCru.

The following cluster contains 6 strains, 67% selected in the Raboso area, while 16,5% are Prosecco strains and 16,5% were isolated from Lison area.

The cluster 14 grouped 13 different strains, 8 detected in Raboso area (the 61,5%), 4 in Prosecco (30,8%) and only 1 (6,7%) in the Lison area. One Prosecco profile, the P13, has 100% of similarity with the commercial wine strain Intec LV10. In this cluster the sequenced strain RM11a is also present.

In the next two cluster, 7 strains are present. In the cluster 15 the 57% of the total strains were detected in Raboso area, while the 43% in Prosecco one. Two commercial strains, Clib219 and NCYC361, and 6 sequenced strains (Sigma1278b, DBVPG6040, S288c, Y9, L1414 and L1374) are also present. So in this cluster there is the 46% of the total sequenced strains considered in the survey.

The last cluster (16) 3 strains are grouped, 2 selected in Lison and one in Prosecco areas. In this cluster 3 sequenced strains (AWRI1631, BC187 and JAY270) and one commercial strain, Mycoferm611, are present. In particular this last strain has 100% of similarity with the H profile detected in Lison area.

3.3.2 Microsatellites analysis

3.3.2.1 Recovery of *S. cerevisiae* strains

The 202 autochthonous yeasts were collected in three different wine areas (DOCG Prosecco of Conegliano Valdobbiadene, DOC Piave and DOCG Lison-Pramaggiore) located in the Veneto Region (north-east Italy). Samples collection was performed in a total of 162 wineries collecting 97 grapes of Glera variety in the Prosecco area, 20 of Raboso Piave variety in the Piave DOC and 45 of Tocai Italico variety in the Lison-Pramaggiore area during the 2004–2010 pre-harvest period. All the isolates were analyzed by their mtDNA RFLP (HinfI) and a pattern profile was attributed to each isolate. When the same profile was found in more than one sample, one strain from each sample was taken into account.

In order to better understand yeasts evolution, 28 commercial wine strains, including 10 strains which sequence is completely available, 2 beer strains (NCYC361 and Clib382), 2 clinical isolates (YJM428 and YJM653), 1 bread (6662), 1 laboratory (S288c), 1 oak (NC02), one sake (UC5) and one ragi (Y9) strains, were analyzed (see materials and methods for details). discorso repliche e mettere frasetta tocai resulting a total of 184 different profiles.

3.3.2.2 Genotypes and strains biodiversity

The microsatellites typing has revealed 191 different genotypes out of the 184 different profiles recovered with the mtDNA analysis, with six strains equal with others in the survey. Only one of them has shown 100% of equal repeats number in all 18 loci with a commercial wine strain. The 18 microsatellite loci recorded from 7 to 33 different alleles per locus. SCAAT1, SCYOR267c, C5 and C4 displayed the highest number of alleles in the global population, which was expected given the length of these repeated motifs and their selection for high polymorphism (Legras et al. 2005). The loci C9 and YKL172w showed the lowest polymorphism rate with respectively 11 and 7 alleles. The number of alleles per locus per strain varied from two to four. The bread strain exhibited four alleles in

one locus and three in four loci. In contrast to the results obtained for the bread strain, 96,5% of wine isolates have presented two alleles maximum for all loci, suggesting a diploid state for most wine yeast strains. Six strains had three alleles in the C4 locus and one strains in the C8 locus. In total 32% of the isolates were homozygous for all loci. The neighbour-joining tree calculated from the Dc chord distance matrix for all pairs of strains is reported in Figure 3.3.2.

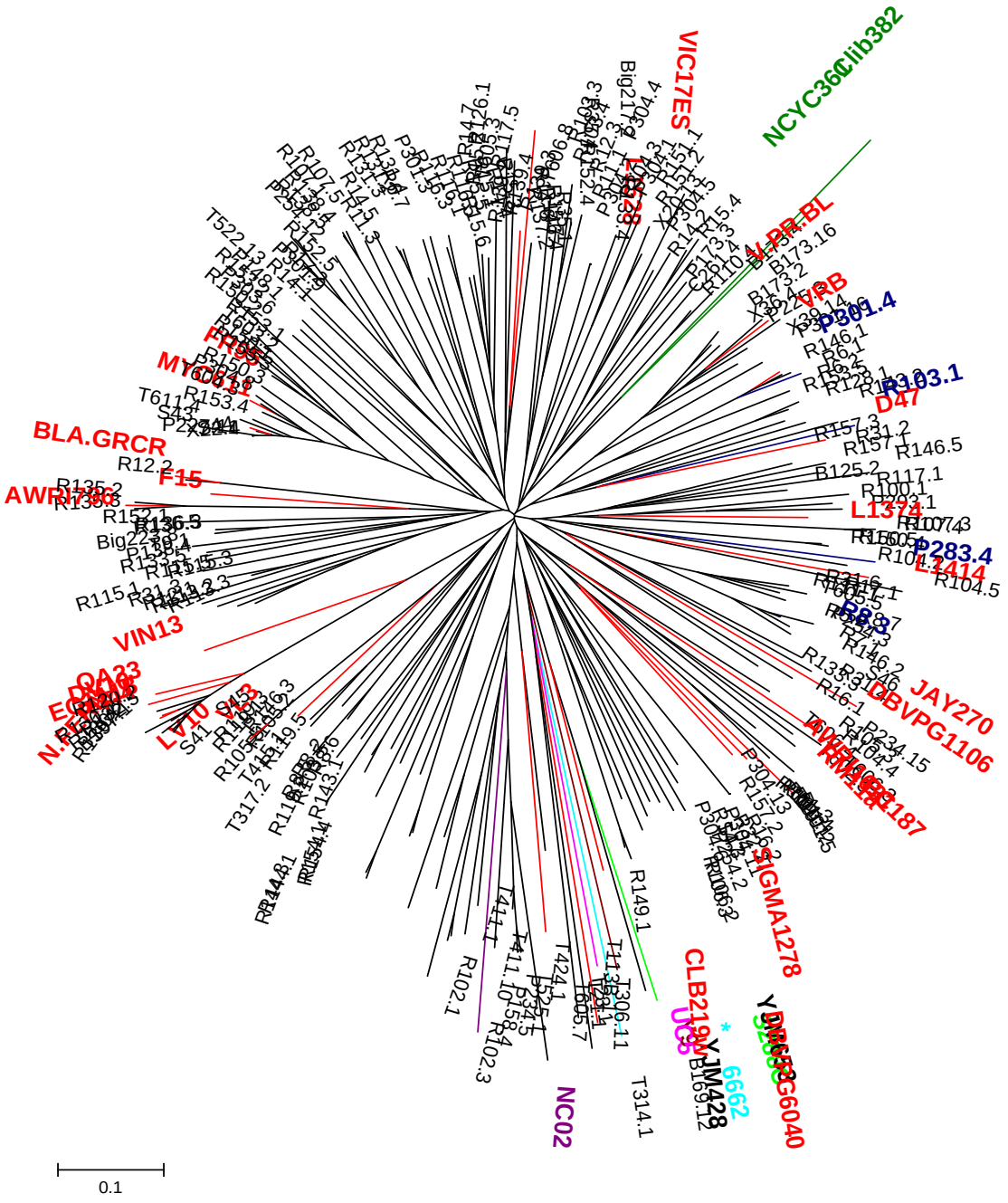


Figure 3.3.2 Neighbour-joining tree showing the clustering of 239 yeast strains isolated from different sources. The tree was constructed from the chord distance between strains based on the polymorphism at 18 loci and is rooted according to the midpoint method. Branches are coloured according to the substrate from which strains have been isolated. All the strains coming from our selection programs are in black (four of them, that are sequenced, are in dark blue). Colour code of commercial strains (in bold font): wine, red; bread, light blue; beer, dark green; sake from Japan, pink; ragi strain, brown; oak tree from America, violet; clinical isolates, black; laboratory strains, light green.

The tree showed a clear cluster of strains coming from substrate different to wine one, like bread, ragi, sake, laboratory, clinical isolates and a strain isolated from fermenting fruit juice (DBVPG6040) All of the commercial wine strains are equally spread all around the tree.

There is also a cluster made up of six commercial wine strains, EC1118, P444, DV10, QA23, VIN13 and NOVEAUX FERMENTS that are related to five yeast strains isolated from Raboso Piave grape variety. One strain coming from the Prosecco variety has the same repeats number as the strain "NOVEAUX FERMENTS" at all the loci that we analyzed.

Eleven strains coming from Raboso, six isolated in Prosecco and one in Tocai varieties are related to the two yeasts isolated from beer, namely strain NCYC361 isolated in Ireland from and Clib382 isolated in Japan.

There is an important cluster formed by ten strains isolated in the Lison area and the NC02 strain. This strain was isolated from oak tree exudates in the Smoky Mountains of North Caroline in 2003. So this cluster can be considered the most particular one because, despite the other autochthonous strains isolated from grape skin, they are poorly related with the commercial wine strains considered in the survey. In particular they have a weak correlation with the Asian vine (Vitis amurensis) strain Clib219w.

All the other autochthonous strains isolated in the three Veneto areas are related to wine commercial yeasts considered in the surveys, including the four sequenced ones (P301.4, P283.4, R8.3 and R103.1).

The reliability of the tree topologies was assayed through a jackknife procedure taking out from the analysis a locus at a time from the sample set. A consensus tree was obtained through the distant matrix re-elaborated with the neighbour joining clustering method using the Mega program (data no show).

The data obtained show the high robustness of the tree branch that confirm the effectiveness of microsatellites method using 18 loci.

3.3.2.3 Yeast population analysis

For population analysis our 202 autochthonous yeast strains were divided into six different populations depending on their geographical isolation area. Therefore the yeasts isolated in the Prosecco area were subdivided into two pseudopopulations (named ProA and ProB), the Piave ones into three (RabA, RabB and RabC) and the Lison ones into two subpopulations (TocA and TocB) (figure 3.3.3).

Figure 3.3.3 Ecotipical yeasts divided into sub- population depending on their geographical isolation area.

A principal coordinate analysis (PCA) of molecular variance was conducted to understand the genetic structure considering distance among major groups. PCA is a multivariate technique that allows to find and plot the major patterns within a multivariate data set (e.g., multiple loci and multiple samples). PCA is a process by which the major axes of variation are located within a multidimensional data set. Each successive axis explains proportionately less of the total variation, such that when there are distinct groups, the first 2 or 3 axes will typically reveal most of the separation among them.

In the analysis we have also considered the populations formed by strains coming from other substrates than the wine ones, like sake, ragi, beer, bread, laboratory and clinical isolates, and for each one we have attribute a distinct population.

The results are reported in the figure 3.3.4.

The plane presented is built from the two first axis which explain 43,6% of the total variance. There is a distinct cluster of strains, in the right upper part of the plane, which came mainly from the population TocA and TocB, and is very close with the oak strain NC02. Another group of strains is located in the top left of the plane and is constituted by ones closely related to the commercial strain Myc611, that is a commercial wine strain used in the Lison-Pramaggiore area. All the other populations in the center of the plane with commercial wine strains and the strains isolated from the different substrates.

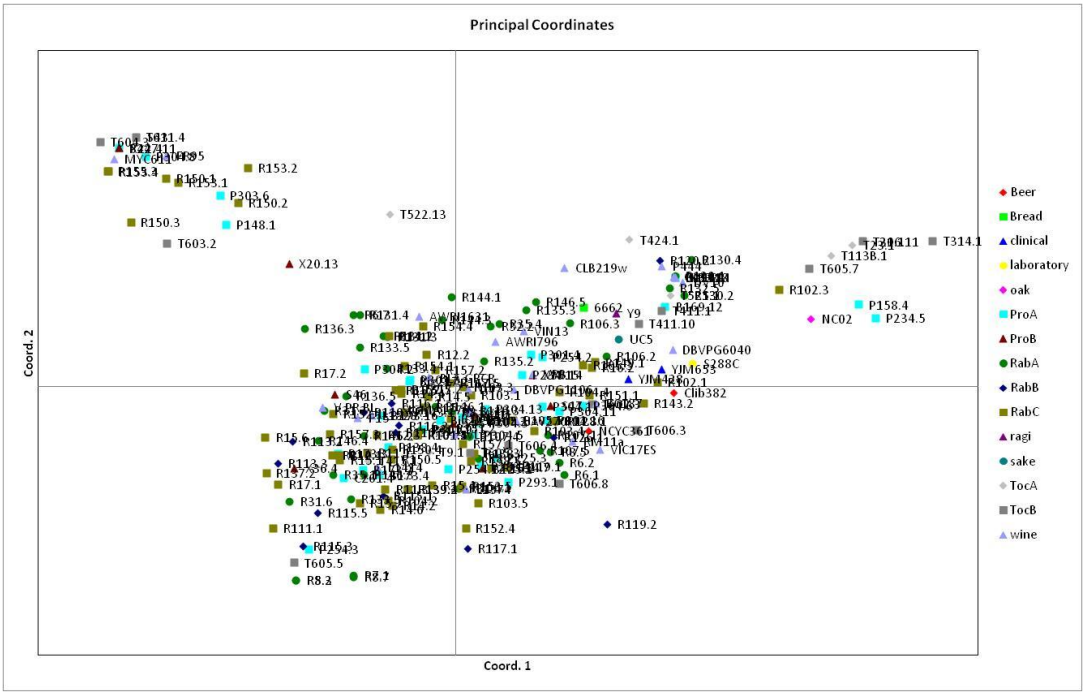
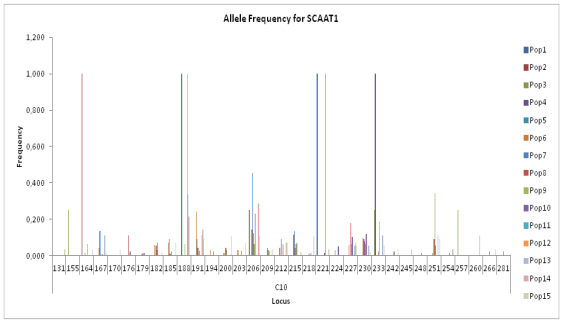
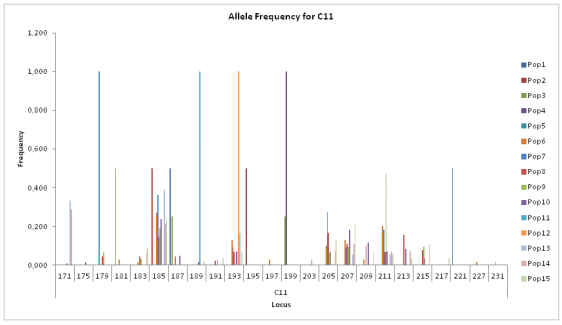


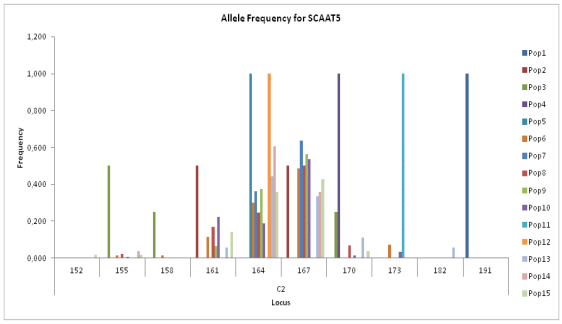
Figure 3.3.4 Principal Coordinates Analysis based on genetic distance. Coord.1 = 22.79%, Coord.2 = 20.81%.

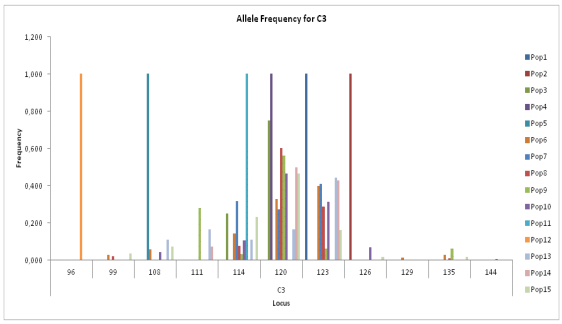
Alleles frequency for the different populations was also calculated for each loci. The histograms reported in figure 3.3.5 represent the distribution of the alleles frequencies for each locus considered in the survey. The low mean allelic frequencies of loci SCAAT1

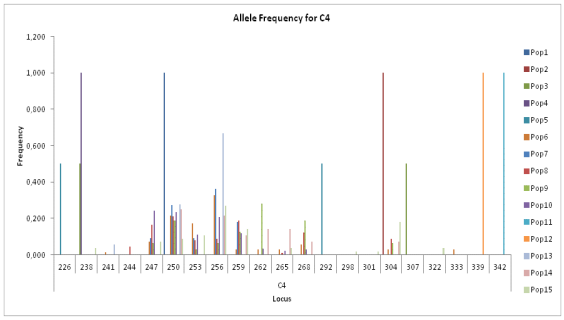
confirms that it is the most polymorphic locus with C11 and C4. The loci C9, YKL172w and YLR are the less ones.

