UNIVERSIDADE DE AVEIRO
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Departamento de Biologia

Sara Sofia Dinis de Sousa

Biodiversidade de estirpes de *S. cerevisiae* vínicas em Portugal.

Biodiversity of wine *S. cerevisiae* strains in Portuguese appellations.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Ana Catarina Batista Gomes, Investigadora da Unidade de Genómica do Biocant e do Professor Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Vinho, leveduras vínicas, *Saccharomyces cerevisiae*, genómica, microssatélites

resumo

A produção de vinho é uma prática ancestral, onde o sumo de uva é fermentado. Este processo baseia-se na ação de um conjunto de microrganismos, dos quais a levedura *Saccharomyces cerevisiae* se destaca. Considerando que atualmente há uma crescente pressão para a diferenciação dos vinhos, suportada sobretudo na valorização das características únicas do vinho, a utilização massiva de estirpes de *S. cerevisiae* industriais limita o potencial de produção de vinhos com perfis metabólicos e aromáticos únicos e diferenciados.

Neste trabalho, o objetivo foi identificar a população de leveduras vínicas fermentativas de diferentes regiões vitivinícolas de Portugal, por forma a explorar a sua biodiversidade natural e, desta forma, avaliar o potencial de diferenciação existente naturalmente nas vinhas Portuguesas.

Para tal, isolaram-se leveduras de 36 fermentações de diferentes castas de quatro regiões vitivinícolas de Portugal. Ao todo foram isoladas 1260 leveduras na sua maioria *S. cerevisiae*. Das 911 *S. cerevisiae* identificadas foram distinguidas 826 estirpes de *S. cerevisiae*.

Com este trabalho, reunimos uma amostragem significativa da diversidade de estirpes de *S. cerevisiae* endógenas nas vinhas portuguesas, que permitirão um estudo mais aprofundado da diversidade genética. Assim, este trabalho representa um avanço significativo na exploração da biodiversidade de *S. cerevisiae* em Portugal, e será a base de futuros trabalhos em que se pretende explorar o potencial enológico destas estirpes.

keywords Wine, yeast, *Saccharomyces cerevisiae*, genomics, microsatellits

abstract

Winemaking is an ancestral practice, where the grape juice is fermented into wine. This process relies on the action of a set of microorganisms where the yeast *Saccharomyces cerevisiae* stands out. Considering that nowadays there is a growing pressure for the wines' differentiation, manly supported by the valorisation of the unique wine characteristics, the massive use of industrial *S. cerevisiae* strains limits the potential of a differential production of wine with metabolic and aromatic unique profiles.

In this work, the main objective was to identify the fermentative wine yeasts population of different Portuguese wine appellations of origin, in order to exploit their natural biodiversity and to evaluate the differentiation potential present at Portuguese vineyards.

Therefore, it is urgent to unveil the yeasts communities present in different Portuguese wine appellations whit a holistic approach and by this explore their natural biodiversity. In this work, yeasts were isolated from 36 fermentations of different grape varieties of four Portuguese appellations of origin. The predominant species from the 1260 yeast isolates was *S. cerevisiae*. In the 911 *S. cerevisiae* identified we found 826 *S. cerevisiae* strains.

With this study we have gathered a significant sampling of endogenous diversity of *S. cerevisiae* that will allow a deeper study on the genetic diversity. Therefore, this study represents a significant progress in the exploitation of the Portuguese *S. cerevisiae* biodiversity, which will be the basis for future work in which we intend to exploit the oenological potential of these strains.

Index

Figures index

Tables index

Annexes index

Introduction

1. Grape must fermentation

1.1. Grape must fermentation pathways

During the last 30 years, major progress was achieved in the understanding of the ecology, biochemistry, physiology and molecular biology of yeasts, and in particular of their importance in wine production. To strategically tailor wines in a changing market, winemakers find the alcoholic fermentation as the key process that they can creatively engineer wine character and value (*16*).

The wine is achieved after two sequential fermentations: the alcoholic fermentation and the malolactic fermentations. The alcoholic fermentation is the foundation of the transformation of grapes into wine and is conducted by yeasts of the genus *Saccharomyces*. The yeasts present on the grape must convert its glucose, fructose and sucrose into ethanol via the process of fermentation. In the overall fermentation, glucose ($C_6H_{12}O_6$) is converted into ethanol (CH_3CH_2OH) and carbon dioxide (CO_2), which is released in the form of gas. During glycolytic pathway, one glucose molecule is converted into two ethanol molecules and two carbon dioxide molecules (Figure 1).

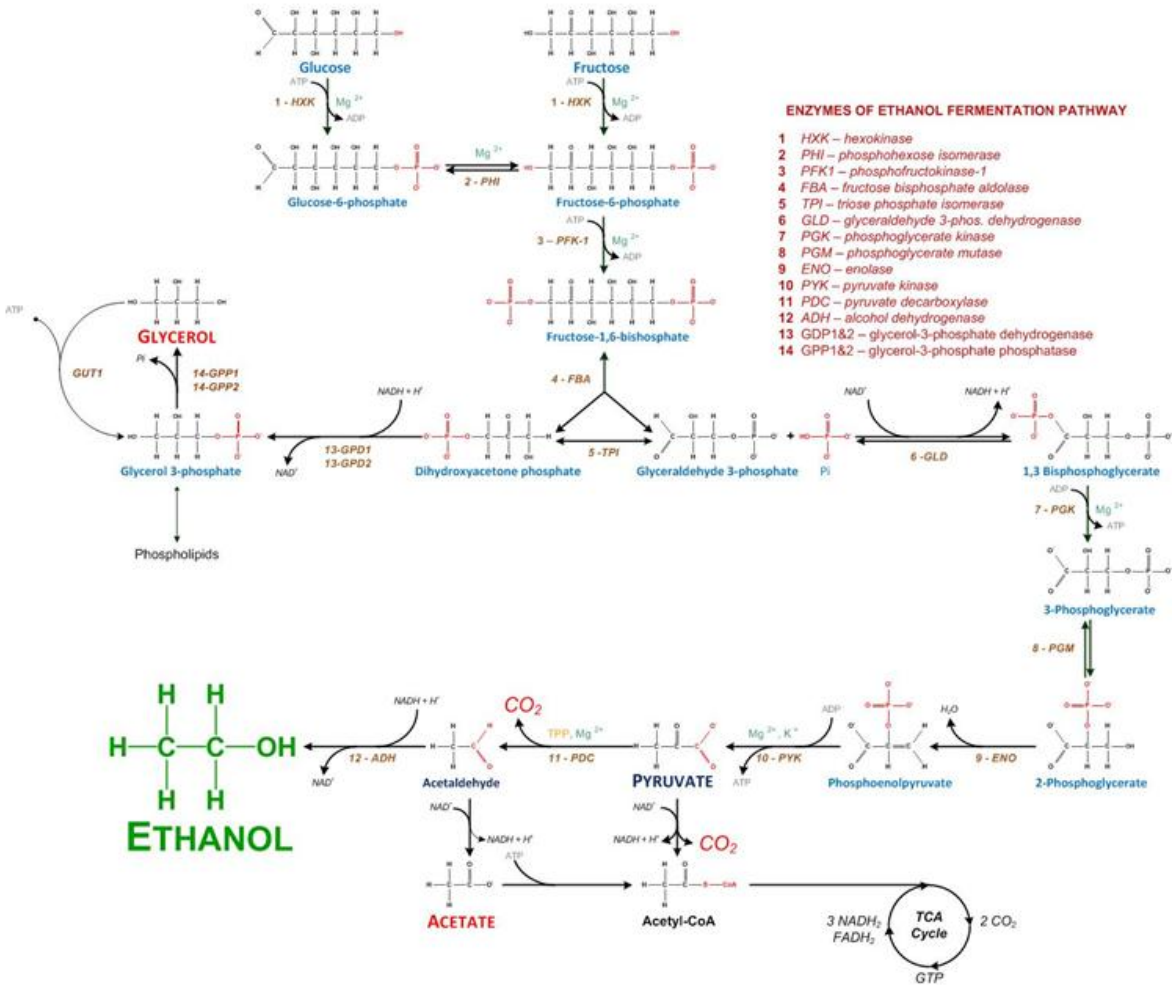


Figure 1. Metabolic overview of the alcoholic fermentation.

In this process, one molecule of glucose is broken down into pyruvate and during this reaction two molecules of NADH and of ATP are generated. Pyruvate is then converted into acetaldehyde and carbon dioxide by pyruvate decarboxylase. Afterwards, the acetaldehyde is reduced to ethanol, using the previously produced NADH molecule, which is converted into NAD^+ (*1*). The malolactic fermentation is carried out by acid lactic bacteria such as *Oenococcus oeni*, *Lactobacillus* ssp., *Leuconostoc* spp.. Indeed *Oenococcus oeni* is the ideal species used to conduct this fermentation due to its tolerance to low pH and flavor metabolites produced. By a process of deacidification it is able to convert the dicarboxylic L-malic acid (malate) into the monocarboxylic L-lactic acid (lactate) and carbon dioxide (Figure 2) (*29*). This fermentation is an additional step to confer a distinct flavor and smooth texture to the wine (*30*, *26*). The bacterial activity plays a role in stabilization of wine and ensures an enrichment of its aromatic composition (*34*).

Since our aim is to study yeasts and this process is done by bacteria it won't be target of our study.

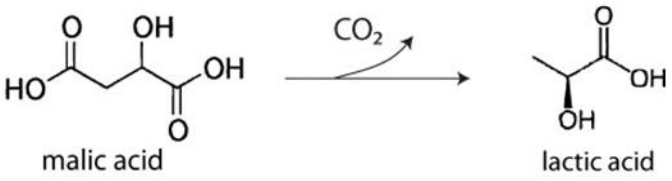


Figure 2. Basic chemical reaction of the malolactic fermentation.