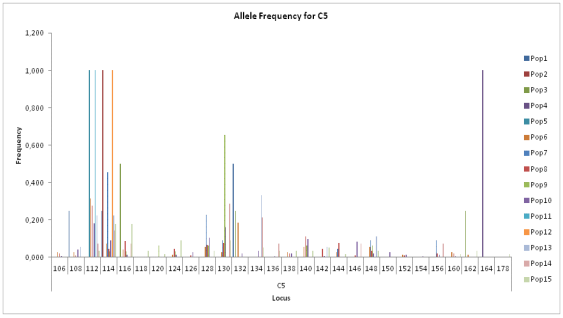


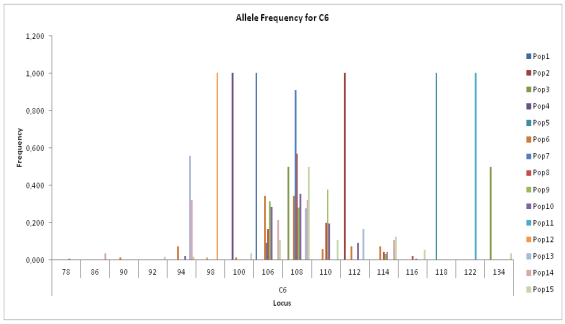


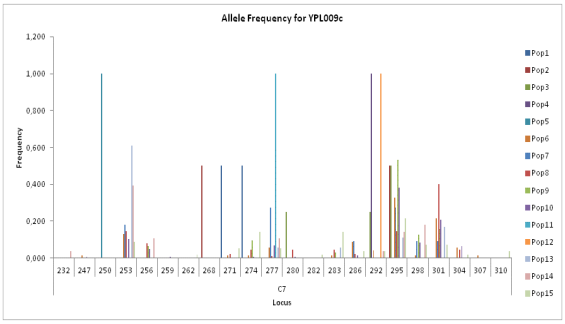


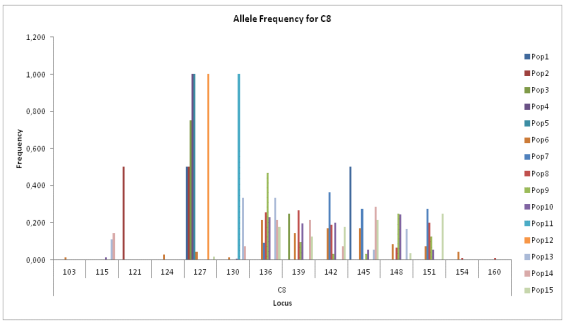











Allele Frequency for C8
Frequency
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1,000
0,800
0,600
0,400
0,200
0,000
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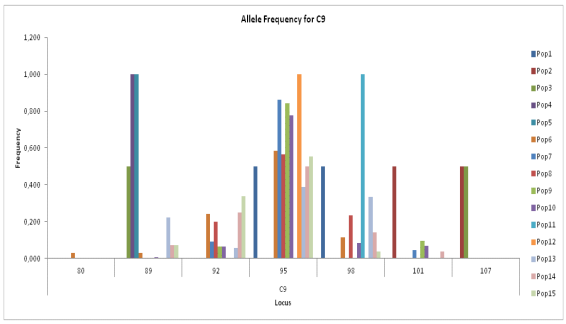
C8
Locus
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15


Allele Frequency for C9
Frequency
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0,800
0,600
0,400
0,200
0,000
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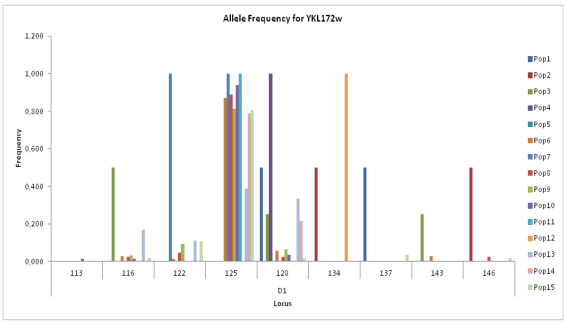
C9
Locus
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15


Allele Frequency for YKL172w
Frequency
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0,600
0,400
0,200
0,000
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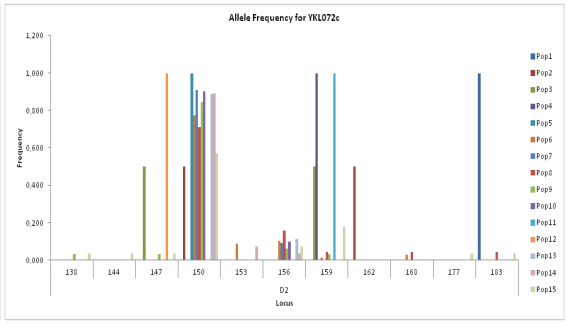
D1
Locus
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15


Allele Frequency for YKL072c
Frequency
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D2
Locus
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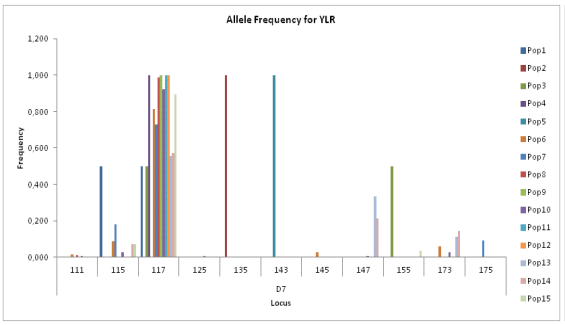
Allele Frequency for YLR
Frequency
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0,600
0,400
0,200
0,000
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D7
Locus
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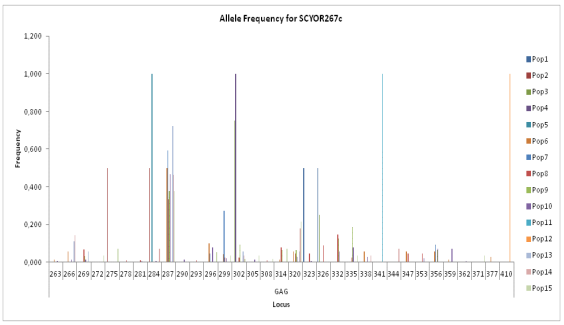
Allele Frequency for SCYOR267c
Frequency
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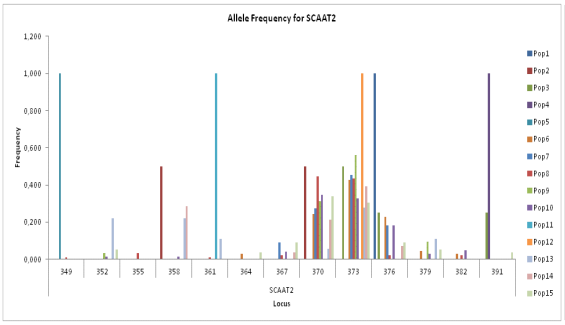
0,600
0,400
0,200
0,000
263 266 269 272 275 278 281 284 287 290 293 296 299 302 305 308 314 320 323 326 332 335 338 341 344 347 353 356 359 362 371 377 410
GAG
Locus
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15


Allele Frequency for SCAAT2
Frequency
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0,600
0,400
0,200
0,000
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SCAAT2
Locus
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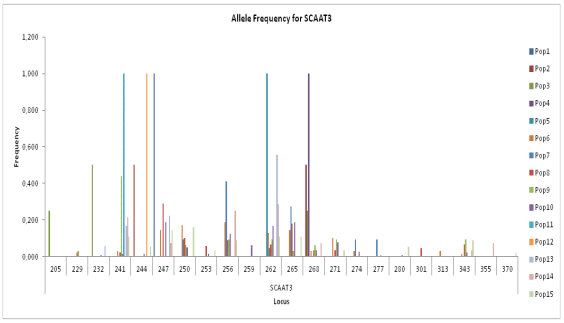
Allele Frequency for SCAAT3
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0,600
0,400
0,200
0,000
205 229 232 241 244 247 250 253 256 259 262 265 268 271 274 277 280 301 313 343 355 370
SCAAT3
Locus
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15

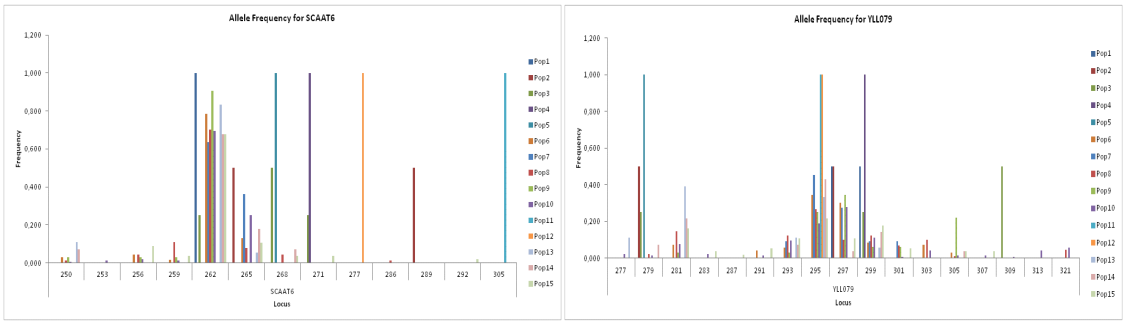


Figure 3.3.5 Alleles frequency distribution through the populations. Legend: Pop1, beer; Pop2, bread; Pop3, clinical isolates; Pop4, laboratory; Pop5, oak; Pop6, ProA; Pop7, ProB; Pop8, RabA; Pop9, RabB; Pop10, RabC; Pop11, ragi; Pop12, sake; Pop13, TocA; Pop14, TocB; Pop15, wine commercial strains.

We then try to analyse this diversity at a population scale. In other to reduce the effect of low sampling on genetic distances, we have not taken in account groups of strain coming from substrates different from wine, for which we had genotyped too few individuals (less than 9), a population comparison was evaluated considering the matrix calculated on the pair wise populations Fst values of ProA, ProB, RabA, RabB, RabC, TocA, TocB and commercial wine strains (Wine population). This statistic provides a measure of the genetic differentiation between subpopulations. That is, the proportion of the total genetic diversity (heterozygosity) that is distributed among the subpopulations. The neighbour-joining tree calculated from the Fst distance matrix for all pair wise populations is reported in Figure 3.3.6.

The population tree reflect the geographical distribution of the populations, in fact the most related RabA, RabB, RabC and ProB are close in the Veneto area while the populations ProA and TocA are most far from the others.

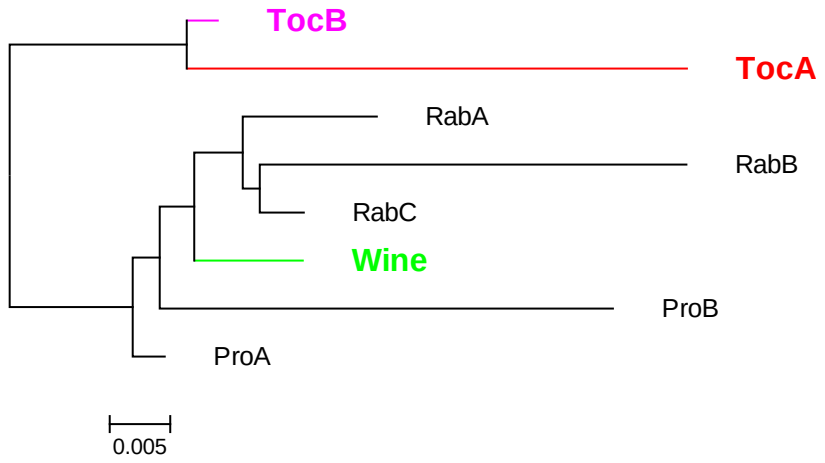


Figure 3.3.6 Neighbour-joining tree showing the clustering of the 8 populations considered. The tree was constructed from the Fst genetic distance matrix for all pair wise populations and is rooted according to the midpoint method.

The tree shows a clear difference between populations TocA and TocB and the others coming from the different Veneto areas. TocA populations show the highest genetic diversity of all populations taken into account, displaying that strains coming from this area are special, in fact they are related to the oak strain NC02 coming from the North Carolina. Moreover, the two subpopulations TocA and TocB are not related with commercial wine strains that constitute the Wine population, reinforcing the idea that the strains coming from these areas have a special geographic or genetic barrier that close them off from the other strains influence. Indeed, despite the geographical closeness the populations TocB and RabB show a high genetic difference between them and the most related population with the TocA and TocB is the ProA, that are very far from them.

The tree displays the high genetic correlation between ProA and ProB, RabA, RabB and RabC populations with the Wine one. The strains coming from these areas are probably much closer from the wine yeast populations from which commercial strains have been isolated. Another possibility would be that these populations contains variants of industrial strains. This could be explained by the widely used of commercially available yeast starters in these winemaking areas that are introduced every year in large quantities in the ecosystem surrounding a winery together with liquid and solid wine-making residues.

In order to test this possibility, we estimated the geneflow between each populations from Fst value. Indeed gene flow between population can be inferred from Fst following equation:

$$Nm = \frac{(1 - Fst)}{4 * Fst}$$

Results presented in table 3.3.1 shows that higher gene flows are encountered between populations of the same area (TocA and B, RabA and C, Pro A and B). However some high gene flow can be observed between populations: ProA and RabA, ProA and rabC, and also ProA and TocB. Interestingly we could also detect high gene flow between the group of wine strains and the populations ProA, RabA and RabC. As these high gene flow rates are detected only in some populations we can make the hypothesis that the they correspond indeed to the introduction of industrial wine strains in these ecosystems. Another consequence of the gene flow caused by industrial wine strains is that it may reduces differences between populations: the high gene flow between wine strains and TocB may also explain the high geneflow between TocB and ProA.

Table 3.3.1 Geneflow between each population from Fst value

	ProB	rabA	rabB	rabC	TocA	TocB	wine
ProA	9.32	9.91	3.81	22.84	3.27	8.49	14.02
ProB		3.98	1.96	6.49	1.74	4.71	7.47
rabA			3.73	12.12	2.04	4.42	13.29
rabB				4.84	1.41	3.13	4.03
rabC					2.14	4.79	7.92
TocA						41.02	2.56
TocB							7.32

The number of heterozygote loci per strain were then compared for the three main populations: the Pro (strains coming from ProA and ProB subpopulations), Rab (strains coming from RabA, RabB and RabC subpopulations) and Toc population (with strains coming from TocA and TocB areas). The relative frequencies are reported in figure 3.3.7.

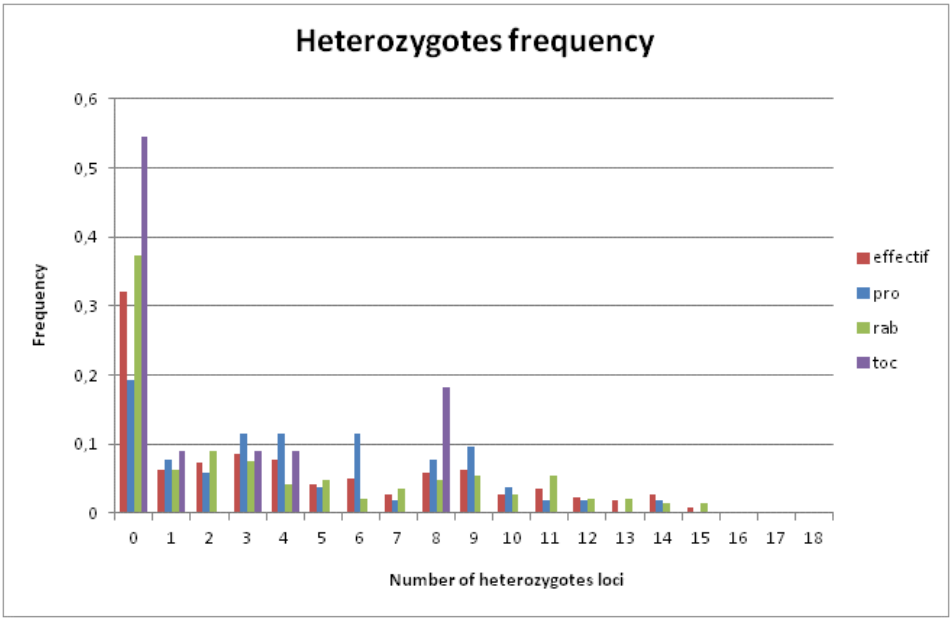


Figure 3.3.7 Heterozygous frequencies in the three populations of Pro, Rab and Toc, and in the total (effective one).

The three populations present different pattern of heterozygosity suggesting different rate of outcrossing. The highest homozygous frequency, was found in the Toc population. This population has at most 8 heterozygous loci, despite the other two populations considered in the survey that have up to 14-15 heterozygous loci.

We estimated the selfing rate from this data, with two methods: the first methods rely on the F statistics as Fis is related to the selfing rate according to the relation:

$$F_{is} = 2 * \frac{s}{1+s}$$

and the second method uses the multilocus heterozygosity structure and is implemented in the software RMES (David et al. 2007). The results are given in table. (N°X) . The estimation obtained for selfing rates in both cases are in quite good agreement for both method, They show quite clearly that Toc populations present a higher selfing rate. Only the selfing rate inferred for population RabA by RMES reaches the level of TocA and B, however the estimate obtained from Fis for RabA is clearly lower than those estimated for TocA and B. In contrast to TocA and B, the estimates obtained for the group of wine strains are the lowest (0.72 and 0.74)

These results confirm that the Toc populations are clearly the most differentiated from other populations, with a different life style as indicated by the higher higher selfing rate. However a significant geneflow exist between Toc population and ProA or industrial wine strains.

3.3.4 Comparison between mtDNA-RFLP and microsatellites analyses

The two methods considered in this survey, point out the different level of biodiversity obtained for the three winemaking regions.

Indeed, the microsatellites typing has revealed 191 different genotypes out of the 184 different profiles recovered with the mtDNA analysis. Only one of them has shown 100% of equal repeats number in all 18 loci with a commercial wine strain. By the microsatellites analysis a clear cluster with non-wine strains, like laboratory strains S228c and Sigma1278 and clinical strains YJM653 and YJM428, was achieved. The mitochondrial DNA analysis does not reflects the territorial origin of strains that has got a clear influence for the microsatellites analysis.

The most important difference is obtained with strains selected in Lison area. The mtDNA analysis does not group the major part of these strains together, but they are all spread along the dendrogram. With the microsatellites survey, on the contrary, there is a clear cluster of Lison strains that have a high polymorphism similarity with the oak strain NC02, that was not highlighted with the mtDNA analysis.

Some strains that have different patterns in the mtDNA analysis, have the same profile for the microsatellites survey, like the R164, R165 and R166 profiles detected for Raboso strains, and also the R203, R204, R205 and R206 selected in the same area. By the microsatellites analysis only 2 different profiles were obtained for these patterns achieved only in two different samples. This result points out that, in this case, the mt DNA analysis is more powerful in strain discrimination. This finding is not a general results as the number of genetic profile found with microsatellite is higher that the one for mtDNA RFLP analysis.

Moreover, only the B197.1 (profile P36) isolated in Prosecco area has shown identical profile with a commercial wine strain (Vason Noveaux Ferments) out of the two other strains selected in Raboso sites.

Similar finding between the two methods were observed, as well. Indeed the sequenced strains EC1118, QA23 and the commercial strains DV10 and P444, are grouped together in both of the analyses. Moreover also AWRI1631, BC187 and JAY270, that are sequenced strains, are very close in both the cluster analyses.

The mitochondrial DNA analysis seems to be a less powerful method to point out the presence of fine genotype difference between strains coming from the same geographic area, which is obtained with the microsatellites analysis.

The mtDNA analysis can be a good method to discriminate natural strains from commercial ones, but revealed to be less powerful to understand phylogenetic correlation and populations dynamics between geographically-related natural strains.

4. Focus on biodiversity of technological yeasts in vineyard: importance of ecological niches on grapevine

4.1 Introduction

Despite the great numbers of indigenous strains constantly recovered from the environment as reported in literature (Shuller, Dequin 2005), grape bunches sampling conducted in the DOC Lison-Pramaggiore did not give appreciable results in terms of biodiversity.

During the last years several studies pointed out the presence of yeasts belonging tothe *Saccharomyces* places different to grapes. The new species *Saccharomyces arboricolus* (Shi-An Wang, Feng-Yan Bai, 2008) was isolated from China oak bark, like the NC02 commercial strain isolated in North Caroline in 2003 (REF). Therefore bark could be a natural niche to preserve yeasts biodiversity throughout the year, while grapes are present only for few months and are subjected to pesticide treatments. Moreover, grapes damaged during harvest could be a nutrient source for yeasts present on bark that could then develop easier.

For all these reasons, a new sampling strategy was carried out in the DOC Lison-Pramaggiore area in post-harvest period, investigating *Saccharomyces* biodiversity on vineyard bark portions.

4.2 Materials and methods

4.2.1 Bark portion isolation

Sampling

The sampling was carried out by collecting bark portions from Tocai Italico vines in the DOC Lison-Pramaggiore area in the post-harvest period (October-November).
Bark portions were collected scratching vine stock with a spatula sterilized each time with denatured alcohol to avoid any kind of contamination.
Samples collected in the vineyard (in quantity to fill a 50 mL Falcon) were transferred to the laboratory where were put into 100 ml-Erlenmeyer flasks sealed with silicon cap and supplied with bowed glass pipettes and filled with 100 ml of synthetic must (Delfini, 1995). 100µl of sulphur dioxide at 5% v/v and 10mL of vaseline oil, to prevent apiculate yeasts and moulds development, were added.
Fermentation process was followed monitoring the weight loss daily. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

Yeasts isolation

After fermentation, 1 mL were took from each flasks, and 6 serial dilutions (1:10) were performed on Ringer solution. 100 µl of the last three dilutions were plated on WL medium.
After 5 days at 25°C, colonies count was performed and 10 colonies with *Saccharomyces*-like morphology were randomly considered.

4.2.2 Culture media and growth condition

Media

Wallerstein Laboratory (WL medium) nutrient agar (Green & Gray, 1950).

Suspend 75 g WL nutrient agar (Oxoid) in a liter of distilled water.

Sterilize by autoclaving at 121 ° C for 15 minutes.

YM agar medium

- 3 g L^{-1} yeast extract (Oxoid);
- 3 g L^{-1} malt extract (Oxoid);
- 5 g L^{-1} vegetatone peptone (DIFCO);
- 10 g L^{-1} glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YM solid agar medium

- 3 g L^{-1} yeast extract (Oxoid);
- 3 g L^{-1} malt extract (Oxoid);
- 5 g L^{-1} vegetatone peptone (DIFCO);
- 10 g L^{-1} glucose (PROLABO)
- 16 g L^{-1} Bacto Agar (DIFCO).

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

YPD (Yeast Extract/Peptone/Dextrose)

10 g L^{-1} yeast extract (OXOID)

20 g L^{-1} vegetatone peptone (DIFCO)

20 g L^{-1} glucose (PROLABO)

Adjust to volume with distilled water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Synthetic nutrient medium (NSM) (Delfini, 1995)

Macronutrients

0,1 g L^{-1}	$CaCl_2$
0,1 g L^{-1}	NaCl
1 g L^{-1}	KH_2PO_4
0,5 g L^{-1}	$MgSO_4 \cdot 7H_2O$
3 g L^{-1}	tartaric acid

Micronutrients

0,2 mg L^{-1}	$NaMoO_4 \cdot 2H_2O$
0,4 mg L^{-1}	$ZnSO_4 \cdot 7H_2O$
0,5 g L^{-1}	H_3BO_3
0,04 mg L^{-1}	$CuSO_4 \cdot 5H_2O$
0,1 mg L^{-1}	KJ
0,4 mg L^{-1}	$FeCl_3 \cdot 6H_2O$
0,4 mg L^{-1}	$MnSO_4 \cdot H_2O$

Vitamins

400 μg L^{-1}	pyridoxine hydrochloride
400 μg L^{-1}	thiamine hydrochloride
2000 μg L^{-1}	Inosite
20 μg L^{-1}	Biotin
400 μg L^{-1}	Calcium pantothenate
400 μg L^{-1}	Nicotinic acid amide
200 μg L^{-1}	P-amino-benzoic acid

Variable components

0,3 g L^{-1}	$(NH_4)_2SO_4$
0,3 g L^{-1}	$(NH_4)_2HPO_4$
200 g L^{-1}	Glucose
0,2 g L^{-1}	Hydrolyzed Casein

Prepare the micronutrients and vitamins in a 100 times concentrated aqueous solution and use the 1%. Dissolve all components in distilled water, adjust the pH with KOH of the resulting solution to pH 3.2. Sterilize by autoclaving at 100 ° C for 5 min.

Solution

Ringer Solution for dilutions (1/4 *strenght*; Dept. of Health & Social Security, 1937).

Dissolve one tablet preparation (LAB M, International Diagnostics Group) in 500 ml of deionised water. Sterilize by autoclaving at 121 ° C for 15 minutes.

Growth conditions

The yeast strains were grown at 25 ° C, the liquid cultures, for fermentation inoculum, were subjected to agitation of 130 oscillations per minute.

4.2.3 Yeast isolates storage and purification

After the colonies determination of *Saccharomyces sensu stricto* group, by the multiplex PCR Sac18-Sac26, all the colonies confirmed belonging to the group were growth on liquid YPD medium for 24h at 25°C, then centrifuged and resuspended in 2 ml of a sterile solution composed of half YPD medium and 40% of glycerol. The vials were stored at -80°C.

4.2.4 DNA amplification

4.2.4.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 μL of sterile deionised water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

4.2.4.2 SAC26-SAC18 multiplex PCR

Saccharomyces sensu stricto colonies identification was performed by adopting the method developed by Nardi *et al*. 2006. The various components of the reaction mixture were used in the following final concentrations:

Table 4.2.1 PCR master mix composition

Primer SAC26F	0,2 μM
Primer SAC26R	0,2 μM
Primer SAC18SF	2 μM
Primer SAC18SR	2 μM
dNTPs (Amersham)	200 μM (each one)
Taq polimerasi (Promega)	0,02 U/μl
Buffer	1X
DNA	2 μl cellular suspension

Primers utilized are reported below (table 4.2.2).

Table 4.2.2 Primers for SAC26-SAC18 amplification

Name	Length	Sequence (5'-3')
SAC26F	22 nt	GAGAGGGCAACTTTGGGRCCGT
SAC26R	27 nt	ACCATTATGCCAGCATCCTTGACTTAC
SAC18F	23 nt	CTGCGAATGGCTCATTAAATCAG
SAC18R	25 nt	CCCTAACTTTCGTTCTTGATTAATG

The thermal protocol was the follows:

Initial incubation at 94°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 94°C for 15 s, annealing at 54°C for 30 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gels with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.
Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

4.2.5 Mitochondrial DNA analysis

4.2.5.1 Yeasts total DNA extraction

Yeasts coat obtained on YM agar medium, growing yeasts for 48 h at 25 °C, was resuspended in 1 ml of sterile water and then centrifuged at 14000 rpm for 3 minutes in an Eppendorf microcentrifuge. After fluid discarding, the cells were resuspended in 500 μl of a solution containing 50 mM Tris-HCl, 20 mM EDTA, pH 7.4 and transferred to a 2 ml Eppendorf containing 0.3 g of glass beads of 425-600 μm (Sigma) and vortex for 3 minutes. 50 μl of 10% SDS were then added to the samples that were incubated in a thermostatic bath at 65°C for 30 minutes. At the end 200 μl of potassium acetate 5M were added and the samples were left on ice for 30 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes. 600 μl of supernatant was transferred to an Eppendorf tube and 600 μl of cold isopropanol were added. The samples were kept at room temperature for 5 minutes, stirring by inversion and then centrifuged at 14000 rpm for 10 minutes. The supernatant was removed and 500 μl of 70% ethanol were added. After centrifugation at 14000 rpm for 10 minutes and the supernatant discarding, the pellet was dried for 1 h at 37 ° C. The samples were resuspended in 50 μl of sterile water, to which 1.5 μl (10 mg / ml) of RNase (Amersham Bioscience E70194Z) were added. The samples were left at room temperature for 15-20 minutes and finally stored at -20 °C.

4.2.5.2 Total DNA enzyme restriction

The total DNA digestions were performed in 15 µl of volumes reaction containing 10 U of *HinfI* enzyme (Fermentas) and 10 µl of extracted DNA. The reactions were performed at 37 °C for 2 h.

4.2.6 Yeasts species identification by ITS1-5,8S-ITS2 region amplification and RFLP

4.2.6.1 Sample preparation for DNA amplification

Yeast colonies (1–2mm diameter), grown for 1–3 days, were picked up with a sterile toothpick from YM plates and resuspended in 20 µL of sterile deionised water in 0.5mL tubes. Two microlitres of the suspension were used for PCR amplification.

4.2.6.2 ITS1-5,8S-ITS2 region amplification

The various components of the reaction mixture were used in the following final concentrations:

Table 4.2.3 PCR master mix composition

ITS1	2 µM
ITS4	2 µM
dNTPs (Amersham)	200 µM (each one)
Taq polimerasi (Promega)	0,02 U/µl
Buffer	1X
DNA	2 µl cellular suspension

Primers utilized are reported below (table 4.2.4).

Table 4.2.4 Primers for ITS1-ITS4 amplification

Name	Length	Sequence (5'-3')
ITS1	19 nt	**TCCGTAGGTGAACCTGCGG**
ITS4	20 nt	**TCCTCCGCTTATTGATATGC**

The thermal protocol was the follows:
Initial incubation at 95°C for 5 min to allow cell lysis and DNA denaturation, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at 53,5°C for 45 s and extension at 72°C for 90 s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1,2% agarose gels with 0,1 μg/ml of ethidium bromide. The running was performed with TBE 0,5X (44,5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) on a potential difference of 50-110 V.
Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

4.2.6.3 ITS1-4 RFLP analysis

The amplification products of the region ITS1-5,8S-ITS2 of rDNA were digested with enzymes HaeIII and MaeI (Amersham). The digestions were performed in 20 μl volumes reaction containing 10 U of enzymes and 10 μl of the amplified. The reactions were conducted at 37 ° C for 16 h.

4.2.7 Fermentation surveys on Synthetic Nutrient Medium (NSM) (Delfini, 1995)

4.2.7.1 Yeasts inoculum preparation

Yeasts were grown for 3 days on YM solid medium. The cultures obtained were used to inoculate 10 ml of YPD liquid medium. The tubes were left in incubation for 30 hours at 25 °C, moved to obtain a culture on stationary phase (approximately 10^7-10^8 cells/ml) measured by spectrophotometry (OD_{600} between 5 and 8).

4.2.7.2 Test preparation

Based on the OD of the respective pre-inoculation, for each strain the culture volumes to obtained a final OD_{600} of 0.5 (approximately 10^5 cells/ml) in 100 ml of medium at the beginning of fermentation, were calculated.

Each strain was inoculated in a 100 ml-Erlenmeyer flask sealed with silicon cap and supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). The advantage to use the synthetic must than the natural, for a first physiological assessment, is to enable a fully control of the development setting, and to facilitate significantly the daily growth monitoring operations.

The medium was sterilized by autoclaving at 100 ° C for 5 minutes.

Alcoholic fermentation development was controlled by measuring the weight loss daily from the beginning to the end of fermentation process. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

4.3 Results and discussion

4.3.1 Biodiversity on bark *versus* grape bunches

With the aim to find out a higher level of biodiversity, 22 bark portions were sampled in two vineyards. sampling was carried out in post-harvest period (October-November) in two different vineyards in the area of Motta di Livenza, to test sampling effectiveness. Small bark portions were collected by scratching vine stock with a spatula sterilized with ethanol.

Samples were transferred to the laboratory where were put into 100 ml-Erlenmeyer flasks closed with silicon caps supplied with bowed glass pipettes and filled with 100 ml of synthetic must (Delfini, 1995). 100µl of sulphur dioxide at 5% v/v was added to prevent development apiculate yeasts. Tenml of vaseline oil was layered on the liquid surface to prevent moulds development.

Fermentation process was followed by monitoring flask weight loss daily. The fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours. At the end of fermentation, suitable dilutions of fermented juice were plated on selective WL media. Indigenous yeasts were isolated and, by means of plate count, yeasts concentration in fermented musts was determined to be 10^6-10^7 CFU/ml. From each fermented sample, up to of 10 colonies showing *Saccharomyces sensu stricto* morphologiy were collected. Finally a total of 79 colonies with *Saccharomyces*-like morphology were isolated.

For unambiguous identification of the *Saccharomyces sensu stricto* yeast isolates, all the 79 colonies collected from the two samples were analyzed by Sac26-Sac18 multiplex PCR and a total of 43 colonies were confirmed to belong to the *Saccharomyces sensu stricto* group.

To obtain a strain-specific characterization of the isolates, mitochondrial DNA restriction fragment length polymorphisms analysis (mtDNA-RFLP) was performed. Restriction profiles obtained were compared by the GelCompareII (Applied Maths) software and the Dice similarity coefficient was used for matrix construction .

The analysis evidenced 7 different profiles from the 43 colonies isolated. Cluster analysis, including yeasts isolated from grape bunches in the Lison-Pramaggiore area and seventy

commercial enological strains, was performed. no similar pattern were found among strains coming from bunches and bark, and among strains fom bark and commercial ones.
The results achieved with the two different sampling methods are reported in table 4.3.1

Table 4.3.1 Comparison between grapes bunches and bark portions samplings.

	Total samples	**Samples containing *S.sensu stricto***	***S.sensu stricto* isolates**	**mtDNA profiles**	**N°profiles/ N°*S.sensu stricto***
Bunches of grapes	208	18 (8,6%)	195	17	1:11
Bark portions	22	4 (18%)	43	7	1:6

As shown, yeast biodiversity in bark portions sampling was higher. The number of samples containing *S.sensu stricto* increased from 8,6% to 18% from bunches to bark. mtDNA-RFLP analysis showed an increase in the number of different profiles, with a ratio (n° of profile/n°of *S. sensu stricto*) of 1:6 compared to 1:11 for grape bunches. All strains investigated resulted belonging to *Saccharomyces cerevisiae* species.
Therefore bark sampling seems to be more effective for isolating new yeast strains in vineyard compared to grape bunches.
To better understand yeasts biodiversity on bark, a second widespread bark sampling was conducted the year after.

4.3.2 Large-scale bark portion sampling in the DOC Lison-Pramaggiore area

Bark samples were collected from eleven vineyards located in four different areas (Lison, Loncon, Motta di Livenza and Pramaggiore), two of which (Lison and Loncon sites)were the most sampled during grapes bunches survey, in the post-harvest period (October). A total of 44 samples were collected.
According to previous bark sampling, these were put into 100 ml-Erlenmeyer flasks filled with 100 ml of synthetic must (Delfini, 1995). The fermentation process was monitored by checking the weight loss daily and was considered completed when loss was lower than 0,1 g within 24 hours.

At the end of the process a total of 170 colonies were isolated on WL medium. The multiplex PCR analysis revealed the presence of 160 isolates belonging to the *Saccharomyces sensu stricto* group, that are the 94% of the total considered.

The strain-specific mtDNA-RFLP analysis evidenced 38 different profiles without similarity with strain profiles from grape bunches or commercial strains.

Moreover, ITS-RFLP analysis for species identification on the 38 different strains, revealed two strains belonging to *S. paradoxus* while the other were *Saccharomyces cerevisiae*.

Comparison of data achieved from grapes bunches sampling and the last one conducted on bark portions is reported in table 4.3.2

Table 4.3.2 Comparison between grape bunches and wide bark portions sampling

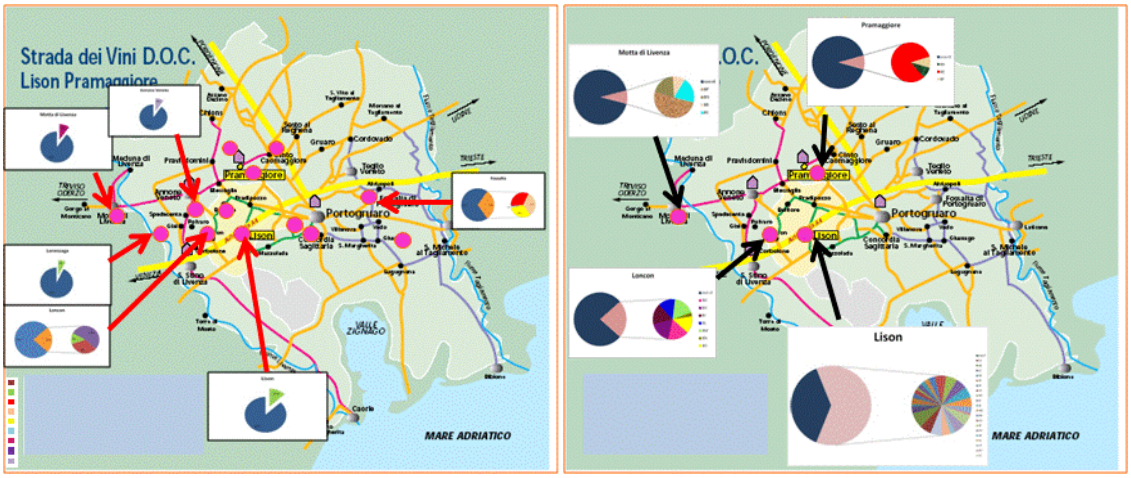
	Samples	**Total isolates**	**Samples with *Saccharomyces***	***Saccharomyces* isolates**	**mtDNA profiles**
Bunches of grapes	208	835	18 (8,6%)	195 (23%)	17
Bark portions	44	170	32 (73%)	160 (94%)	38

Data reported in table 4.3.2 confirm that bark sampling is more effective both in terms of number of strains rescued and yeast biodiversity. Indeed, despite the small number of samples collected (44 bark portions compared to 208 grape bunches), a higher number of *Saccharomyces* isolates was collected, that reached 94% compared to 23% found on grape bunches. Moreover ratio between the number of profiles on the number of total colonies considered as *S.sensu stricto* on WL plates revealed that there is a different profile every 4 isolates from bark portions compared to a profile every 11 strains selected from grape bunches.

Observing the geographic distribution in the Lison-Pramaggiore area of the different mtDNA profiles isolated from grape bunches and bark portions, an increase in biodiversity was noted.

As reported in figure 4.3.1 only two sites sampled for grape bunches gave 3 different profiles (4.3.1 A), while concerning bark portion, all 4 sites gave many different profiles

(4.3.1 B). In particular in the Lison site, 24 different profiles were found from bark portion while just one was observed from grape bunches.