1.2. Grape must microflora and its impact on wine organoleptic character

A spontaneous wine fermentation is carried out by the sequential action of different genus and species of yeasts and bacteria (*22*). The fermentative microflora is reliant on a diverse network of factors such as the origin of the grapes, the winemaking technique and phytosanitary conditions of the grapes and cellars. Indeed, in our previous studies, we have already demonstrated that grape berries are a great reservoir for *S. cerevisiae*. The alcoholic fermentation is usually started by the non-*Saccharomyces* yeasts and then completed by *S. cerevisiae*, who can resist to high ethanol concentration and have high fermentative power. Yeast species within *Hanseniaspora*, *Candida*, *Kluyveromyces*, *Zigosaccharomyces* genera are the non-*Saccharomyces* yeasts most commonly present at the beginning of the alcoholic fermentation (Figure 3).

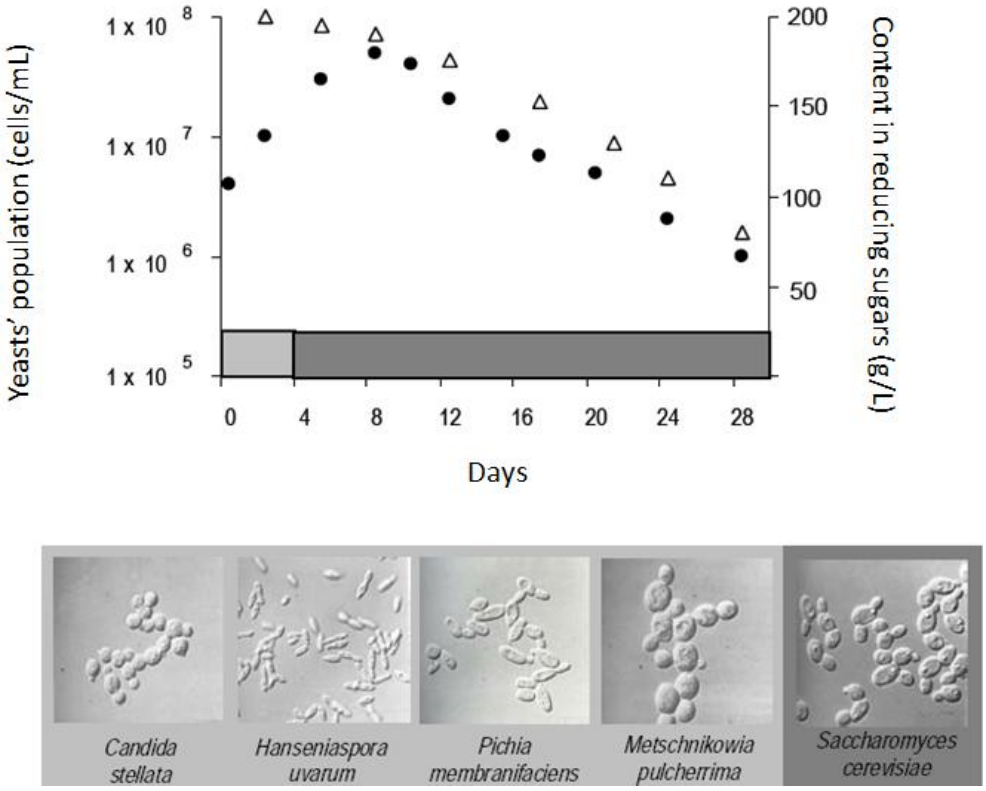


Figure 3. Yeast population dynamic and the content of reducing sugars during fermentation (8). Legend: Δ - Content in reducing sugars, • - Yeast´s population.

Yeasts affect wines' sensorial properties by different mechanisms that extend far beyond the glycolytic pathway, namely the grape juice and the nitrogen metabolism, enzymatic hydrolysis of grape components that affect wine aroma, flavor and clarity, yeasts autolysis and yeast bioadsorption properties. Nowadays, it is accepted that the metabolic profiles from different yeast species and strains are singular, so it is important to carry out an extensive strain screening to understand the positive and negative contributions of each strain (*16*). Also, such a wide screen will allow us to unveil the metabolic biodiversity which can afterwards bring value to wines.

Ethanol is the second most abundant compound in wine, being water the first. Ethanol's affinity to water and its solubility, by forming hydrogen bonds, makes it a powerful dehydrator. This property is useful in flocculating hydrophilic colloids, proteins and polysaccharides. It also gives ethanol disinfectant properties that are particularly valuable in aging wines. The combination of ethanol and acidity makes it possible to keep wine for a long time without any noticeable spoilage. The addition of ethanol to stabilize certain wines is a long-standing winemaking tradition seen in the Port winemaking. Ethanol's

solvent properties are involved in solubilizing certain odoriferous molecules and definitely contribute to the expression of aromas in wine (*36*). Several monoterpene alcohols occur naturaly on grapes such as citronellol, geraniol, linalool and nerol providing distinct fruity, estery, spicy and vegetative aromas. Yeasts are able to produce glycosidases that break down the covalent link between trepenes and glucose so that volatile trepenes are released and impact on wine organoleptic character. The production of glycosidases varies with species and strains, and some authors suggest that non-*Saccharomyces* like *Hanseniaspora*, *Debaryomyces* and *Dekkera* are stronger producers of such enzymes when compared to the *S. cerevisiae* (*16*). *Saccharomyces* also produces acetic acid but generally not in a concentration above the threshold for detection. Nevertheless, many commercial strains are available with little to no acetic acid production.

There are numerous wine aroma precursors at different fermentation stages and they can have a positive or negative impact on wine aroma (Figure 4). There are several spoilage species, which produce metabolites that give bad flavor to the wine. The most common species are *Brettanomyces*/*Dekkera bruxellensis* and *Zigossacharomyces bailii*. The yeast *Brettanomyces*/*Dekkera* produces 4-ethyl phenol which confers an unpleasant horse seat aroma. Some examples of spoilage bacteria are: *Lactobacillus* (*4*), some species of *Acetobacter* (*3*) and *Glucanobacter oxydans*. A case of a bad flavor precursor is the acetic acid produced via ethanol consumption by acetic acid bacteria, giving a vinegar flavor to wine.

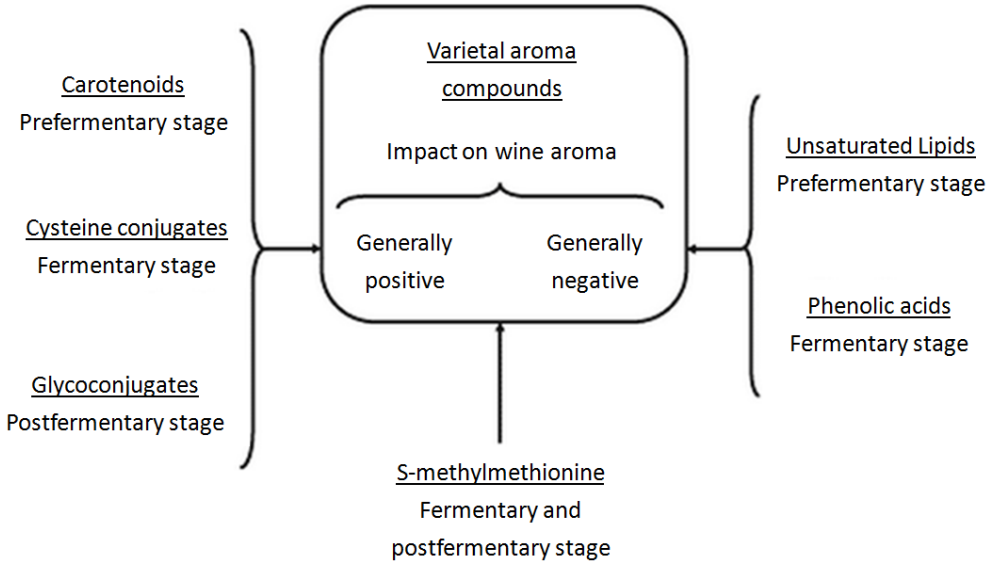


Figure 4. Wine aroma precursors, their main stages of degradation during the wine biotechnological sequence and general impact on wine aroma of the odorants generated (*40*)**.**

1.3. Commercial *S. cerevisiae* strains

A constant risk for the producer are stuck fermentations, this origins great lost of investment. To guarantee a successful fermentation, winemakers currently apply dry yeast to the grape must (*40*). They have several characteristics that make them chosen, for example, its high fermentative power, tolerance to high alcohol concentration, synthesis of metabolites that originate desired sensorial properties and the absence of production of undesirable compounds. Thus, these yeasts provide greater efficiency and speed of fermentation. One of the most significant advantages of commercial yeast usage is that they can add a desired organoleptic characteristic. However, this procedure has serious consequences on the production of different regional wines and it leads to a gradual replacement of the indigenous microflora. This leads to a standardization of organoleptic characteristics of wines, which become more similar to popular wines in international markets and this can originate a loss of competitiveness of local wines (*7*).

1.4. Critical factors on grape must fermentation

Non-*Saccharomyces* yeasts grow during the early stage of the alcoholic fermentation as they have a low fermentative power and low resistance to high ethanol concentrations (*21*). As the ethanol concentration rises up to about 5%, it denatures proteins leading to cell death. Nevertheless, the ethanol tolerance varies from strain to strain. At the physiological level there are some traits that are particularly important for wine strains of *Saccharomyces*, such as, reasonable rate of fermentation, predictable fermentation characteristics, high ethanol and good temperature tolerance, sulfur dioxide tolerance, no production of hydrogen sulfide, acetic acid and ethyl carbamate, killer factor resistance and production of desired aroma and flavor characters.

Considering fermentation kinetics, it is problematic if they are too fast as well as too slow. If the kinetics is too fast, the fermentation may reach a high temperature, due to the rate of heat released from metabolism and may also lead to an increased loss of volatile components. Conversely, if kinetics is too slow, then it will take longer to the ethanol conversion increase, and allows for growing of non-*Saccharomyces* organisms, which can

produce unwanted metabolites and also becomes difficult to distinguish from a problematic fermentation. Temperature is another key factor that affects directly the microbial ecology of the grape must and its biochemical reactions (*45*). Temperature is a growth modeler, since *Saccharomyces* and non-*Saccharomyces* show differential growth rates. Some non-*Saccharomyces* have a better ability to grow at low temperature fermentations than the *Saccharomyces*. This may happen because they have an increased tolerance to ethanol at low temperatures (*17*, *45*). Grape must is frequently fermented at extremes of temperature to which *Saccharomyces* is tolerant. It is critical that if commercial strains are used they must not be inhibited by heat or by cold conditions. White wines are generally fermented at a temperature lower than the optimal to *S. cerevisiae* growth (12-14°C), while many red wines are fermented at temperatures at the upper limit of *S. cerevisiae* growth (35-42°C). If a commercial yeast is used it is also important that the yeast strains are tolerant to sulfur dioxide, which is used as an antimicrobial agent. *Saccharomyces* is able to survive in the presence of sulfur dioxide by metabolizing the SO_2 via the formation of acetaldehyde adjuncts.

2. Genomics overview

Understanding biological diversity at the whole genome level will provide insights into the origins of individual traits. Since we are now living in a post-genomic era progress at the comparative, structural, evolutionary and functional genomics level is seen. Nevertheless, bioinformatics is giving a great help as the number of results is increasing and the analysis is complex. New generation genomic tools were developed in order to speed up and improve the results obtained. The implementation of molecular tools has rapidly enhanced our knowledge of prokaryote abundance, diversity and function (*9*). Since new yeasts genome fingerprinting techniques are now available, there has been an exponential increase in yeasts genome studies accompanied by great progress in the understanding of yeasts strains diversity.

2.1. Genomic techniques overview for yeasts' identification by molecular methods

MET2 gene analysis is based on the principle of RFLP and Hansen & Kielland-Brandt (1994) used it for the identification of wine yeasts, *S. cerevisiae* and *S. bayanus (23).* RFLP technique comprises the breaking of the DNA by restriction enzymes in certain regions with base sequences and the separation of the resulting fragments by electrophoresis. Different profile bands or polymorphisms can be observed, thus exposing a genetic fingerprint of the organism. The MET2 gene codes for synthesis of homoserine acetyltransferase and the DNA sequences of this gene differ for these two species. Complimentary oligonucleotides amplify a part of the gene located on the outer flanks and a 580 bp amplicon is obtained. Restriction endonucleases, EcoRI and PstI, are used to cleave the MET2 gene amplicon of *S. cerevisiae* and *S. bayanus*, respectively (*32*). In the case of EcoRI, two fragments (369 bp, 211 bp) are obtained when the MET2 gene product of *S. cerevisiae* is cleaved (Figure 5). EcoRI doesn't cut *S. bayanus*' MET2 gene. For PstI the reverse effect occurs, whereby two fragments (365 bp, 215 bp) for *S. bayanus* are visible and no fragment is visualized in *S. cerevisiae*.

Figure 5. Recognition site of EcoRI.

Also the fact that the rDNA occurs in multiple copies, reflecting the need to produce large quantities of ribosomal subunits in cells, has facilitated its RFLP analysis, and the polymorphism allowed the differentiation of both species and strains. It should be noted that almost all the restriction sites that allow discrimination of individual strains are located in the hyper variable region of the intergenic spacer (*13*). The non-transcribed areas from ribosomal genes such as 18S gene, ITS region and 26S gene have been widely used by various authors to identify species in the *Saccharomyces sensu stricto* group (*2, 43, 46, 8, 6, 47, 13*). These transcription units are repeated between 100-200 times in the genome. Other regions include the internal transcribed spacer (ITS) (Figure 6) and external transcribed spacers (ETS), which are areas that are transcribed, but not processed. The transcription units are also separated by non-transcribed spacer (NTS).

These ribosomal regions have developed into the chosen target regions to identify phylogenetic relationships between yeasts (*20, 27, 11*).

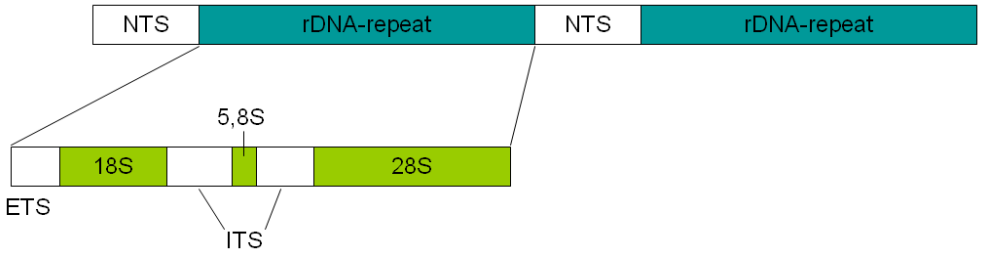


Figure 6. Amplification region of ITS1 and ITS4 primers (*20*).

2.2. Genomic techniques for differentiation of *S. cerevisiae* strains

In order to unveil the wide diversity of *S. cerevisiae* strains observed in wine fermentations there are several genotyping techniques that can be used to differentiate them, such as, pulsed field electrophoresis, mitochondrial DNA restriction, interdelta element amplification (*28*) and microsatellite fingerprint (*41*).

δ-sequences are present in the genome of *S. cerevisiae*, about 150-300 sequence repeats with a size of 300 bp. The interdelta sequences are repetitive DNA sequences present in the *S. cerevisiae* genome that have an intrinsic variability in number and location. By using specific primers it is possible to amplify the regions flanked by δ–sequences. The result of the PCR reaction is a mixture of fragments of sizes between 200 and 2000 bp. Since the inter-δ sequences distribution profile is highly variable in different *S. cerevisiae* strains, the molecular typing method has been used to differentiate oenological strains. These sequences have a good discriminatory power on commercial strains however they seem to have a lower discriminatory power on indigenous strains (*41*).

Another genotyping technique is the fingerprint of microsatellite or simple sequence repeat loci that are small DNA sequences tandem repeats (1-10 nucleotides) dispersed throughout the genome. Microsatellites present a high degree of variability which lead to their great discriminatory potential on the *S. cerevisiae* strains (*41*). Microsatellites are highly abundant in eukaryotic genomes but their function and evolution haven't yet been well understood. Their elevated mutation rate makes them ideal markers of genetic differentiation.

The complete sequence of the *S. cerevisiae* genome (*25*) has allowed the identification of these regions For the genotyping of *S. cerevisiae*, some of the most frequently utilized loci include YOR267C, SC8132X, SCPTSY7 (*44*); ScAAT1-ScAAT6 (*41*); YPL009C, C4, C5, C9 and C11 (*28, 39*). These loci can also be used for multiplex-PCR reactions where two or more loci are amplified as we can see in the GeneMapper software (Figure 7) (*48, 39*). Results are expressed as a number of repeats of the loci. These loci have been identified and used in studies to effectively discriminate between *S. cerevisiae* strains (*14, 37, 44, 31*) and evaluated to distinguish between commercially available yeast strains (*41, 28, 5, 48*). This technique has the same discriminatory resolution as interdelta regions, but less than electrophoretic karyotyping and has higher discriminatory power and it is more reproducible than RAPD and AFLP (*12*). The advantages of this technique are: the same method can be used in different organisms, computer compatibility, and highly reproducibility.

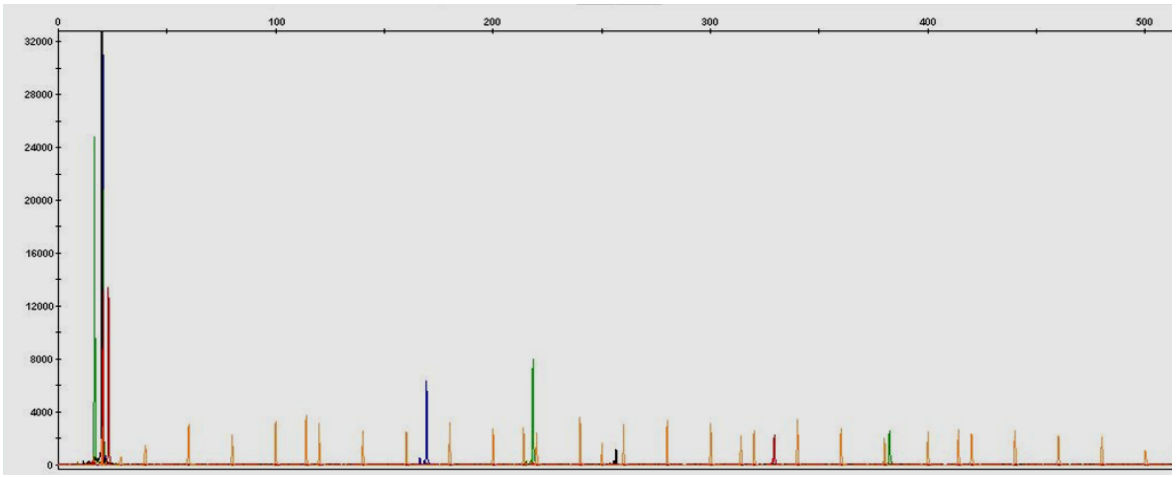


Figure 7. Results obtained with GenneMapper analyze software.

3. Objectives

In this work we aim to identify and cluster Portuguese endogenous wine yeasts in order to uncover the natural richness of fermentative yeasts. We also will create a *S. cerevisiae* strain collection contributing to the preservation of *S. cerevisiae* genetic resources. To achieve this, we have established two specific objectives:

i) to carry out spontaneous wine must fermentations and isolate the microorganisms responsible for their fermentation;
ii) to characterize wine yeasts, by microsatellites amplification and other molecular approaches.