A **B**

Figure 4.3.1 geographic distribution of mtDNA profiles from grape bunches (A) and bark portion sampling (B).

4.3.2.1 Technological strains characterization in synthetic must

After isolation and genetic characterization, identification of strains with interesting technological characters was performed. To evaluate the fermentative performance of the isolates, a representative of each mtDNA profile was inoculated in synthetic must (Delfini, 1995) under conditions that simulate enological setting. Also the first 7 strains isolated on bark in the first trial were analyzed. A total of 45 strains were inoculated at a concentration of about $5*10^5$ CFU/ml in a 100 ml-Erlenmeyer flask closed with silicon cap supplied with a bowed glass pipette and filled with 100 ml of synthetic must (Delfini, 1995). Fermentations were considered completed when weight loss was lower than 0,1 g within 24 hours.

Fermentative vigor, glucose consumption after 7 days and at the end of the process, and the fermentation length were observed.

To better compare fermentation kinetics of yeasts selected on bark portion to those of strains from grape bunches, a Principal Component Analysis (PCA) was performed (figure 4.3.2).

On the positive x-axis are reported fermentative vigour and glucose consumption after 7 days, while on positive y-axis is reported glucose consumption at the end of fermentation, and on the negative y-axis the fermentation length. As showed in figure, the strains selected on grape bunches (red circles) cluster together on the upper left part of the plane, distinctly separated from the strains coming from bark portion (blue circles).

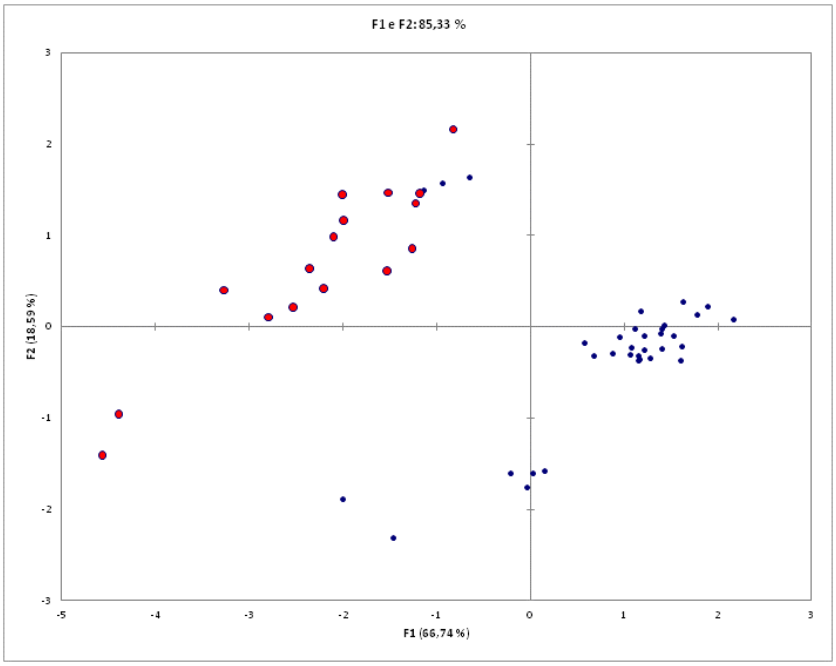


Figure 4.3.2 Fermentative performance PCA of strains selected on bark portions (blue circles) and grape bunches (red circles)

The PCA highlights the better fermentative performances achieved by bark strains that display the best fermentative vigour and glucose consumption after 7 days. They leave a higher glucose residue but close the fermentation process in the same number of days.

The strains tested in synthetic must were further evaluated also in Lison natural must to analyze their fermentation attitudes in an enological contest.

4.3.2.2 Physiological characterisation of yeasts in Lison natural must

Strains were grown in a 200 ml-Erlenmeyer flask closed with silicon cap supplied with a bowed glass pipette and filled with 200 ml of Lison must. The fermentation process was monitored daily by weight loss. Together with the 45 strains, the commercial strain mostly used in the Lison-Pramaggiore area (Mycoferm611), was also tested.

In this contest, fermentative vigour, glucose consumption after 7 days and fermentation length were evaluated. The results obtained were subdivided into relative frequency classes. Concerning fermentative vigour, lower values were detected compared to those achieved with strains selected on grape bunches. Strains from bark portions seem to have some difficulties to start the fermentative process in natural must, compared to synthetic must. Indeed the highest fermentative vigour value observed was 2,5g/100ml (figure 4.3.3) reached also by the commercial strain Myc611.

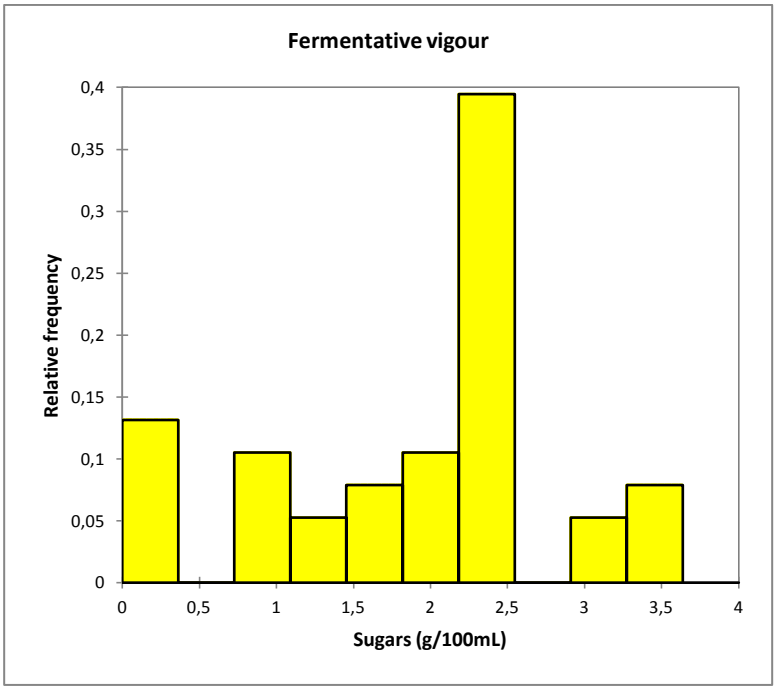


Figure 4.3.3 Relative frequency of strain fermentative vigour in natural must, in g/100mL

A similar result was achieved for sugar consumption after 7 days of fermentation (figure 4.3.4).

The mean value was 9g/100ml that is lower than the value achieved in synthetic must. Only one strain and the commercial one reached 10g/100ml. Moreover fermentation length was longer, with a mean value of 25 days compared to 20 days of the commercial strain and 18 days mean value obtained in synthetic must.

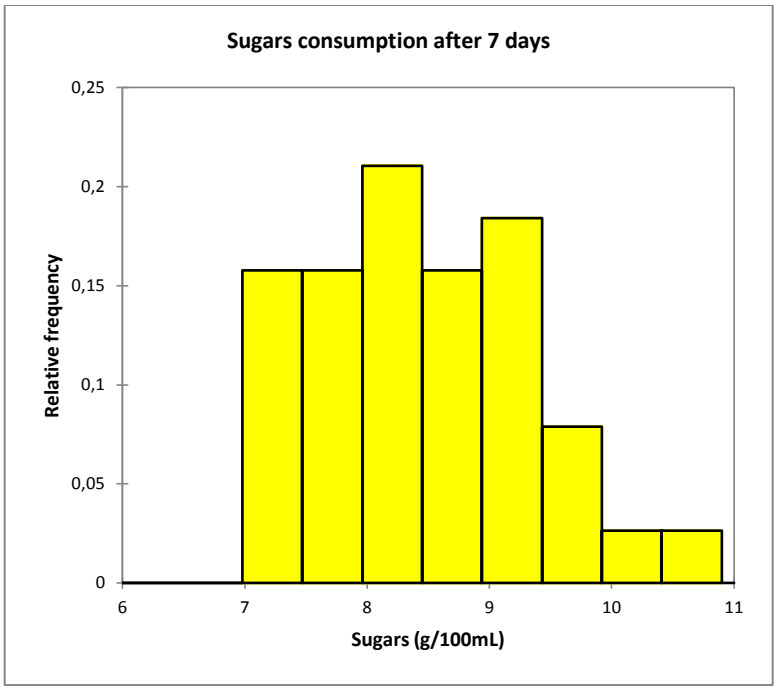


Figure 4.3.4 Strains relative frequency of sugar consumptions after 7 days.

Finally the fermented musts obtained were tasted by a panel of four expert wine taster chosen by the Lison-Pramaggiore Consortium.

Only general characters, such as positive and negative organoleptic notes were took into account.

The evaluation was performed by expressing a preference and by choosing some strains with the best characteristics..

At the end, 6 strains emerged for their interesting organoleptic qualities and were considered for microvinification test.

Despite worse fermentation kinetics achieved on natural must by strains selected on bark portion compared to those from grape bunches, they expressed better organoleptic notes.

4.3.3 Conclusions

The results achieved with bark portions samplings highlight the great yeasts biodiversity present on this substrate with respect to that obtained on grape bunches.

Bark could be considered a natural ecological niche that preserves and promotes yeast biodiversity throughout the year, since bunches are present only for few months. Therefore sampling bark portions of white vineyard variety could be more efficient and representative in terms of *Saccharomyces* isolation.

Moreover, strains tested on synthetic mustshowed better fermentative vigour and greater glucose consumption after 7 days than those observed for strains selected on grape bunches.

Notwithstanding fermentative kinetics obtained on natural Lison must were worse, 6 strains were considered for their good organoleptic notes.

5. Identification and characterization of wine yeasts isolated during early stages of high sugar fermentation

5.1 Introduction

5.1.1 Ecology of yeasts in vineyard

Wine is the product of complex interactions between fungi, yeasts and bacteria that commence in the vineyard and continue throughout the fermentation process until packaging. Although grape cultivar and cultivation provide the foundations of wine flavour, microorganisms, especially yeasts, impact on the subtlety and individuality of the flavour response. Consequently, it is important to identify and understand the ecological interactions that occur between the different microbial groups, species and strains. Grapes are a primary source of yeasts that enter the winery environment. Consequently, the ecological interactions that occur on grapes will contribute to the species diversity in the winery. Generally, very few yeasts (10–100 cfu/g) are detected on immature grape berries, but they increase to populations of 104 –106 cfu/g as the grapes mature to harvest. During ripening, sugars leach or diffuse from the inner tissues of the grape to the surface, thereby encouraging yeast growth. Unripe grapes harbour a predominance of *Rhodotorula* , *Cryptococcus* and *Candida* species, along with the yeast-like fungus *Aureobasidium pullulans*. Most of these species are also isolated from mature, ripe grapes but, at this stage, species of the apiculate yeasts, *Hanseniaspora* (anamorph *Kloeckera*) and *Metschnikowia* , are mostly predominant. Damage to the skin and surface of grapes increases the availability of nutrients for microbial growth, and encourages a greater population (>10^6 cfu/g) and diversity of yeasts that need to co-exist with various filamentous fungi, acetic acid bacteria and lactic acid bacteria that also develop under these conditions (Fleet et al., 2002). Damaged grapes have increased incidence of *Hanseniaspora* (Kloeckera), *Candida* and *Metschnikowia* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* . It is not uncommon to find damaged grapes in vineyards, but the impact of this factor on the yeast ecology of wine production has been underestimated in earlier literature. Obviously, the extent of this impact will be determined by the relative proportions of damaged to undamaged fruits. The principal wine yeast, *S. cerevisiae* , is not prevalent on wine grapes. Generally, it occurs at populations less than 10–100 cfu/g and is best isolated by enrichment culture than direct agar plating (Fleet et al., 2002; Mannazzu et al., 2002).

Some researchers have not been able to isolate this species from healthy, mature grapes, and these observations have raised speculations and controversies as to its origins in wine production (Martini et al., 1996; Mortimer and Polsinelli, 1999) . Certain yeast species (e.g. *Hanseniaspora* /*Kloeckera*, *Metschnikowia*) predominate on wine grapes, and others (e.g. *S. cerevisiae*) are absent. Influencing factors that determine this specific yeast behavior include: physiological and biochemical compatibility of the species with the surface chemistry of the grape (e.g. adhesion to grape surface, metabolise available nutrients); tolerance of the natural stresses of temperature, sunlight, irradiation, periodic desiccation; tolerance to chemical inhibitors, from the grape itself and from the application of agrichemicals; and interactions with other species (yeasts, bacteria, filamentous fungi) (Fleet et al., 2002; Andrews and Buck, 2002) . Yeast–yeast interactions could be important, but these require investigation. There are suggestions that M. pulcherrima , commonly found on grapes, is inhibitory to a range of other yeasts, including S. cerevisiae (Nguyen and Panon, 1998).

5.1.2 Yeasts of the alcoholic fermentation

During alcoholic fermentation yeasts are responsible for ethanol production and make a positive contribution to wine flavour (Henschke, 1997). They do this by several mechanisms: (i) utilising grape juice constituents, (ii) producing ethanol and other solvents that help to extract flavour components from grape solids, (iii) producing enzymes that transform neutral grape compounds into flavour active compounds, (iv) producing many hundreds of flavour active, secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds), and (v) autolytic degradation of dead yeast cells (Cole and Noble, 1997; Lambrechts and Pretorius, 2000). These reactions, especially the production of secondary metabolites, vary with the species and strain of yeast. Thus, the uniqueness and individuality of the flavour contribution by yeasts depends on the species and strain ecology of fermentation and the many factors that determine this ecology (Fleet and Heard, 1993; Fleet, 2001). Alcoholic fermentation is a complex microbial process that involves the interactive growth and biochemical activities of a mixture of yeast species and strains. These yeasts originate from (i) the flora of the grapes,

(ii) the flora associated with the surfaces of winery equipment and the winery environment (e.g. air, insects), and (iii) added starter cultures, if used. Generally, species of *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* initiate the fermentation, and largely originate from the grapes. Sometimes, species of *Pichia* , *Issatchenkia*, *Kluyveromyces* and *Rhodotorula* may also grow at this stage. These yeasts grow to about 10^6 –10^7 cfu/ml but, by mid-fermentation, begin to decline and die off. At this time, *S. cerevisiae* becomes predominant (10^7 –10^8 cfu/ml) and continues the fermentation until its completion. Occasionally, the fermentation may not proceed to completion and unacceptably high amounts (>2–5 g/l) of unfermented sugars remain in the wine. These fermentations are often termed sluggish or stuck and present major practical problems to the winemaker (Bisson, 1999) . In addition to the successional growth of different yeast species throughout the course of fermentation, there is an underlying successional development of strains within each species. This latter revelation became most evident with the use of molecular techniques that enabled strain differentiation and recognition (Pretorius, 2000; Fleet, 2001). Up to five or more strains of *S. cerevisiae* have beenfound in the one ferment, and similar findings have been reported for some non-Saccharomyces species(Schulz and Gaffner, 1993; Henick-Kling et al., 1998;Sabate et al., 1998 ; reviewed in Fleet, 2001). Many factors affect the occurrence and growth of yeasts during alcoholic fermentation. These include the initial population and diversity of species and strains in the grape juice, inoculation of the juice with selected starter cultures, chemical composition of juice including any fungicide/pesticide residues, processing conditions such as concentration of sulphur dioxide addition and temperature of fermentation, and interactions between the different yeast species and strains (Fleet and Heard, 1993; Bisson, 1999; Fleet, 2001) . The following sections focus on the significance of interactions in determining the ecological outcome. Ethanol production by *S. cerevisiae* is considered to be a major factor that governs the growth and influence of non-*Saccharomyces* species during fermentation. Generally, the species of *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia*, *Issatchenkia* and found in grape juice are not tolerant of ethanol concentrations exceeding 5–7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Heard and Fleet, 1988; Gao and Fleet, 1988) . However, low temperatures decrease the sensitivity of these species to ethanol and, consequently, wine fermentations

conducted at temperatures less than 15–20 C° may show a greater contribution from *Hanseniaspora* and *Candida* species. On such occasions, these species may equal *S. cerevisiae* as the predominant species at the end of fermentation and, accordingly, would have a greater impact on wine flavour (Heard and Fleet, 1988; Erten, 2002) . There are recent reports of some strains of *Candida* species that may have ethanol tolerances similar to *S. cerevisiae* (Cocolin et al., 2001; Mills et al., 2002). Strains of *Candida stellata* fall into this category and have been used in co-culture with *S. cerevisiae* to enhance the glycerol content and other flavour characteristics of wines (Ciani and Ferraro, 1998; Soden et al., 2000). *Schizosaccharomyces pombe*, *Zygosaccharomyces bailii* and Zygosaccharomyces fermentati are well known for their tolerance of high ethanol concentrations (>10%) and occur in winery environments (Fleet, 2000; Romano and Suzzi, 1993). Surprisingly, they are rarely reported as contributors to grape juice fermentations and the reasons for this require investigation. Possibly, they grow slower than other wine yeasts and, consequently, are out-competed, or they may be inhibited by factors produced by these yeasts. All three species have the ability to utilise malic acid, which is a positive attribute in many winemaking instances. While some strains of these species are known to produce off-flavours, a program of selection and evaluation could reveal strains with desirable flavour attributes. The increase in ethanol concentration during alcoholic fermentation could also explain the sequential growth of strains within a species. Strains of *S. cerevisiae* , as well as those of other species, vary in their tolerance to ethanol stress (Fleet, 1992; Bauer and Pretorius, 2000; Bisson and Block, 2002). Strains with higher ethanol tolerance are more likely to dominate at later, rather than earlier stages of fermentation. This behaviour has been demonstrated experimentally, along with the interactive effect of fermentation temperature (Torija et al., 2002) , and becomes an important consideration in designing mixtures of yeast strains (oligo strains) for use as cultures to enhance the complexity of wine flavour (Grossman et al., 1996). Short- to medium-chain fatty acids, such as hexanoic, octanoic and decanoic acids, are produced during alcoholic fermentation and, above certain thresholds, become inhibitory to S. cerevisiae and, probably, to other species (Viegas et al., 1989; Edwards et al., 1990; Bisson, 1999). Production of these acids varies significantly with yeast species and strain (Lema et al., 1996; Lambrechts and Pretorius, 2000) and could influence the sequential

growth of yeasts during fermentation. However, further research is needed to assess the full impact of these acids on the conduct of alcoholic fermentation. Nutrient availability and nutrient limitation are likely factors that modulate the yeast ecology of fermentation, as one yeast species or strain produces or utilises a nutrient relevant to another species or strain. Evidence for these types of interactions is scant, but various possibilities could be proposed. The non-*Saccharomyces* yeasts appear to be less tolerant of very low oxygen availability than S. cerevisiae . Removal of residual oxygen from fermentating grape juice by the vigorous growth of *S. cerevisiae* could contribute to the early death of these non-*Saccharomyces* species (Hansen et al., 2001) . Non-*Saccharomyces* species growing early in the fermentation could utilise amino acids and vitamins, and limit the subsequent growth of strains of S. cerevisiae . There are reports that *Kloeckera apiculata* could strip the grape juice of thiamine and other micronutrients, leading to deficient growth of S. cerevisiae (Bisson, 1999; Mortimer, 2000) . However, some non-Saccharomyces species, such as *K. apiculata* and *M. pulcherrima* are significantly proteolytic (Charoenchai et al., 1997; Dizzy and Bisson, 2000) and could generate amino acids for use by S. cerevisiae . The early death and autolysis of these non-Saccharomyces yeasts (Hernawan and Fleet, 1995) is another possible source of nutrients for S. cerevisiae , and spoilage yeasts. Cell wall polysaccharides, principally mannoproteins, are also released by yeast autolysis and these could combine with tannins and anthocyanins to impact on wine astringency and colour (Escot et al., 2001). So far, the studies on polysaccharide release relate only to *S. cerevisiae*, and need to be extended to include the non-*Saccharomyces* species.