With this we expect to gather valuable information to apply in winemaking.

Methods

1. Wine must collection and fermentation

In order to have a representative sample of the Portuguese endogenous biodiversity, grape and wine samples were collected in different Portuguese wine appellations, such as, Alentejo, Bairrada, Dão and Douro. The different grape varieties studied here are described in Table 1.

Table 1. Grape variety from Portuguese appellations.

Wine appellation	Grape variety
Alentejo	Touriga Nacional
	Tinta Roriz
	Trincadeira
Bairrada	Baga
	Bical
	Maria Gomes
Dão	Alfrocheiro
	Touriga Nacional
	Jaen
Douro	Touriga Nacional
	Touriga Franca
	Tinta Roriz

Grape and wine samples were collected, by the producers, in an appropriate bag and bottle, respectively, and sent to the laboratory in a cooled box. In the laboratory, grapes were crushed in a bag in aseptic conditions. Then the resulting must was placed in a sterile 500 mL erlenmeyer. All fermentations were carried out at room temperature except for some of the Bical and Maria Gomes fermentations that were tested with different fermentation temperatures, described in Table 2. The fermentation kinetic was followed every day by measuring the weight loss. Grape must samples were collected from each fermentation at three different moments: the initial must, the beginning of the fermentation (5 g/L loss), and the end (70 g/L loss). Samples from fermentations occurring in the cellar were collected at the beginning and at the end of the fermentation process. For each sampling stage 100 mL were filtered into falcon tubes and centrifuged at 4000 rpm. The pellet was collected and cryopreserved in 40% w/V glycerol at -80ºC.

Table 2. Fermentation temperature and code.

Grape variety	Fermentation temperature (°C)	Fermentation code
Bical	12	BBi0817
		BBi0917
		BBi1117
	18	BBi0818
		BBi0918
		BBi1118
	25	BBi0819
		BBi0919
		BBi1119
Maria Gomes	12	BM1217
		BM1317
		BM1417
	18	BM1218
		BM1318
		BM1418
	25	BM1219
		BM1319
		BM1419

2. Yeasts isolation and cryopreservation

At the end of the fermentation, the filtered must, from the previous step were diluted to 10^{-4} times and plated in agar YPD medium (1% w/v yeast extract, 2% w/v glucose, 2% w/v peptone) and placed in an incubator at 30ºC during 48 hours so that yeasts could be isolated. For yeasts' isolation from the 12°C fermentations three incubation temperatures were tested: 30°C, 12°C and 4°C for 48h. Pure cultures were isolated and grown in the same conditions and preserved in YPD liquid medium with 40% w/V glycerol at -80ºC.

3. Genomic DNA isolation

DNA isolation was done on 1 mL pure cultures, cells were collected by centrifugation and washed twice with ultrapure water. Cells were then resuspended in 100 µL of lysis buffer (1 M sorbitol, 0,1M Na_2-EDTA, 7,5 pH) and then 5 µL of lyticase (5U/µL) were added. The mixture was briefly vortexed and incubated at 37ºC during 1 hour. Then 100 µL of a second solution (50mM Tris-HCl, 20mM EDTA-Na_2, 7,4 pH) and 5 µL of 10% w/v SDS were added, the mixture was vortexed and incubated at 65ºC during 10 minutes. After that, 80 µL of 5 M potassium acetate were added and the samples were incubated on ice during at least 10 minutes. The cellular debris and proteins present were pelleted by centrifugation and the supernatant, containing the DNA was collected into a new tube with 1 volume of isopropanol. The solution was mixed and incubated for 10 minutes at -20ºC allowing for DNA precipitation, which was then recovered by another centrifugation step. The supernatant was discarded and the DNA pellet was washed with 70% ethanol and dried for 3 minutes under vacuum. Finally, DNA was resuspended in 50 µL of TE (10mM Tris-HCl, 1mM Na_2-EDTA, 7,5 pH) and stored at -20ºC (*38*).

4. Yeasts identification

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces* yeasts, MET2 gene amplifications were performed in an Eppendorf thermal cycler using synthetic oligonucleotide primers for MET2. Amplifications were carried out in 25 µL reaction volumes containing 100 ng DNA, 1x reaction buffer (100mM Tris-HCl (pH 8,8 at 25 ºC, 500 mM KCl, 0,8% igepal), 1,7 mM $MgCl_2$, 0,2 µM of each primer (MET2_F: CGA AAA CGC TCC AAG AGC TGG and MET2_R: GAC CAC GAT ATG CAC CAG GCA), 0,2 mM of each dNTP (Bioron) and 0,5 U/µL taq DNA polymerase. The cyclic program is described in Table 3. The EcoRI digestion was done on reactions of 50 µL where 20 µL of the PCR product, 5 µL of 10x NEbuffer EcoRI (Fermentas) and 0,5 µL of the EcoRI enzyme (20 U/µL) (Fermentas) were used. MET2 gene and resulting restriction fragments were analyzed on LabChip.

Table 3. MET2 gene PCR run program.

Step	Temperature (ºC)	Time	Number of cycles
Initial denaturation	94	3 minutes	1
Denaturation	94	1 min	
Annealing	60	1 min	35
Extension	72	1 min and 30 sec	
Final extension	72	5 min	1

Those isolates that showed no amplification by PCR of MET2 were classified as non-*Saccharomyces* species, and were then identified by amplification of ITS gene region, in 25 µL reaction volumes containing 100 ng DNA, 1x reaction buffer (100mM Tris-HCl (pH 8.8 at 25 ºC, 500 mM KCl, 0,8% igepal), 2 mM $MgCl_2$, 0,4 µM of each primer (ITS1: TCC GTA GGT GAA CCT GCG G and ITS4: TCC TCC GCT TAT TGA TAT GC), 0,2 mM of each dNTP (Bioron) and 2,5 U/µL taq DNA polymerase. The cyclic program is described in Table 4. The PCR product was analyzed on LabChip® 90 and the size of the fragments compared with the values presented on Table 5.

Table 4. ITS PCR run program.

Step	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	6 minutes	1
Denaturation	94	40 sec	
Annealing	53	40 sec	35
Extension	72	1 min	
Final extension	72	5 min	1

Table 5. ITS fragment size and corresponding species (adapted from (*20, 18*)).

Species	Amplicon (bp)
Schizosaccharomyces pombe	970
Saccharomyces bayanus *Saccharomyces cerevisiae* *Saccharomyces pastorianus*	880
Candida colliculosa	810
Torulaspora delbrueckii	803
Candida glabrata	800
Hanseniaspora uvarum *Kloeckera apiculata*	760
Saccharomyces ludwigii	758
Zygosaccharomyces baiili	750
Candida boidinii	700
Kluyveromyces thermotolerans	682
Candida tenuis	680
Candida famata *Debarymoces hansenii*	656
Candida zeylanoides	620
Pichia guilliermondii	606
Candida norvegica	580
Candida albicans *Candida tropicalis*	550
Candida parapsilosis	520
Dekkera anómala	514
Brettanomyces bruxellensis *Candida stellata* *Dekkera intermédia* *Pichia membranaefaciens*	500
Issatchenkia orientalis	494
Candida sake *Pichia fermentans* *Pichia kluyveri*	470
Dekkera bruxellensis	459
Metschnikowia pulcherrima	390

5. Genomic fingerprinting by microsatellite amplification

In order to group *S. cerevisiae* strains the amplification of microsatellites was done. The eleven microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5, ScAAT6, SYOR267c, C4, C5, C9 and C11 (Table 6) were amplified in two multiplex reactions using 25-100 ng of template DNA (*28*). The first multiplex reaction (A) contained 0,016 µM of ScAAT1F (F- forward), ScAAT1R (R-reverse), ScAAT3F and of ScAAT3R, 0,0068 µM of ScAAT2F, 0,039 µM of ScAAT2R, 0,015 µM of ScAAT4F and of ScAAT4R, 0,00078 µM of ScAAT5F and 0,013 µM of ScAAT5R. The second multiplex reaction (B) contained 0,0115 µM of ScAAT6F, 0,015 µM of ScAAT6R, 0,01 µM of SYOR267cF, SYOR267cR, C5F, and of C5R, 0,02 µM of C4F, 0,025 µM of C4R, 0,009 µM of C9F and of C9R, 0,015 µM of C11F and 0,02 µM of C11R. The forward primer of all microsatellite was labelled with fluorescent dye. In both multiplexes the total volume of the reaction was 10 µL and the run was done in an Eppendorf thermal cycler. The run program for each reaction is described on Table 7 and Table 8.

Table 6. Characteristics and polymorphism of 11 loci (*28*).

Name	Motif and type	Primers	Size/number of repeats
SCAAT1	TAA	FW: AAAGCGTAAGCAATGGTGTAGATACTT RV: CAAGCCTCTTCAAGCATGACCTTT	240/35
SCAAT2	TAA	FW: CAGTCTTATTGCCTTGAACGA RV: GTCTCCATCCTCCAAACAGCC	373/20
SCAAT3	TAA	FW: TGGGAGGAGGGAAATGGACAG RV: TTCAGTTACCCGCACAATCTA	199/24
SCAAT4	TAA+TAG (i)	FW: AGGAGAAAAATGCTGTTTATTCTGACC RV: TTTTCCTCCGGGACGTGAAATA	235/13
SCAAT5	TAA	FW: AGCATAATTGGAGGCAGTAAAGCA RV: TCTCCGTCTTTTTTGTACTGCGTG	168/12
SCAAT6	TAA	FW: TGGCTACAGCACTTGCTGAACAT RV: GGGAAAACTAGATCCAGGATTGG	172/19
C9	TAA	FW: AAGGGTTCGTAAACATATAACTGGCA RV: TATAAGGGAAAAGAGCACGATGGC	92/9
C4	TAA+TAG (i)	FW: AGGAGAAAAATGCTGTTTATTCTGACC RV: TTTTCCTCCGGGACGTGAAATA	235/13+5
C5	GT	FW: TGACACAATAGCAATGGCCTTCA RV: GCAAGCGACTAGAACAACAATCACA	165/30

Name	Motif and type	Primers	Size/number of repeats
C11	GT	FW: TTCCATCATAACCGTCTGGGATT RV: TGCCTTTTTCTTAGATGGGCTTTC	203/20
SYOR267c	TGT	FW: TACTAACGTCAACACTGCTGCCAA RV: GGATCTACTTGCAGTATACGGG	186/21

(i) – imperfect motif.

Table 7. Multiplex A PCR run program.

Step	Temperature (ºC)	Time	Number of cycles
Initial denaturation	94	15 minutes	1
Denaturation	95	15 seconds	
Annealing	58*	1 min 30 sec	10
Extension	72	30 sec	
Denaturation	94	30 sec	
Annealing	48	1 min 30 sec	25
Extension	72	30 sec	
Final extension	72	5 min	1

* - decrease of one degree at each cycle.

Table 8. Multiplex B PCR run program.

Step	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	94	30 sec	
Annealing	53	30 sec	34
Extension	68	1 min	
Final extension	68	10 min	1

The amplified PCR products were then analysed on the Sanger sequencer. For this, 7,5 µL of formamide and 0,5 µL of molecular size standard (Liz Genescan 600 Rox Applied Biosystems) were added to 1 µL of the PCR product. This mix was transferred to a Bioplatics 96x0,2 ml plate and denatured for 3 minutes at 95ºC and then kept on ice to cool down. The plate was then loaded on the AB 3500 genetic analyzer (Applied Biosystems) and the program Fragment Analysis Assay run. The analysis was done using the BioNumerics (Applied Maths) with the clustering Jaccard correlation UPGMA and the Principal component analysis (PCA).

Results and Discussion

1. Introduction

Given that the alcoholic fermentation is a key process on winemaking and that yeasts have the major role at this process, it is the objective of this work to monitor the biodiversity of the fermentative yeasts and genotype *S. cerevisiae* since they represent the majority fermentative population. In order to do so, we studied the wine cellar biodiversity and white wine spontaneous fermentations at low temperatures.

2. Wine cellar biodiversity study

2.1. Yeasts' isolation and identification

Yeasts were isolated from wine cellar fermentations in order to exploit the endogenous biodiversity of the wine cellar. This study was done in four Portuguese wine appellations: Alentejo, Bairrada, Dão and Douro. From all the 12 fermentations 30 yeasts were isolated at the end of the fermentation to gather information of the fermentative microflora.

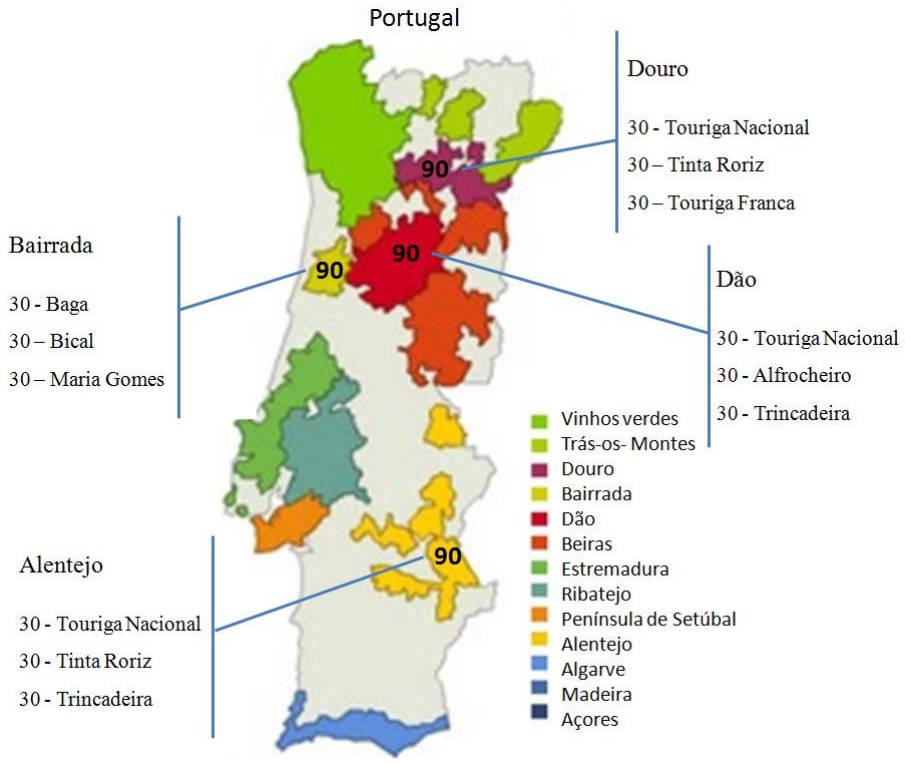


Figure 8. Number of yeast isolates from cellar fermentations in Portuguese wine appellation.

From the three grape varieties from the four regions, 30 yeasts were isolated each makes a total of 360 yeasts from wine cellars fermentations (Figure 8). The isolation was done by inoculating a wine sample in YPD medium. Wine yeasts were isolated following classical microbiology procedures. We successfully achieved the isolation of pure cultures of the fermentative population (Figure 9).

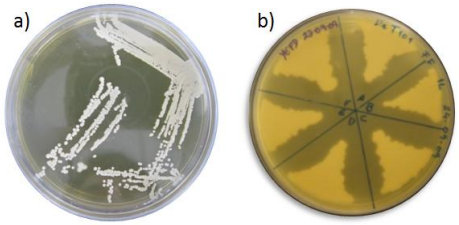


Figure 9. Wine yeasts isolation.

a) Wine yeast isolation on YPD medium. **b)** Pure yeast colonies isolated from the first plate.

After having effectively extracted the DNA from each yeast isolate and diluted it to 100 ng, the identification was attempted by the MET2 gene amplification and enzymatic restriction. In *S. cerevisiae* the MET2 gene amplification generates a fragment of 580 bp and its EcoRI restriction profile corresponds to two bands, of 369 and 211 bp each. The PCR product of the MET2 gene amplification followed by the enzymatic restriction with EcoRI, run in Labchip (Figure 10), showed a high number of *Saccharomyces cerevisiae* present at the end of the fermentation as expected (*35*, *22*, *15*).

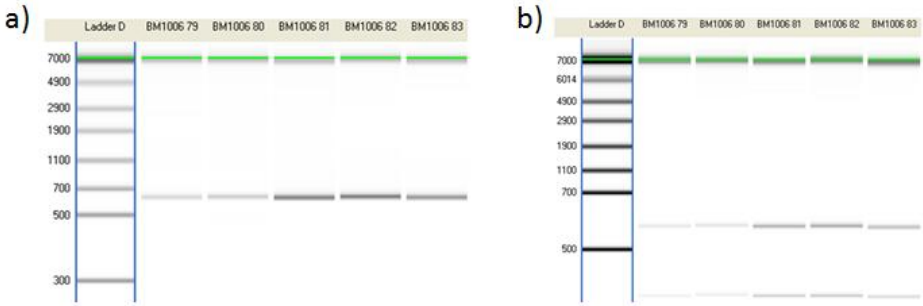


Figure 10. Eletrophoretic band profile on Labchip.

a) MET2 gene amplification eletrophoretic band profile. **b)** Restriction of MET2 gene with EcoRI eletrophoretic band profile.

All the fermentations carried out at the cellars had an homogeneous population of *S. cerevisiae*, which is in line with the described yeast populations in wine cellars. At Douro appellation, commercial strains of *S. cerevisiae* were added to the Touriga Nacional and Tinta Roriz fermentations, so the *S. cerevisiae* abundance found was also expected.

2.2. *S. cerevisiae* genotyping by microsatellite amplification

All *S. cerevisiae* strains isolated from wine cellar fermentations were tested with the 11 loci and then clustered in order to have a strain differentiation. The clustering was done with all microsatellite loci except for the ScAAT4 since its amplification in the multiplex A reaction was not favored, and not fully optimized, specially for poor DNA quality samples.

From Alentejo wine appellation three different grape varieties were studied. A total of 58 strains were identified by clustering the three fermentations. Touriga Nacional variety had 16 *S. cerevisiae* strains, Trincadeira had 21 strains and Tinta Roriz had 25 strains discriminated by microsatellites amplification. For a more global understanding of the proximity of the strains we made a Principal Component Analysis (PCA). Below we can observe that the majority of the strains are very close when we look from the three dimensional point of view, except for three isolates from Tinta Roriz fermentation, showing a similar endogenous yeast population, but the strains are still distinct (Figure 11).

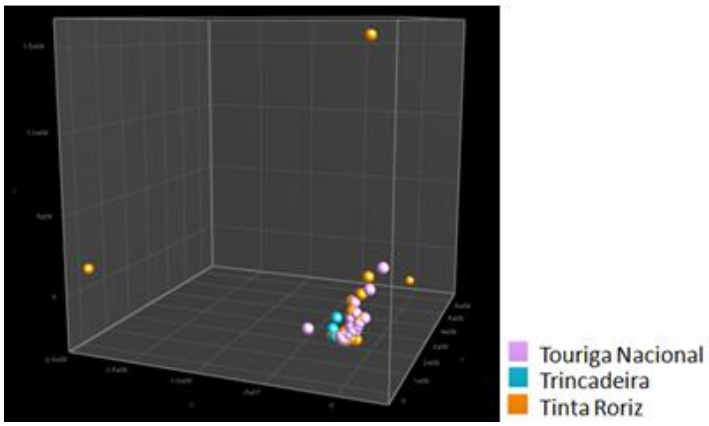


Figure 11. PCA of *S. cerevisiae* strains present at Alentejo fermentations.