There is an extensive literature on the isolation of killer toxin producing strains, killer-sensitive strains and killer neutral strains of *S. cerevisiae* from fermenting grape juice (van Vuuren and Jacobs, 1992; Shimizu, 1993; Musmanno et al., 1999; Gurie´rrez et al., 2001) . Although many winemaking variables affect the expression of killer and killer-sensitive phenotypes, there is good evidence that killer interactions may determine species and strain evolution during fermentation. Killer strains of *S. cerevisiae* sometimes predominate at the completion of fermentation, suggesting that they have asserted their killer property and taken over the fermentation. Killer strains have been found within wine isolates of *Candida*, *Pichia* and *Hanseniaspora* and some of these can assert their killer action against wine strains of *S. cerevisiae* (Fleet and Heard, 1993). However, properly designed

studiesare needed to connect killer interactions and an impact on wine flavour. We have demonstrated how killer interactions between strains of *S. cerevisiae* could be used to manipulate the autolytic response of wine yeasts, and give increased protein content in wine (Todd et al., 2000).

5.1.3 Yeast spoilage of wines

Yeasts can spoil wines at several stages during production. Unacceptable flavours can be produced if inappropriate yeast species or strains grow during the alcoholic fermentation. These defects include wines with excessive concentrations of hydrogen sulfide and other sulphur volatiles, acetic acid, various esters, and volatile phenols (Sponholz, 1993; Fleet, 1992, 1998; Fugelsang, 1997; Du Toit and Pretorius, 2000). Such occurrences highlight the importance of understanding and managing the yeast ecology of fermentation, as mentioned already. Bulk storage of wines in tanks and barrels prior to packaging is another critical point where yeast spoilage may develop. Wine that is exposed to air, as in incompletely filled tanks or barrels, quickly develops a surface flora of weakly fermentative or oxidative yeasts, usually species of *Candida* and *Pichia* (e.g. *Pichia membranifaciens*). These species oxidise ethanol, glycerol and acids, giving wines unacceptably high levels of acetaldehyde, esters and acetic acid. Bulk wines, as well as bottled wines, are also spoiled by fermentative species of *Zygosaccharomyces*, *Dekkera* (anomorph *Brettanomyces*), *Saccharomyces* and *Saccharomycodes* . In addition to causing excessive carbonation, sediments and haze, these species produce estery and acid off-flavours (Sponholz, 1993). Species of *Dekkera*/*Brettanomyces* are also associated with the production of unpleasant mousy and medicinal taints, because they can form tetrahydropyridines and volatile phenolic substances such as 4-ethylguaiacol and 4- ethyl phenol (Grbin and Henschke, 2000; Du Toit and Pretorius, 2000) . Management of these types of spoilage is generally done by following good manufacturing practice and hygiene. However, some yeast interactive phenomena may be relevant. Yeast autolysis after alcoholic fermentation could be a significant source of micronutrients for the growth of these spoilage species (Charpentier and Feuillat, 1993), especially those of *Dekkera*/*Brettanomyces* (Guilloux-Benatier et al., 2001). Consequently, removal of yeast sediment soon after the completion

of alcoholic fermentation may minimise this risk. Some authors have suggested a more proactive control by selecting or engineering strains of *S. cerevisiae*, or other desirable species, with killer activity directed against key spoilage species (Shimizu, 1993; Du Toit and Pretorius, 2000).

5.1.4 Dried-grape musts and high-sugar fermentation

The fermentation of high sugar grape musts could occur in winemaking for wine production from dried, botritized or late-harvest grapes, for ice-wine production or in processing industry that used grape juice concentrate. Using must with high sugar concentrations stuck or sluggish fermentations are probable because of the high osmotic pressure and ethanol toxicity for yeast cells. Moreover, an early arrest of fermentation in these musts produces wines of low quality and stability favouring the production of high volatile acidity and the growth of spoilage micro-organisms *Saccharomyces cerevisiae* is routinely used as starter culture for the wine production from grape musts containing usually 16–26% (w/v) sugars. In dried and late-harvest grape musts sugar concentrations may be easily over 30% (w/v) and in frozen grape musts as much as 50% (w/v) (Malacrinò et al., 2005).

Due to the difficulty in producing this peculiar wines understanding the composition of dried-grape must microflora is of great interest both to select the most adapted strains to use as starter culture and to identified the contaminant yeasts that cause the wine spoilage. Unfortunately there are a very limited number of studies that report the microbial characterization of dried-grape must. Most of the yeasts isolated in this condition belonge to species of *Metschnikowia*, *Hanseniaspora* and *Candida*, and a smaller number of species of Pichia, Torulaspora, Debaryomyces, Zygosaccaromyces and Saccharomyces were found, as well. Regarding the genera *Candida* and *Hanseniaspora*, the species *C. zemplinina*, *C. apicola* and *H. clermontiae/uvarum* were the most abundant (Hiero et al. 2006; Toffalo et al., 2009;).

On the contrary several studies report information concerning single yeast strains isolated from dried-grape surface that show antifungal activity and can be used as biocontrol agent (reviewed in Fleet, 2003). The diffusion of this activity among yeast growing in such

environment could be due to the great presence of moulds that characterize the grape over-ripening process. Such yeasts include *Metschnikowia pulcherrima* and *fructicola* , various species of *Candida*, *Pichia*, *Cryptococcus*, and some *Saccharomyces* and *Zygosaccharomyces* species. One species, *Candida oleophila*, is registered for commercial use (Droby et al., 1998). The antifungal effect, generally, is due to the antagonist behaviour of yeasts, although some of these yeasts produce 1,3-beta glucanases that can destroy fungal walls. The possibility that the killer toxins of yeasts may also inhibit filamentous fungi needs more exploration (Walker et al., 1995) .

The aims of this work was to characterized yeast populations present in dried grape musts and during the early stage of alcoholic fermentation that occur in manufacturing of Friularo Passito wine produced in north –east of Italy, Veneto.

By means of conventional and molecular methods yeast species were identified and phenotypically characterized checking technical traits that influence the quality of the wine produced with high-sugar grape must.. The effect of added sulfites on yeast population was also evaluated.

5.2 Materials and methods

5.2.1 Wine fermentations and sampling procedure

Raboso Piave grapes were harvest and carried to the winery where they were dried in controlled chamber to reach a wine yield of about 45%(v/v). Whole clusters were destemmed, crushed and must was poured in two 4hl-capacity tanks in one of which must was supplemented by 50mg/ml SO_2. Both tanks were saturated with CO_2 and spontaneous fermentations were run to obtain 14% v/v ethanol at 14°C. Pumping-over operation was performed after 2 days. Five replicate for each sampling were collected for microbiological and chemical analyses. Sampling were performed after grape crushing (T0) and after 7 (T7) and 8 (T8) days of fermentation activity.

5.2.2 Chemical analyses

Reducing sugars (glucose and fructose), acetic acid, tartaric acid, were determined by HPLC according to Schneider et al. (1987). Ethanol was calculated from sugar concentration considering a average fermentation yield and using the conversion factor of 0.61 (sugar percentag/etanol concentration, ml/100ml) according to Defini (1995).

5.2.3 Microbiological analyses

For microbiological determination plate-count analysis was performed., in peptone water were made. For all samples 100g of must or fermenting juice were diluted in 100 ml of peptone water (0.1% bacteriological peptone, Oxoid, Milan, Italy), then, using the same solution, decimal dilutions were made. The following microbiological analyses were performed in duplicate agar plates: (1) yeasts on WLN agar (Oxoid) incubated at 25 °C for 2–5 days; (2) moulds on Malt Extract agar (Oxoid) and incubated at 25 °C for 3 days. After counting, means and SDs were calculated. During plate count analysis, 100 colonies of yeasts from T0 isolation plates, 30 colonies from T7 isolation plates, 30 colonies from T8 isolation plates were streaked on the Yeast and Mould agar (Oxoid) and then stored at -80

°C in YPD broth (20 g/l glucose, 20 g /l bacteriological peptone, 10 g/l yeast extract, all from Oxoid), supplemented with glycerol (30% final concentration, Sigma-Aldrich, Milano).

5.2.4 Microbiological assays

Growth in presence of high and low sugar concentration and acetic acid production

Each isolates was grown on YDP Broth and 20 µL of 24-hour culture was poured on GYC –L (10 g/l glucose, 3 g/l yeast extract, 16 g/l agar, all from Oxoid, 3g/l $CaCO_3$ Sigma-Aldrich) and GYC-H (175 g/l glucose, 175 g/l fructose, 3 g/l yeast extract, 16 g/l agar, all from Oxoid, 3g/l $CaCO_3$ Sigma-Aldrich). After 2-7 days at 25°C. The diameters of the cell growth and the calcium carbonate solubilization halo were measured. The longer was the halo diameter, the higher was the acetic acid production.

H_2S production

A frozen-culture aliquot of each isolate was streaked to obtain single cell separation on Biggy Agar (Oxoid), after 2-7 days at 25°C the colony colour was evaluated. The higher was the colour intensity, the higher was H_2S production.

SO_2 production

A frozen-culture aliquot of each isolate was streaked to obtain single cell separation on Fuchsin Agar (10 g/l glucose, 5 g /l bacteriological peptone, 3 g/l malt extract, 16 g/agar, all from Oxoid, 0,002 g/l fuchsin Sigma-Aldrich), after 2-7 days at 25°C the colony colour was evaluated. The higher was the colour intensity, the lower was the SO_2 production.

5.2.4 ITS region analysis

For ITS region primers ITS1 and ITS4 (Guillamon et al., 1998) were used to amplify a region of the rDNA repeat unit which includes two non-coding regions, designated as the internal transcribed spacers (ITS1 and ITS2), the 3′ part of the 18S, the 5′ portion of the 26S and the entire 5.8S rDNA genes. A 3 µL aliquot of cell suspension, prepared as

described above, was heated at 94 °C for 2.5 min and then subjected to PCR amplification using 35 cycles with initial denaturation at 94 °C for 30 s, annealing at 53.5 °C for 30 s and extension at 72 °C for 30 s.

The restriction reactions were performed at 37 °C for 2 h, in a 15 μL mixture containing 5 U of HinfI, enzyme (Fermentas International inc., Canada) set up according to manufacturer's instructions. Restriction fragments were run on 2% (w/v) agarose gel containing 0.1 μg/mL of Gel-Red TM (Biotium, Hayward, CA). Bands were visualised by UV trans-illumination; digital images were acquired with the EDAS290 capturing system (Kodak, USA) and restriction profiles were analysed using BioNumerics (Applied Maths, Belgium) software.

5.2.5 D1/D2 region sequence analysis

Amplification of D1/D2 domain within the 26S rDNA sequence was performed using primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAAAAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) according to the protocol described by Kurtzman and Robnett (1998). Amplification products were checked for purity by agarose gel electrophoresis and then subjected to sequencing. DNA sequencing (Sanger reaction with fluorescent nucleotides, run and analysed by capillary electrophoresis) was performed by BMR-genomics (http://www.bmr-genomics.it/). PCR products were purified from primers and short polynucleotides using the ExoSap™ Cleanup system (USB—United States Biochemical, USA) by adding 1 μL of ExoSap enzyme to 5 μL of PCR products and incubating at room temperature for 30 min. Species identification was done after BLASTN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequences with those present in the GenBank public database. A minimum sequence similarity level of 98% was considered for taxonomic attribution.

5.3 Results and discussion

5.3.1 Quantification of yeast and mould populations during early stage of alcoholic fermentation

By means of plate count analysis we determined the microbial evolution during early stage of fermentation of Friularo passito wine. This is a traditional product obtained from the fermentation of dried-grape belonging to Raboso Piave variety. Starting in steel tanks, generally the fermentation finished in small barrel, where the wine is aged for a total period of 36 months. In this study after grape crushing the must was poured in two 4hl-capacity tanks in one of which the must was supplemented by 50mg/ml SO2. The first sampling (T0) was performed just after grape crushing, while the other two sampling were carried on after 7 (T7) and 8 (T8) of fermentation from both tanks. The chemical characteristics of the must is shown in Table 5.3.1. The sugar concentration present in the must allowed to reach 14-14,5% ethanol (v/v) required by this kind of wine.

Table 5.3.1 Chemical composition of the must of Raboso Piave grape variety.

pH	**Reducing sugars (g/l)**	**Volatile acidity (g/l)**	**Total acidity (g/l)**
3.05	238	0.06	9

After one week the ethanol concentration was still low both in natural and SO_2-supplemented must (Table 5.3.2), revealing a slow rate of fermentation due to the high sugar concentration and the low fermentation temperature chosen by the winery (14°C).

Table 5.3.2 Ethanol production and volatile acidity in the fermentation samples

	T7		T8	
	No SO_2	**SO_2**	**No SO_2**	**SO_2**
Ethanol (%,v)	3.6	1.2	6.1	4.6
Volatile acidity (g/l)	0.13	0.18	0.15	0.18

Yeast population in the must (T0) reached $3,8*10^5$ UFC/ml (Figure 5.3.1). This value is very low compared with the concentration generally found in not-dried must, that generally is 1log higher (Ribereau-Gayon et al., 2006). This could be due to the presence of a consistent concentration of moulds ($1,1*10^5$ UFC/ml) that competed with yeast population in colonizing grape berry. In fact, during drying procedure, the grape bunches are kept in chamber with high level of humidity, condition that promotes mould grown. After 7 days (T7) the fermentation activity that provoked CO_2 saturation of the must contributing to impede oxygen diffusion, reduced the mould concentration under the detection limit of the plate count method (10^2 CFU/ml). The presence of SO_2 delayed yeast growth that reached a lower concentration. During the following 24 for hours (T8) both populations reached the same value. The slower growth rate in presence of sulfite is proven by the ethanol concentration values that were dramatically lower for both sampling times.

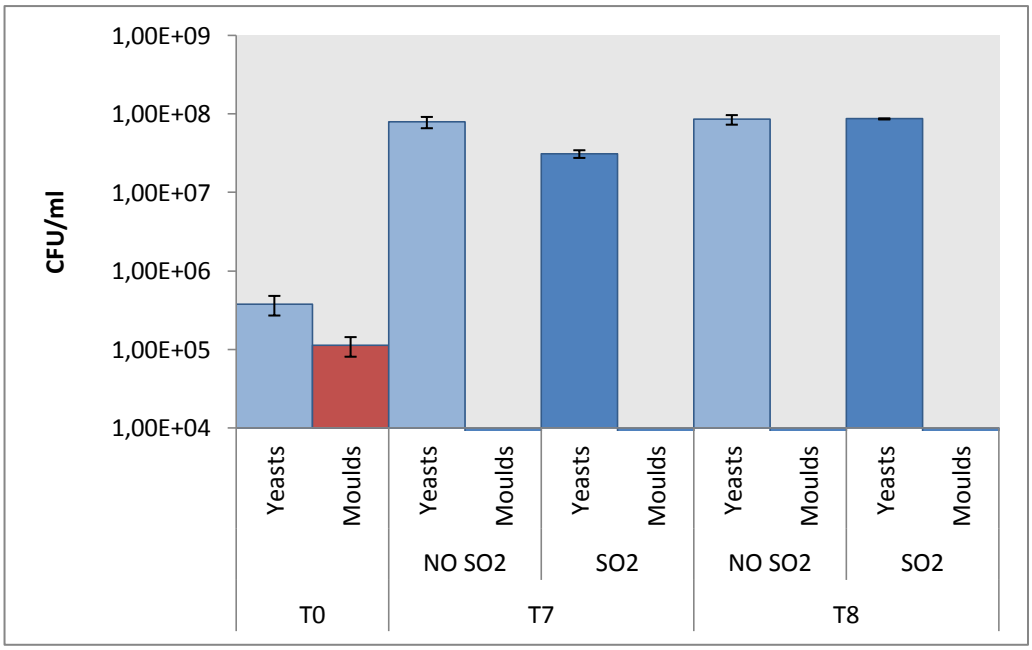


Figure 5.3.1. Yeast and mould concentrations in must (T0) and after 7 (T7) and 8(T8) days of fermentation.

5.3.2 Colony morphology analysis

During plate count analysis, 100 colonies of yeasts from T0 isolation plates, 30 colonies from T7 isolation plates, 30 colonies from T8 isolation plates were collected for further analyses. Before streaking each isolate on new growing medium for storage, the colony morphology on WLN was observed. Differential media for the enumeration of *Saccharomyces* and non-*Saccharomyces* strains has been proposed (Heard and Fleet, 1986), but involves plating on two or more selective media to obtain quantitative information. Wallerstein Laboratory Nutrient Agar (WLN) medium was designed for use in brewing and industrial fermentation processes to observe microbial populations and is not a very selective medium (Green and Gray, 1950). Cavazza et al. (1992) showed that the majority of yeast species typically found in wine fermentations could be distinguished on the basis of colony color and/or morphology on WL medium. This medium contains Bromocresol green a dye used as pH indicator. Moreover this molecule can be differentially adsorbed by yeast giving an additional morphological trait. In this way we can identified 8 different morphology types (Figure 5.3.2) After 7 and 8 days (T7, T8) the numbers of morphologies dramatically reduced to 3 .

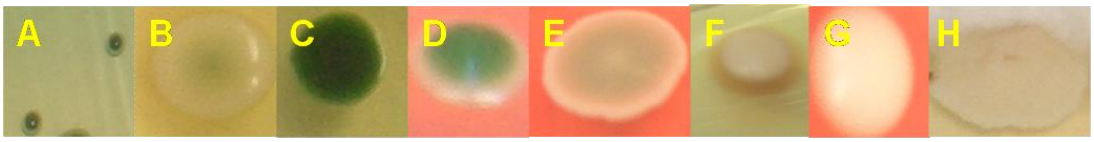


Figure 5.3.2 Morphology types identified in must and during early stage of fermentation.

The morphology type B can be ascribed to the species *S. cerevisiae* since the colonies appered knoblike with a smooth and opaque surface, a cream-to-green color and a consistency of cream. Yeast colonies that corresponded to this description have been isolated mostly at T7 and T8, when alcoholic fermentation was ongoing.

The morphology type A e C were the most abundant in the must before alcoholic fermentation to start (T0). Then, they dropped down at T7 and disappeared at T8. All the other morphology types were identified only at T0 at low concentrations.

5.3.3 RFLP-ITS genetic analysis

The complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8s rRNA gene (coding and conserved) are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than the 18s and 25s rRNA genes (Cai et al., 1996; James et al., 1996). Because ribosomal regions evolve in a concerted fashion, they show a low intraspecific polymorphism, and a high interspecific variability. For that reason this method is very useful to identified yeast species that characterize a natural environment. The DNAs from the 160 isolates were digested with *Hinf*I enzyme and the electrophoretic patterns were determined. Although it is known by previous studies that more the one enzyme is needed to correctly identified some of the species generally present in the enological environment (i.e. the ones of the genus *Hanseniaspora*)(Esteve-Zarzoso *et al*., 1999), we tried, using the most discriminating enzyme, *Hinf*I, to find out how suitable it is to describe yeasts coming from high sugar concentration fermentation. We obtained 14 genetic profiles, among which only 3 were present after 7 and 8 days of fermentation (T7 and T8).

5.3.4 D1/D2 sequenging genetic analysis

Sequencing analysis of the D1/D2 ribosomal region is the most reliable methods to identified yeasts (Kurtzman & Robnett, 1998), nowadays. Infact this sequence, as it is taxonomical relevant, was deeply invesigated and became of great interest because of the huge correlated sequencing database. At the moment of grape crushing 10 species belonging to 6 genera were found out (Table 5.3.3). *Cryptococcus* (37%), *Hanseniaspora* (26%) e *Candida* (29%) were the most represented, while *Torulaspora*, *Metschnikowia* e *Saccharomyces* were present only at low concentration.

Table 5.3.3 Yeast species and colonization percentages in must (T0) and fermenting samples after 7 (T7) and 8(T8) days of fermentation.

	T0		T7		T8	
D1/D2 sequencing			**No SO2**	**SO2**	**No SO2**	**SO2**
Candida californica	8%					
Candida oleophila	3%	29%				
Candida zemplinina	18%					
Cryptococcus carnescens	29%	37%	7%	7%		
Cryptococcus sp.	8%					
Metschnikowia fructicola	4%					
Torulaspora delbrueckii	2%					
Hanseniaspora uvarum	24%	26%	20%	13%	20%	7%
Hanseniaspora vineae	2%					
Saccharomyces cerevisiae	2%		73%	80%	80%	93%

Among the specie of the genus candida *C. zemplinina* was the most present. This new species (type strain 10-372T=CBS 9494T=NCAIM Y016667T), is closely related to *Candida stellata*, a yeast already found on overripe grapes and in sweet fermenting wines. It was isolated for the first time from fermenting botrytized grape musts in the Tokaj wine region of Hungary and represent a new osmotolerant and psychrotolerant species **(**Sipiczki, 2003). *Candida californica*, previously *P. membranifancies*, has as synonimous *Cryptococcus californicus* and was isolated from grapes in USA. It can weackly ferment glucose but has a strong oxidative metabolism, and shows a osmotollerant attitude (Wu and

Bai 2006). *Candida oleophila* has been isolated form flower of *Phacelia* sp., cider, rotten wood, fruit of *Olea europea* (olive), oil-pipe in food-processing plant and unripe strawberry. It has an oxidative metabolism but can ferment glucose. From this species, strains used as biocontrol angent against fungi have been found. Generally these yeast are characterized by a strong capability to rapidly colonize and grow in surface wounds, and subsequently to out compete the pathogen for nutrients and space. Competition for nutrients and space is believed to be the major component of the mode of action of antagonistic yeasts (Lima et al. 1997). Moreover *C. oleophila* is capable of producing and secreting various cell wall-degrading enzymes, including exo-beta-1,3-glucanase, chitinase and protease that can destroy hyphal wall structures (Dobry et al., 2002). Among strains belonging to this species there are some that can produce large amounts of citric acid and are propose for industrial production of citric acid with a new competitive fermentation process for continuous citric acid production (Anastassiadis, 2006). Among genus *Criptococcus* belonging to the Basidiomycetes group, the species *C. carnescens* was found at high concentration. It is an encapsulated yeast isolated the first time from Grape. Biochemically, the members of the genus *Cryptococcus* are unable to ferment sugars, but do assimilate inositol and produce urease. It is characterized by highly pronounced aerobic type methabolism. Carotenoid pigment production is extremely variable. Amomg the species belonging to this genus *C. neoformans* is the only pathogenic, the most common clinical form of cryptococcosis is meningoencephalitis. Cryptococcosis is rare in immunocompetent hosts. Among *Criptococcus* genus antagonistic yeasts able to compete with phytopatogenic fungi were found (Takashima et al., 2003). Another genus found at high concentration was *Hanseniaspora*. We identified two species. The most present was *H. uvarum* (*Kloeckera apiculata*). It represents the dominant native species present on grape at harvest. Historically its activity was thought to be restricted to pre-fermentation and early stage of alcoholic fermentation (as it is inhibited by 4-5 % of ethanol). Depending on temperature Saccharomyces population becomes quickly established and produces sufficient alcohol to inhibit further growth of *Kloeckera*. However despite it is ethanol sensitive at low temperature (10°C) this species was found at high population density (near 10^7 CFU/ml) over the course of the fermentation It is capable of producing acetic acid and its esters at high concentration before and during early stage of fermentation (Fulgensan,

1996). *H. vineae* is strongly related to *H. osmophila* and many strains exhibit remarkable resistance to high sugar concentration. As *H. uvarum* it is characterized by weak fermentation with low alcohol production. Due to its beta-glucosidase activity that can enhance varietal flavour recently some co-inoculation fermentation trial has been reported (Bujdoso et al. 2001). Among the genus present at low concentration *Metschnikowia* is represented by only one species *M. fructicola.* This microorganism, able to ferment glucose, was isolated by first time from grapes grown in central Israel. Phylogenetic analysis of domain D1/D2 26S rDNA sequences showed *M. fructicola* to be a sister species of *M. pulcherrima (Kurtzman and Droby, 2001)*. This species shows biocontrol activity against *Botrytis* rot of stored grapes (Sipiczki, 2006). A commercial product is available on the market containing the strains NRRL Y-30752 (Droby. 2006). *T. delbrueckii* and *S. cerevisiae* are the two last species found, the first one only after crushing (T0). *T. delbrueckii* has good fermentation capacity, it is osmotollerant and described as a slow acetic acid producer. It is considered promising as co-inoculant with *S. cerevisiae* for the production of sweet wine. In fact conventional wine yeasts produce high concentrations of volatile acidity, mainly acetic acid, during high-sugar fermentation. This alcoholic fermentation by-product is highly detrimental to wine quality and, in some cases, levels may even exceed legal limits. *T. delbrueckii*, often described as a low acetic producer under standard conditions, retained this quality even in a high-sugar medium (Tokuoka, 1993; Bely et al., 2008). Considering the evolving of the species during early stage of the fermentation, a strong selection effect due to the starting of the alcoholic fermentation was reported, since only three species (*H.uvarum*, *C. carnescens* and *S. cerevisiae*) out of 10 were present after 7 days (T7) of fermentation and only two (*H. uvarum*, S. cerevisiae) after 8 days (T8). Despite the low alcohol concentration after 7 days (T7), S. *cerevisiae* was the dominant species whose presence increased further on (up to 80-93%) after 8 days (T8). As aspected, the non-Saccharomyces species *H. uvarum* is disadvantaged by the presence of sulphites, as its concentration is lower when SO_2 is present. This is not the case for *C. carnescens*, the other non-Saccharomyces species present at T7.

Comparing the two identification methods (RFLP-ITS and D1/D2 sequencing), using RFLP-ITS analysis 4 different enzymes are recommended, mainly to identified species from *Candida* and *Hanseniaspora* genera (Nisiotou et al, 2007). Using *Hinf*I the most

discriminating enzyme, we succeeded in clustering *Candida* species that showed a highest level of heterogeneity, but we failed to identify *Cryptoccus* species (Table 4.3.4). Unexpectedly, we succeed to cluster *Hanseniaspora* and we found two different profiles that D1/D2 sequencing assigned to S. *cerevisiae*. This was the first case of sequence polymorphism linked to the species *S. cerevisiae*. These results suggest that the restriction enzyme discriminatory ability is strongly dependent on the specific environment analyzed.

Table 5.3.4 RFLP-ITS and D1/D2 sequencing analyses of the isolates collected in must (T0) and fermenting samples after 7 (T7) and 8(T8) days of fermentation

		T0	T7		T8		Total
***Hinf*I profile**	**D1/D2 sequencing**		**No SO2**	**SO2**	**No SO2**	**SO2**	
e	*Candida californica*	8					8
d	*Candida oleophila*	2					3
g	*Candida oleophila*	1					
b	*Candida zemplinina*	17					18
n	*Candida zemplinina*	1					
c	*Cryptococcus carnescens*	29	1	1			31
c	*Cryptococcus sp.*	1					8
f	*Cryptococcus sp.*	4					
m	*Cryptococcus sp.*	3					
a	*Metschnikowia fructicola*	4					4
l	*Torulaspora delbrueckii*	2					2
p	*Hanseniaspora uvarum*	24	3	2	3	1	33
i	*Hanseniaspora vineae*	2					2
o	*Saccharomyces cerevisiae*	1					51
h	*Saccharomyces cerevisiae*	1	11	12	12	14	
	Total	100	15	15	15	15	160

5.3.5 Physiological characterization

Growth in presence of high and low sugar concentration and acetic acid production.

One of the most important factor that infuences the quality of wine obtained from high sugar grape must is the high level of volatile acidity, mainly due to strong production of acetic acid by wine yeasts. *Saccharomyces cerevisiae* is routinely used as starter culture for the wine production from grape musts containing usually 16–26% (w/v) sugars. Although this microorganism is well adapted to high osmotic pressure at this sugar concentration it produces high level of acetic acid from acetaldehyde derived by glucose to rapidly consume sugar and to lower the must osmolarity. So, in situation of high osmotic stress the high level of volatile acidity is the consequence of general yeast adaptation mechanism. For this reason the same isolates that previously have been taxonomically identified were grown on a medium containing high (GYC-H) and low (GYC-L) sugar concentration, miming, with the second medium the environment that yeasts can find when they grow high sugar grape must. We choose two incubation conditions, with oxygen to mimic the first phase of fermentation when the oxygen is present due to the must high density and without oxygen to mimic a second phase when alcoholic fermentation takes place and CO_2 saturation inhibits oxygen diffusion. The production of acetic acid is evaluated by measuring the halo of solubilization of $CaCO_3$ added in the media. To take account of the different growth ability shown by the isolates the ratio between the size of the halo and the size of the colony was considered. When no growth was detected the ratio was considered equal to 0. The results for isolates belonging to the Genus *Candida* are shown in Figures 5.3.3 and 5.3.4

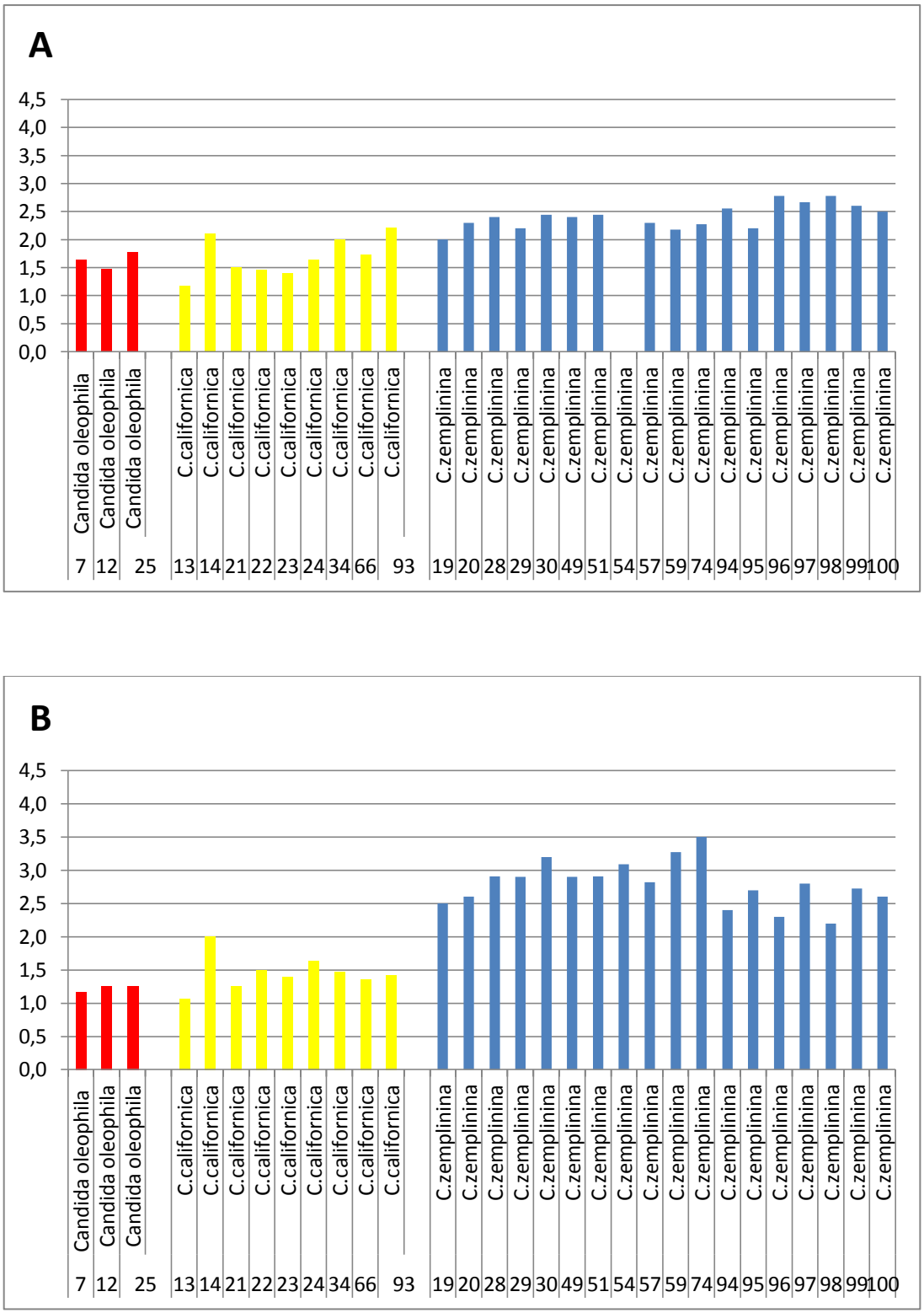
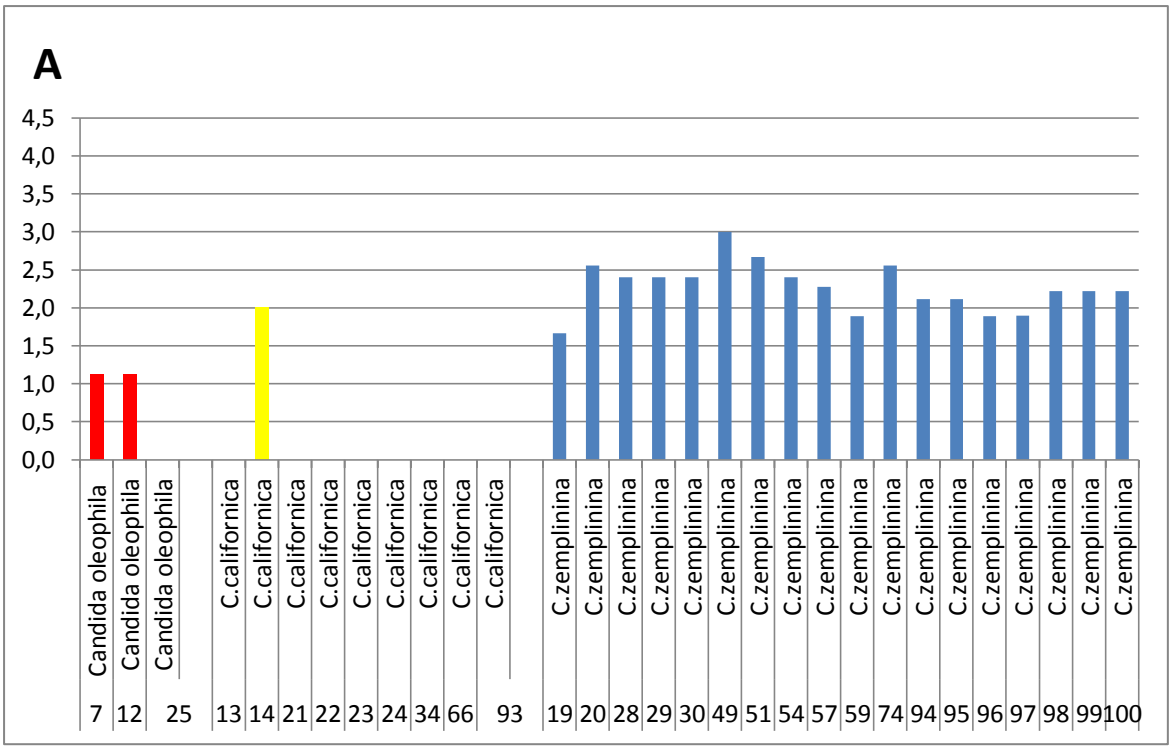


Figure 5.3.3 Acetic acid production in the genus *Candida*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated aerobically.