From Dão wine appellation a total of 79 strains were identified by clustering the three fermentations. Touriga Nacional fermentations had 29 *S. cerevisiae* strains, Jaen had 28 strains and Alfrocheiro had 24 strains discriminated. The discriminative power was also very high but in fact some strains have the same microsatellites, differing only in one of the microsatellites tested. The strains from Dão fermentations seem to be all somehow related, not clustering according to the grape variety (Figure 12). Only some of the

Alfrocheiro strains are clustering with strains from the same fermentation. This result shows the great diversity of the endogenous population from the wine cellar.

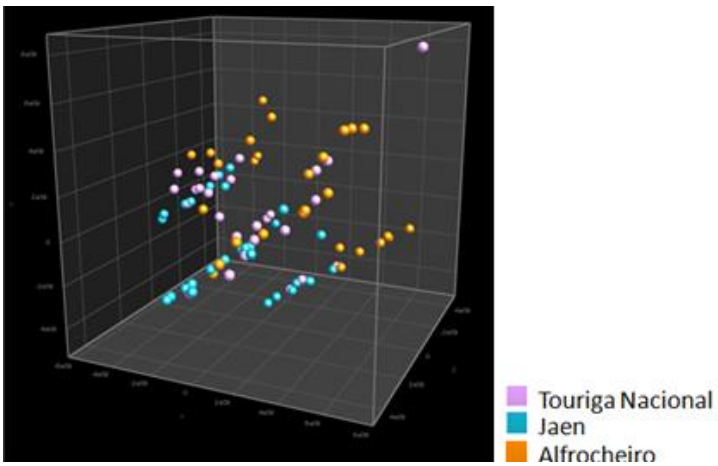


Figure 12. PCA of *S. cerevisiae* strains present at Dão fermentations.
Strains isolated at the end of the fermentations of the grape varieties Touriga Nacional, Jaen and Alfrocheiro.

From Douro wine appellation three different grape varieties were also studied. A total of 71 strains were identified by clustering the three fermentations. At the Touriga Nacional fermentation were found 28 *S. cerevisiae* strains, at Touriga Franca 29 strains and at Tinta Roriz 21 strains. The discriminative power was very high but still some of the strains are related (Figure 13). This result was expected since different commercial strains were used in each fermentation of this wine appellation. In fact, only one different *S. cerevisiae* strain was applied to each one of the Douro's fermentation and at the end of the fermentation we observed large strain diversity. Indeed, the initial strain suffered several divisions along the fermentation and during this divisions can occur genomic phenomena that can origin new related strains. For what concerns the fermentations from this appellation, we should have compared the microsatellite profile of the commercial yeast inoculated to conclude if the fermentation was carried out by the applied yeast, but unfortunately we didn't have access to it.

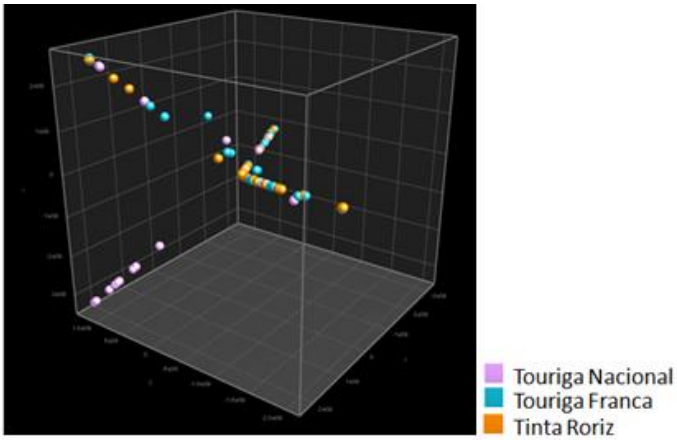


Figure 13. PCA of *S. cerevisiae* strains present at Douro fermentations.
Strains isolated at the end of the fermentations of the grape varieties Touriga Nacional, Touriga Franca and Tinta Roriz.

Comparing all the Touriga Nacional fermentations from Alentejo, Dão and Douro wine appellations we observe that there are strains close to each other, but there are also strains characteristic of their wine appellation (Figure 14). Alentejo strains already clustered together, so here it was already expected to observe this result. These results show the importance of the preservation of endogenous population.

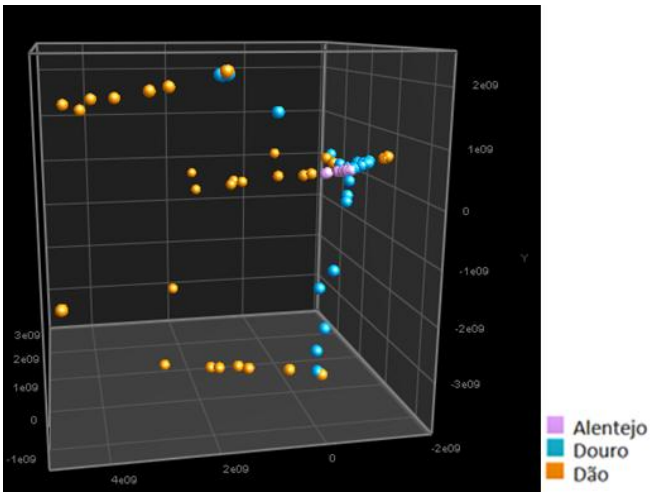


Figure 14. PCA of *S. cerevisiae* strains present at Touriga Nacional variety from Alentejo, Douro and Dão fermentations.

Comparing all the strains of the fermentations from Alentejo, Dão and Douro, it is clear that each population clusters apart, but still, there are some strains that are closely related to some of the strains from other appellations (Figure 15). Strains from Dão seem to be divided in three baselines but each one does not represent isolates from just one fermentation.

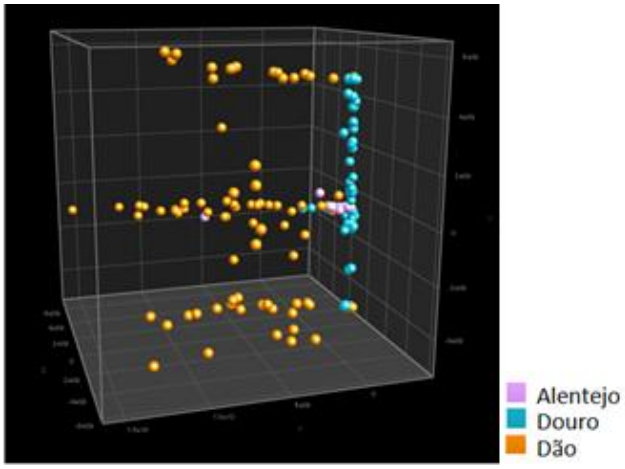


Figure 15. PCA of *S. cerevisiae* strains present at Alentejo, Douro and Dão fermentations.

When comparing all *S. cerevisiae* strains form the same three wine appellations, by clustering them with the Bionumerics UPGMA tool, we observe that the majority of the strains seem to cluster according to the wine appellation (Figure 16). There are no strains in common in these wine appellations, so there is a characteristic population of *S. cerevisiae* strains of each wine appellation.

For what concerns the wine cellar fermentations from Bairrada wine appellation, at Baga variety fermentation we observed 27 different *S. cerevisiae* strains (Annex A 1). In the Maria Gomes variety fermentation 19 strains were differentiated from the 30 yeasts isolated (Annex A 2). In the Bical variety fermentation 25 strains were obtained (Annex A 3). The majority of the strains are very similar, showing a connected population for each one of the grape varieties.

Each one of the *S. cerevisiae* population found at the fermentations on the cellar could be explained by the resident yeast population at the winery (*33*). The samples studied from wine cellar fermentations gave us key information about the microflora responsible for the metabolic profile of each variety and wine appellation. In the fermentations that didn't suffer the appliance of commercial strains the biodiversity found represents the autochthonous yeast population characteristic from each fermentation, wine cellar and wine appellation.