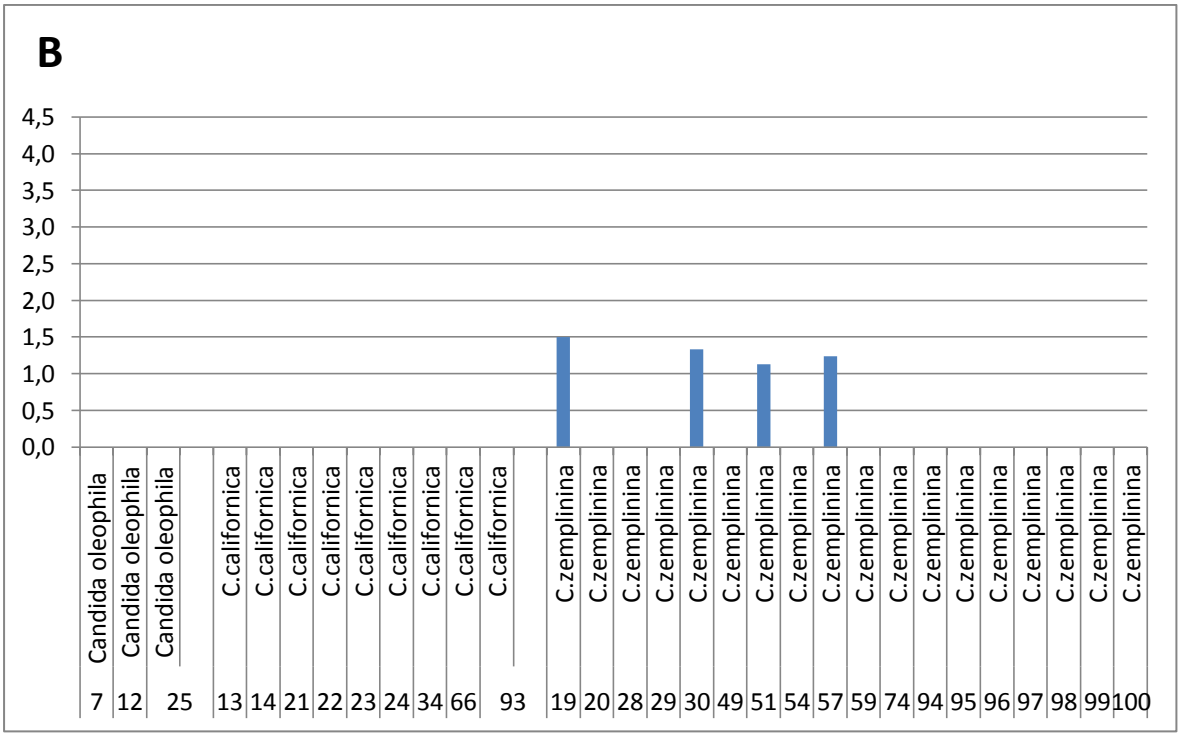
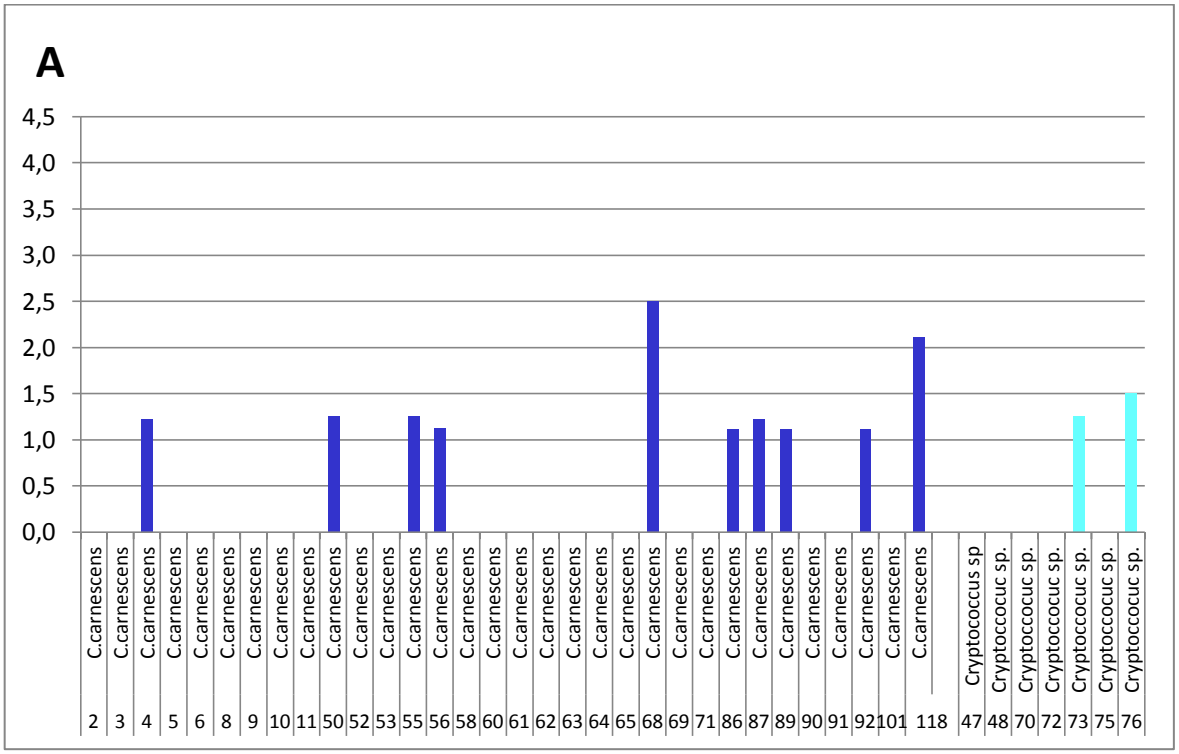


Figure 5.3.4 Acetic acid production in the genus *Candida*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated anaerobically.

There were no difference between high and low sugar concentration when the isolates were incubated aerobically, but when anaerobic incubation was performed no growth on low sugar medium was observed for almost of the isolates. Interestingly only isolates belonging to *C. zemplinina* species, the species that produce the highest level of acetic acid, showed a production comparable to the one obtained aerobically.



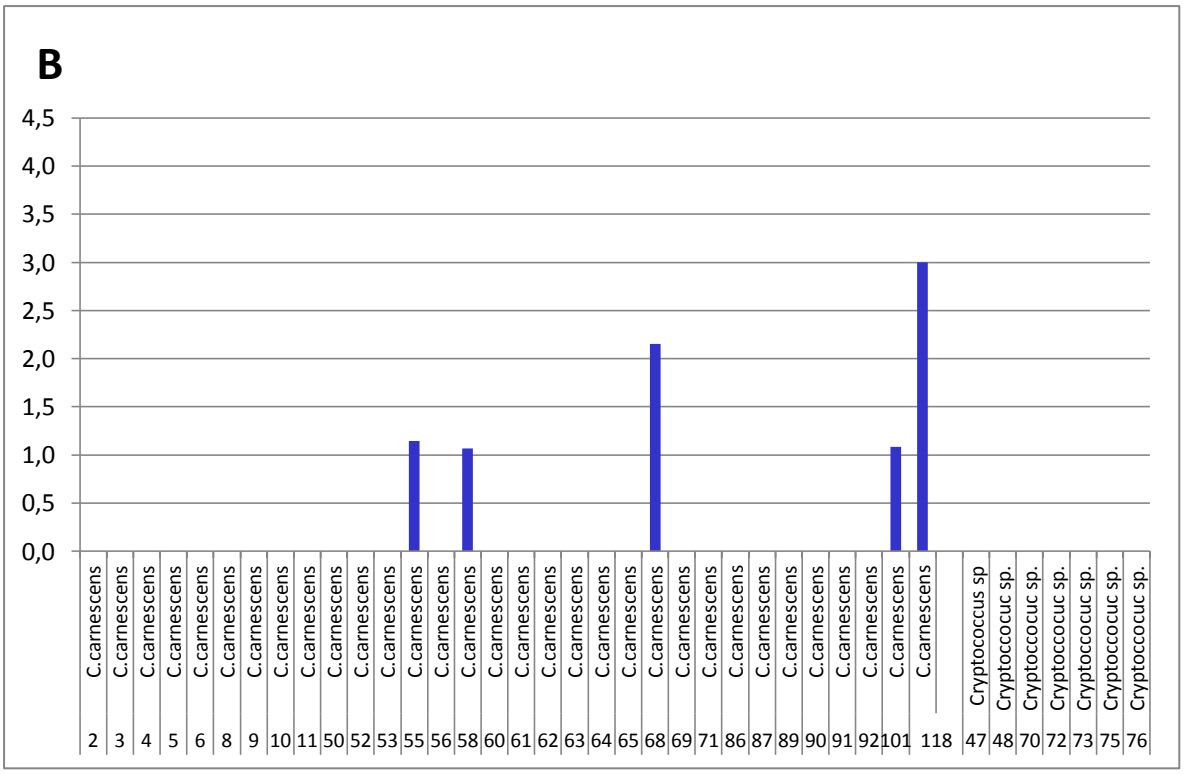
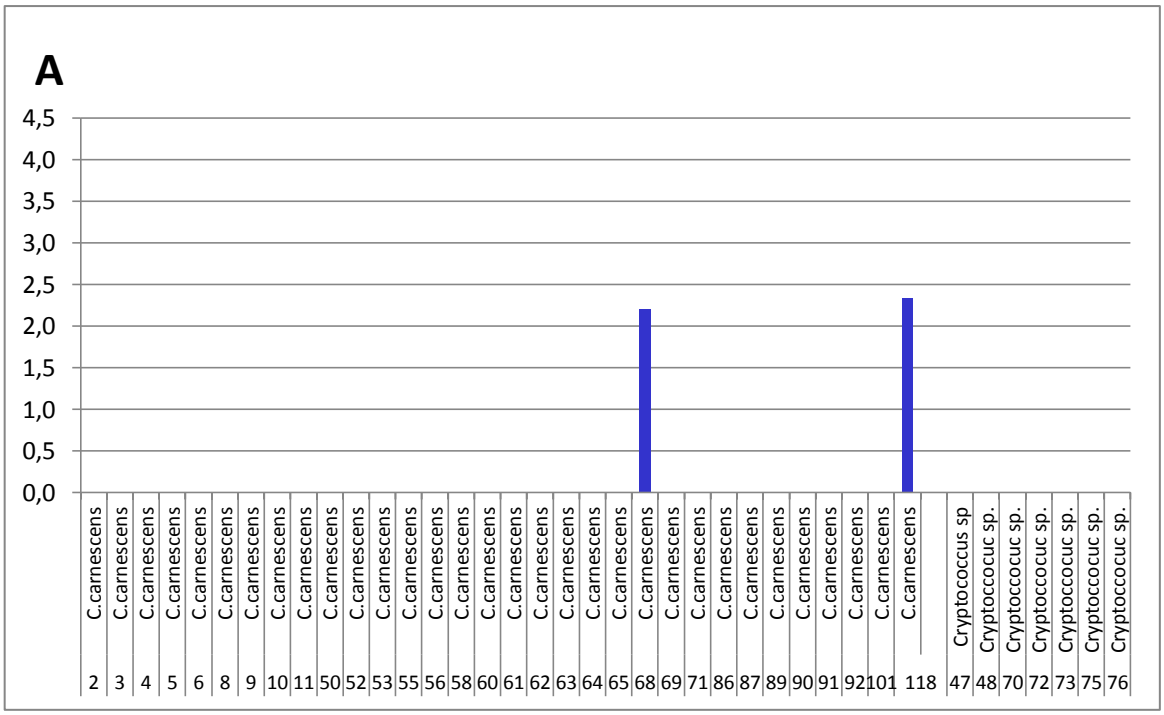


Figure 5.3.5 Acetic acid production in the genus *Cryptococcus*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated aerobically.



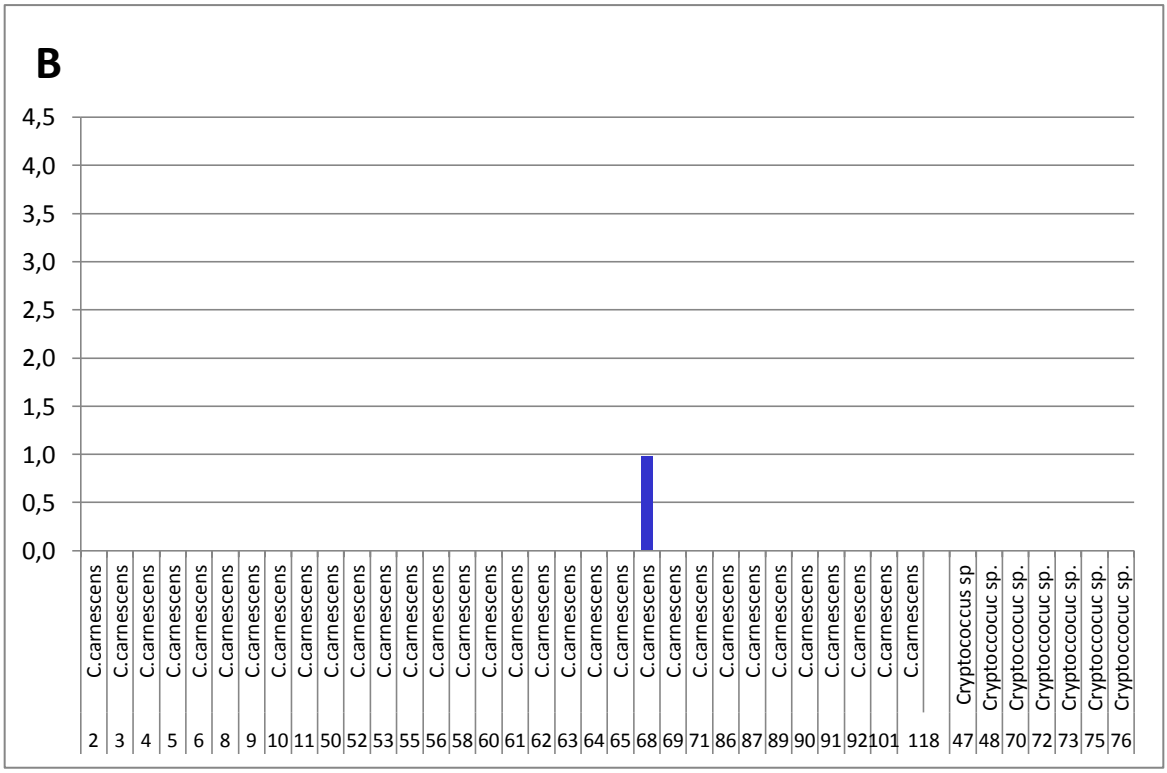
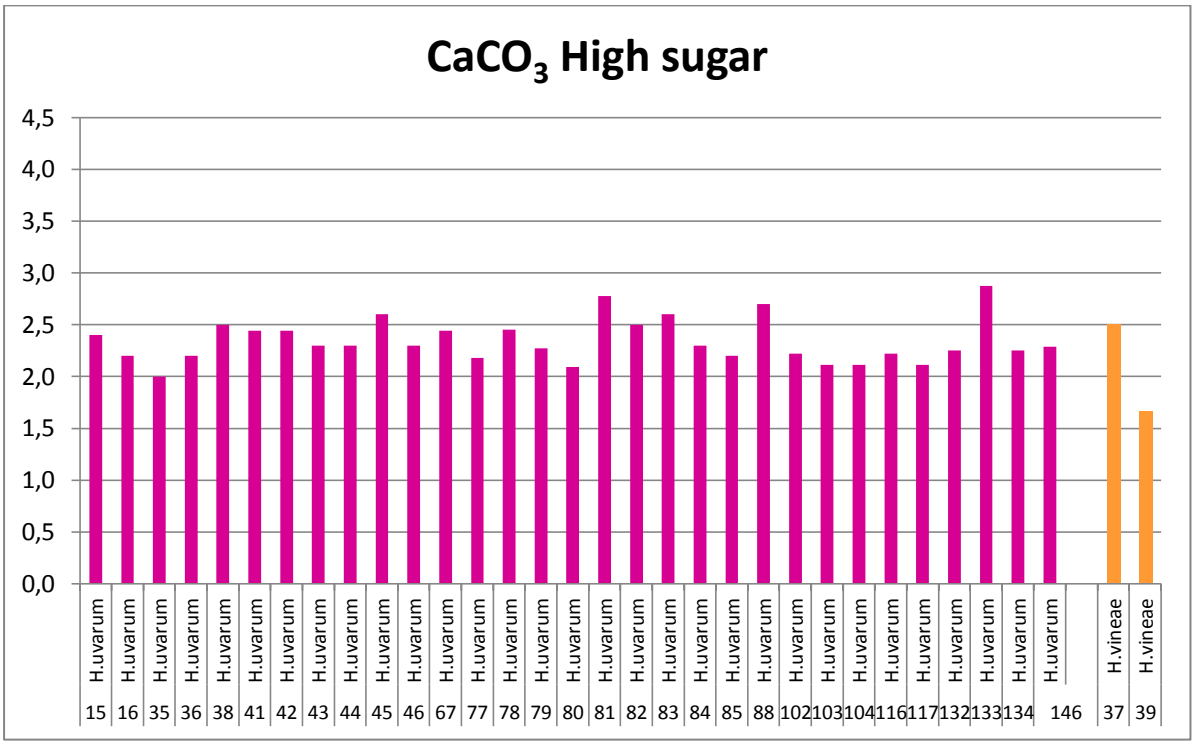


Figure 5.3.5 Acetic acid production in the genus *Cryptococcus*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated anaerobically.

Surprisingly isolates from genus *Cryptococcus* (figures 5.3.4 and 5.3.5) showed a poor ability to growth on GYC both with high and low sugar concentration. The higher number of isolates able to growth was found in GYC-L incubated aerobically. Only the *C. carnescens* isolate 68 was able to growth in all the condition tested.