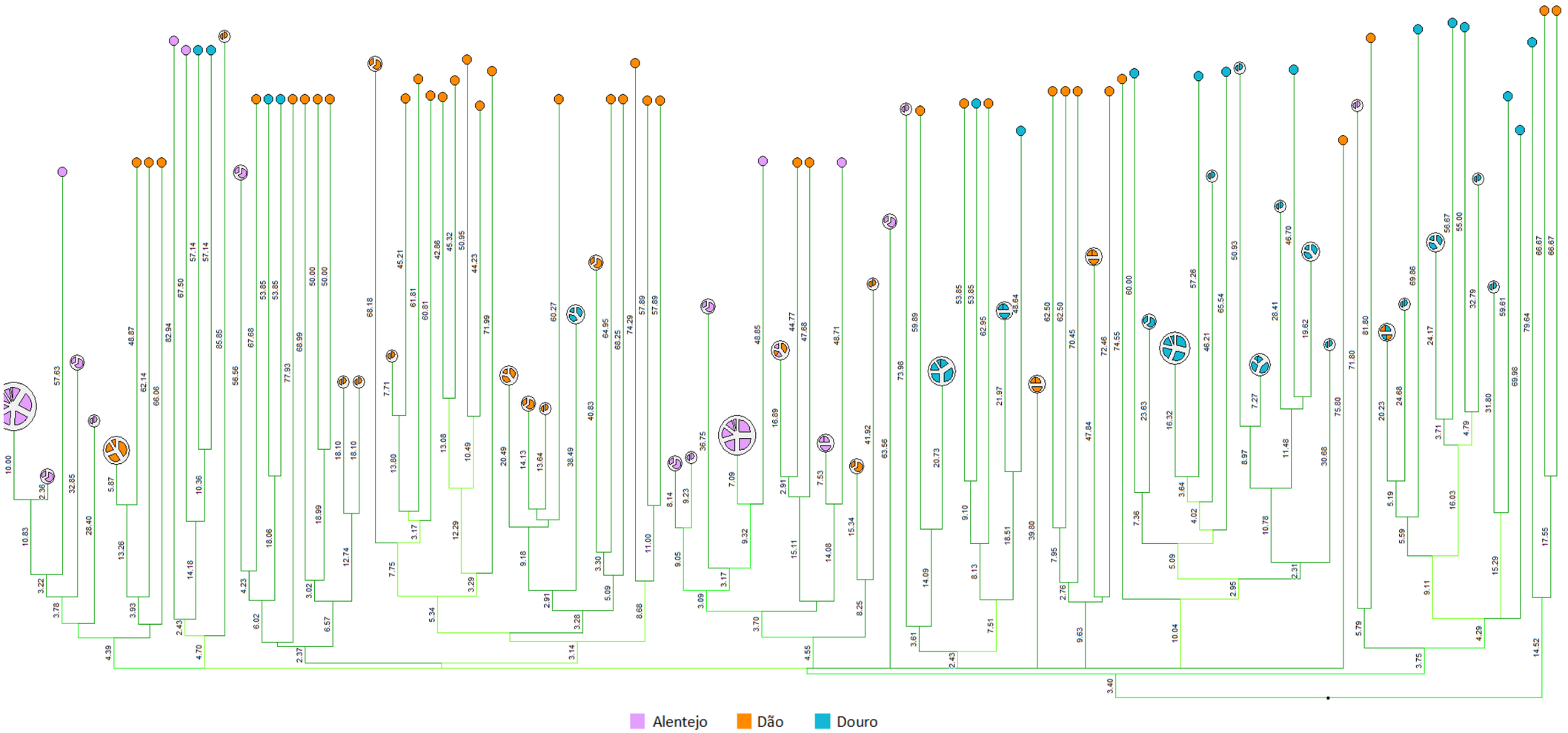


Figure 16. Cluster of *S. cerevisiae* strains from Alentejo, Dão and Douro wine appellations.

Cluster done using the Bionumerics UPGMA tool.

3. Biodiversity of white wine fermentations at low temperatures

White wines fermentations are carried out at low temperatures to maximize the organoleptic properties of the wines and to preserve their floral and fruity properties (1). Therefore, it is necessary to use yeasts strains that are able to carry out the fermentations at such low temperatures. Considering this, we have carried out spontaneous fermentations of white grapes (Bical and Maria Gomes), at different temperatures (12°, 18° and 25°C), and then isolate the present microorganisms that were responsible for their fermentation.

3.1. Spontaneous fermentations kinetics

In the spontaneous fermentations that were carried out in the laboratory, kinetics was followed by monitoring the weight loss: we have considered a 2 g/L loss the beginning of the fermentation and a 70 g/L loss the end of the fermentation. Almost all fermentations reached the end, except for the two Bical fermentations at 18°C (Figure 17). This was due to a calculation error that led us to think that the fermentation had already reached the end, so these two samples represented the middle of the fermentations. The wine must fermentations were successfully followed until the end where we were able to isolate the fermentative yeasts. As expected the fermentations at 25°C had faster kinetics than those carried out at 12°C. It should be noted that the BBi1119 was the fermentations with faster kinetics, which corresponds to fermentations at 25°C of the Bical grape variety. As Bical, the Maria Gomes fermentation at 25°C (BM1219) was the fermentation with faster kinetics.

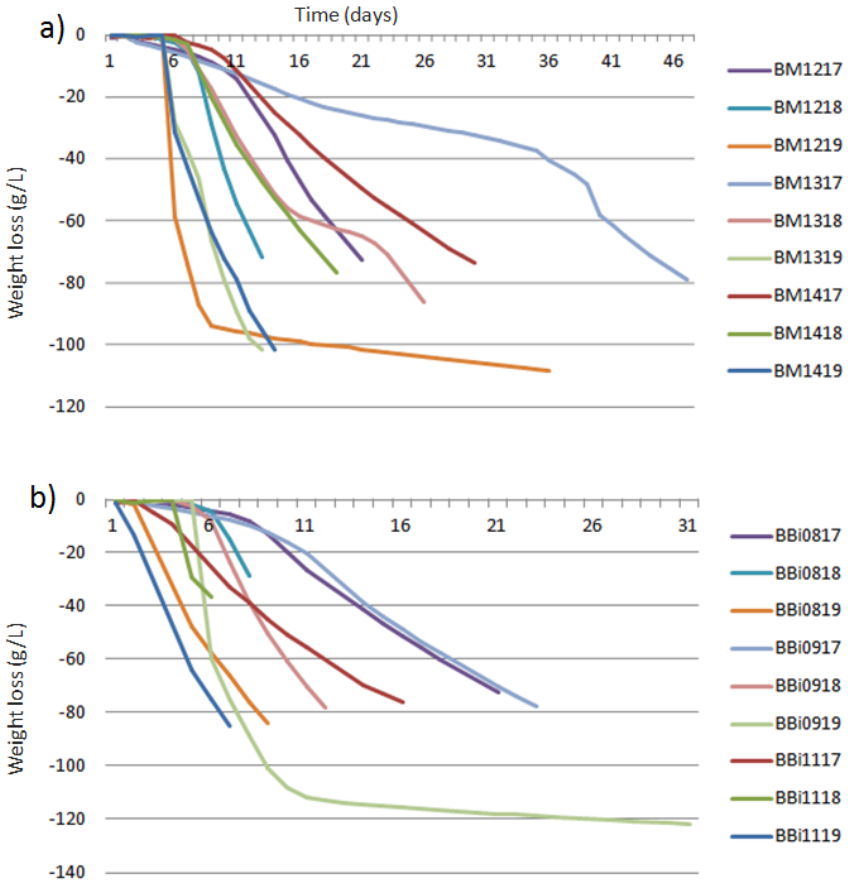


Figure 17. Fermentations kinetics.
a) Fermentation kinetics of Maria Gomes variety fermentations. **b)** Fermentation kinetics of Bical variety fermentations. 17 represent a fermentation that occurred at 12°C, 18 a fermentation at 18°C and 19 a fermentation at 25°C.

3.2. Yeasts' isolation and identification

We then studied the yeast biodiversity of these fermentations. The yeasts' isolation was according to the usual grow rate at 30°C, but for the 12° and 4°C colonies took more days to grow. For 12°C isolation took approximately 15 days. At 4°C colonies took in most cases a month to grow. This was predictable since by lowering the temperature we are increasing the yeasts' stress. The Bical fermentations that occurred at 25°C were carried out by a population of only *S. cerevisiae* (Figure 18). Likewise, the fermentations at 18°C were mostly carried out by *S. cerevisiae*, except in the Bical 08 fermentation where one isolate of *Candida zemplinina* was present. For the fermentations that were carried out at 12°C isolated yeasts were grown at three different incubation temperatures: 30°C, 12°C and 4°C. In Figure 18 we can observe the differences in the fermentative population, while at 30°C we are favouring the *S. cerevisiae* growth, when decreasing the incubation

temperature, we are favouring the non-*Saccharomyces* growth. At the incubation temperature of 12°C we have isolated *H. uvarum* from Bical variety (BBi08). From this fermentation, we have also isolated *M. pulcherrima* and *C. zemplinina* (grown at 4°C) and *S. cerevisiae* (grown at 30°C). On the Bical 09 fermentation, we have isolated *M. pulcherrima* when isolating at 4° and 12°C, and *S. cerevisiae* when yeasts were isolated at 30°C.

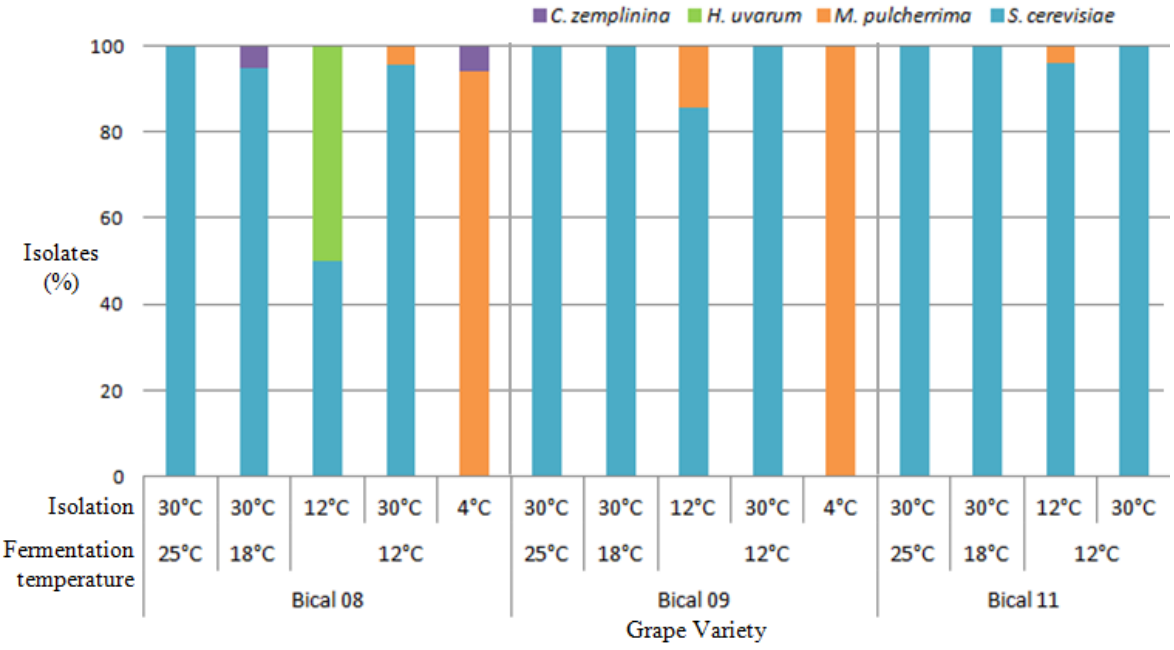


Figure 18. Biodiversity of Bical spontaneous fermentations.
Biodiversity of Bical spontaneous fermentations that occurred at different fermentation and isolation temperatures.