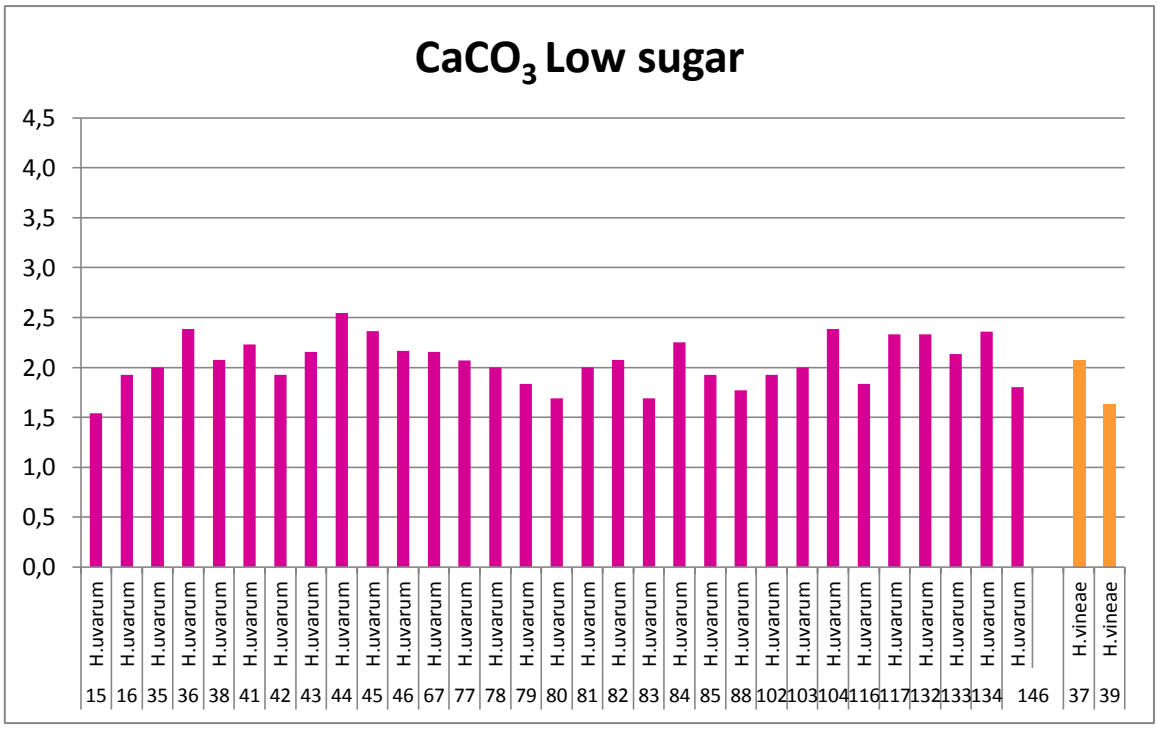


Figure 5.3.6 Acetic acid production in the genus *Hanseniaspora*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated aerobically.

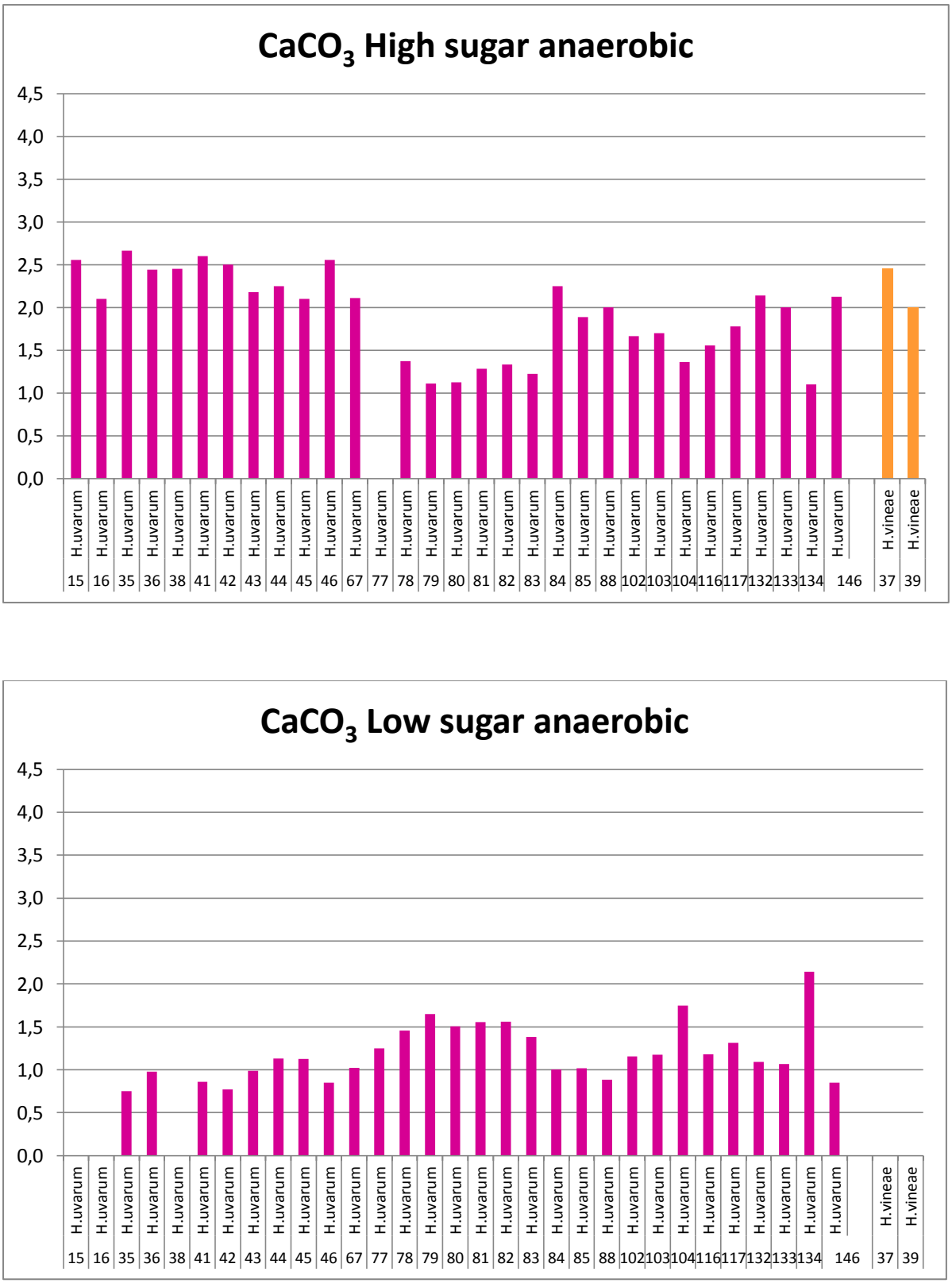
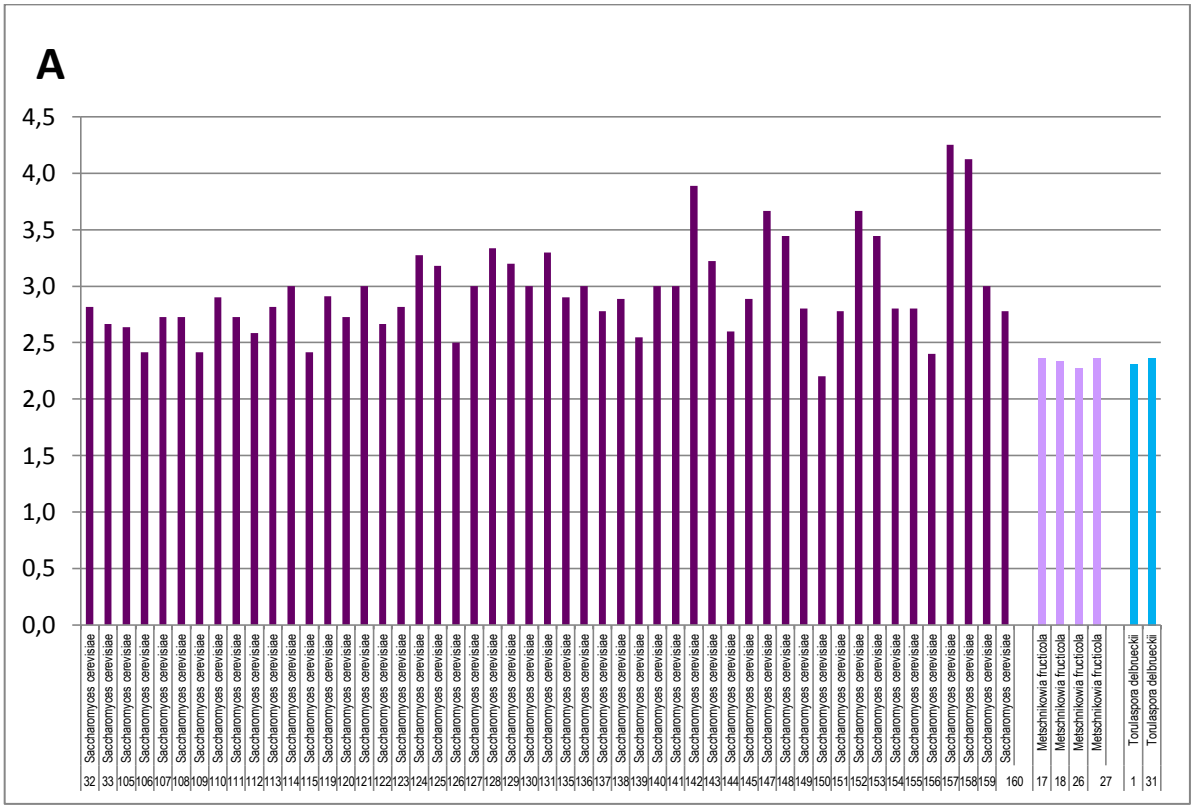


Figure 5.3.7 Acetic acid production in the genus *Hanseniaspora*, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated anaerobically.

The genus *Hanseniaspora* (Fugures **5.3.6 and 5.3.7)** was characterized by a high level acetic acid production mainly when aerobically growth occurred. *H. vineae* isolates, as few isolates of *H. uvarum*, were not able to growth at low sugar concentration anaerobically. In this growing condition the lowest acetic acid production was recovered.



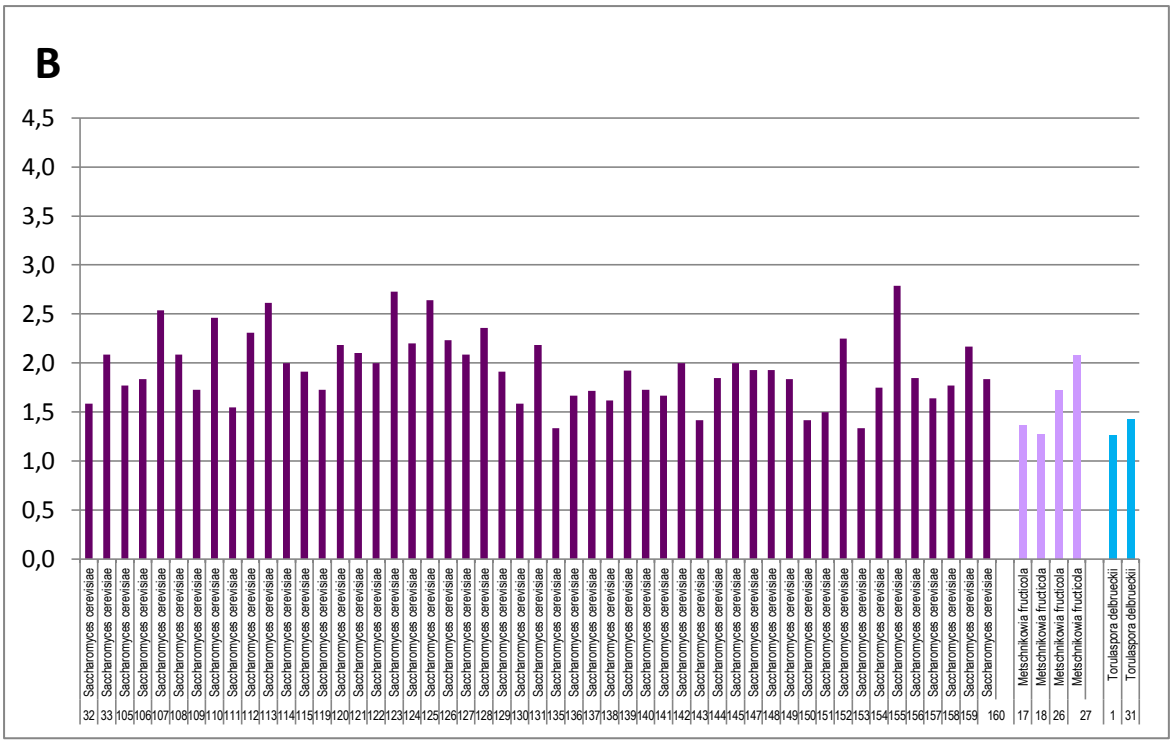


Figure 5.3.8 Acetic acid production in the species *S. cerevisiae*, ***M. fructicola, T. delbrueckii***, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated aerobically.

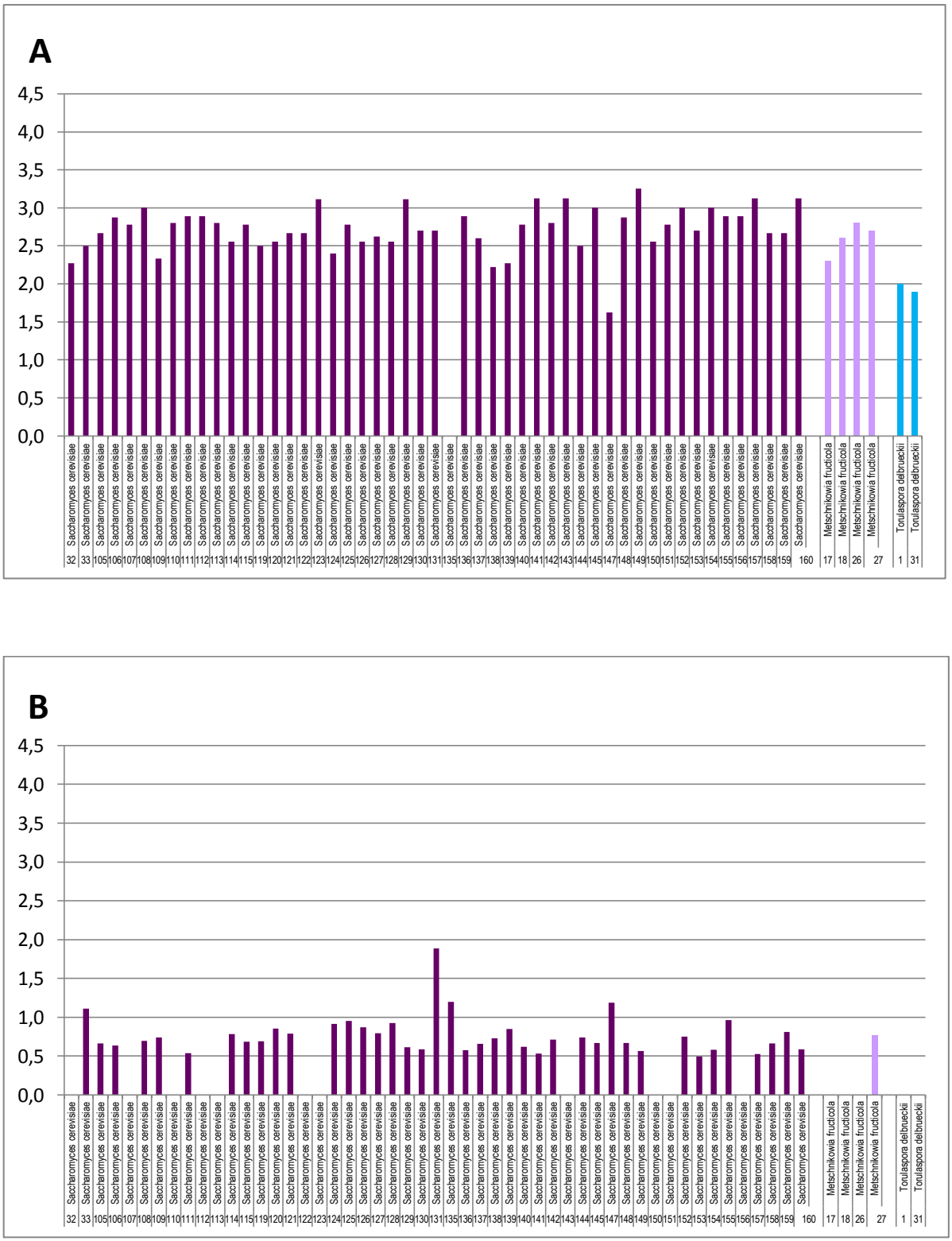


Figure 5.3.8 Acetic acid production in the species *S. cerevisiae*, ***M. fructicola, T. delbrueckii***, quantified as the ratio between the size of the halo and the size of the colony, on GYC-H (A) on GYC-L (B) incubated anerobically.

S. cerevisiae and *M. fructicola* (figures 5.3.7 and 5.3.8) revealed to be the higher producers species, with consistent presence of solubilization halos also in GYC-H under anaerobic condition.

H_2S and SO_2 production

Sulfite and sulfuric acid production are two important enological traits that influence not only the wine quality, but, concerning SO_2, the health of the wine consumers. The effects of sulfites on human health are clearly detrimental, especially for alcoholic drink consumption since ethanol itself contributes to intensify allergic and allergic-like reactions to other molecules (Vally et al., 2009). Moreover wines obtained by dried-grape must, that usually have a higher ethanol concentrations, contain abundant sugar residues, therefore they need to be more protected against microbial spoilage. Consequently higher SO_2 dose are generally used. Considering that the total SO_2 is due to the added fraction and to the quantity naturally produced by yeast, it is of great interest to know how much the native yeasts contribute to the final concentration present in wine.

Table 5.3.6 Sulfite and sulfuric acid production by yeasts isolated in must (T0) and after 7 (T7) and 8(T8) days of fermentation.

	SO2 production (%)			H2S production (%)			
	+	++	+++	+	++	+++	++++
Crytococcus carnescens	53	37,5	9,5	6,3	21,9	68,7	3,1
Cryptoccocucs sp.	100				14,3	71,4	14,3
Candida californica		77,7	22,3	11,1			88,9
Ccandida zemplinina		61,1	38,9		5,5	39	55,5
Candida oleophila		100		33,3	66,7		
Hanseniasopora uvarum		50	50	50	47	3	
Hanseniaspora vineae	50	50				50	50
Torulaspora delbrueckii		100			50		50
Metschnikowia fructicola		100			100		
Saccharomyces cerevisiae		100		29,4	56,9	13,7	

+ low, ++medium, +++high, ++++ very high production

The results of the semi-quantitative assays used to evaluate both technological traits are reported in table 5.3.6. The genus *Candida* mostly contributed to the production of both the molecules as the isolates that synthetized higher quantity of SO_2 and H_2S belonged to this genus. *C. carnescens* showed a greatest variability that seemed to be strain specific. On the contrary *T. delbrueckii*, *S. cerevisiae* and *M. fructicola* showed the lowest. *H. uvarum* and *Cryptococcus sp.* showed the higher number of isolates with low ability to produce SO_2 and H_2S.

In conclusion yeasts that seems to be able to colonize dried-grape must belong to genera and species that carry osmotolerants and fungi antagonists. High sugar concentration and competition with mould that are strongly present in this environment seems to be the two causes of the selective pressure that guides the yeast population dynamics in this environment. *S. cerevisiae* that is the most abundant species already in the early stage of fermentation strongly increases the acetic acid production that is one of the most important factor that negatively affected the quality of this kind of wine.

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