The Maria Gomes variety fermentations were also studied on samples from three different grape producers and at three different fermentation temperatures (25°, 18° and 12°C). The fermentations that occurred at 25°C and 18°C were all carried out by a population of *S. cerevisiae* (Figure 19). However, in the fermentations at 12°C was also found the *M. pulcherrima* which represent the majority of the non-*Saccharomyces* population. Again, yeasts' isolation from the fermentations at 12°C was also made at three different incubation temperatures 30°C, 12°C and 4°C. From the isolations at 12°C we have observed *K. thermotolerans* and *M. chrysoperlae* in a very low account (1 and 2 yeasts isolates, respectively). It should be noted that at the fermentations at 12°C were also isolated *S. cerevisiae* which for the Mª Gomes 12 represents 50% of the fermentative population and around 80% for the sample 12 and 14 at the 12°C isolation temperature.

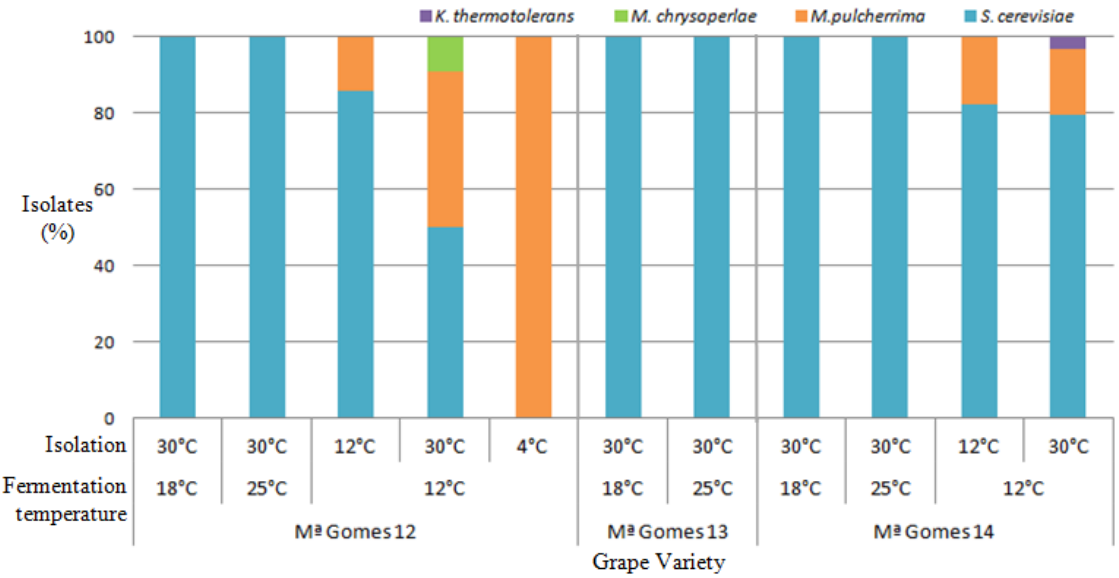


Figure 19. Biodiversity of Maria Gomes spontaneous fermentations.
Biodiversity of Maria Gomes spontaneous fermentations that occurred at different fermentation and isolation temperatures.

Regarding the identification, only few yeast isolates were not identified because in that cases the DNA didn't have good quality. The results obtained for the yeasts identification were in accordance with the literature since at the end of the fermentation where the pH is low and the ethanol concentration is high *S. cerevisiae* represents the majority of the population (*15*). This can be explained by their ability to tolerate high ethanol concentrations. Besides the high presence of *S. cerevisiae* strains on the studied fermentations, on the fermentations at 12°C the non-*Saccharomyces* stood out being able to achieve the end of the fermentations of Bical and Maria Gomes varieties. Several authors suggest that non-*Saccharomyces* can enhance their tolerance to ethanol at low temperatures and this could explain the fact that they appear at the end of the mentioned fermentations (*42*, *17*, *24*, *45*). In Bairrada fermentations, the non-*Saccharomyces* that stand out is *M. pulcherrima* being present in almost all the spontaneous fermentations. The interest on the use of mixed starter cultures of *Saccharomyces* and non-*Saccharomyces* has increased. The aim of this method is to enhance the quality and complexity of wines. Different mixed starter cultures, such as *S. cerevisiae* and *M. pulcherrima* have been experimented and the result was a positive interaction that result in a significant reduction of the volatile acidity (*10*). Therefore, the *M. pulcherrima* isolated here could have enological potential for Bairrada wines.

These results highlight that, despite of being mostly carried out by *S. cerevisiae*, in those fermentations the non-*Saccharomyces* populations also plays an important role, which could be of particular importance for the production of unique white wines.

3.3. *S. cerevisiae* genotyping by microsatellite amplification

In the laboratory, the *S. cerevisiae* isolates from the spontaneous fermentations from Bairrada were genotyped. The microsatellites analysis was done in the 529 *S. cerevisiae* isolated strains. At Bical fermentative population a total of 247 *S. cerevisiae* strains were differentiated and from Maria Gomes fermentative population 145 strains were differentiated (Table 9).

Table 9. Amount of strains from Bairrada spontaneous fermentations.

Grape variety	Fermentation temperature (°C)	Isolation temperature (°C)	Number of *S. cerevisiae*	Number of strains
Bical	25	30	85	77
	18	30	78	71
	12	30	68	57
		12	58	55
Maria Gomes	25	30	90	54
	18	30	79	60
	12	30	34	17
		12	37	18

Strains from the grape samples of Bical variety fermentations seem to cluster, based in microsatellite profile, randomly, only the strains from the sample BBi09 cluster closely (Figure 20). This result was expected since fermentations from the same grape sample fermented at distinct temperatures.

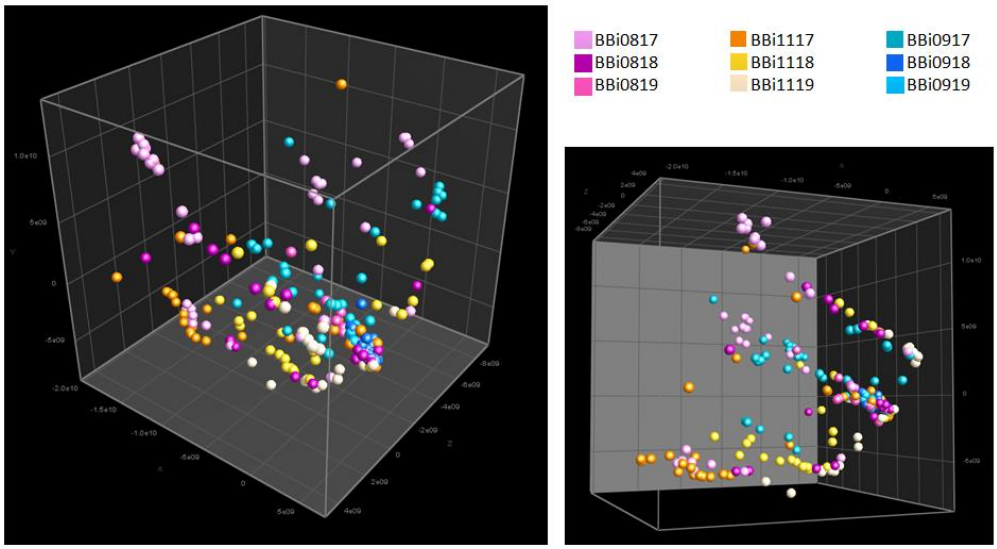


Figure 20. PCA of *S. cerevisiae* strains present at Bical fermentations.
17 represent a fermentation that occurred at 12°C, 18 a fermentation at 18°C and 19 a fermentation at 25°C.

At the Maria Gomes fermentation the strains seem to cluster according to the grape sample particularly 13 and 14 (Figure 21). This reveals that the endogenous yeast population from each vineyard is characteristic of each one.

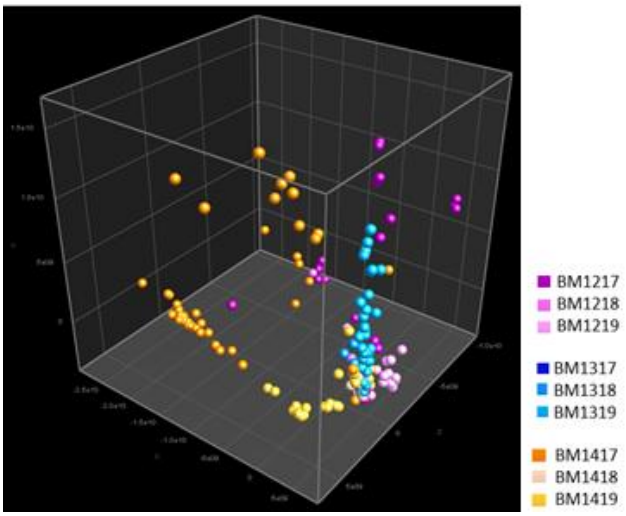


Figure 21. PCA of *S. cerevisiae* strains present at Maria Gomes fermentations.
17 represent a fermentation that occurred at 12°C, 18 a fermentation at 18°C and 19 a fermentation at 25°C.

3.2.1. Effect of temperature on strains diversity

We analyzed the variability of *S. cerevisiae* strains that were present at the end of the fermentations that occurred at 12ºC in Bical and Maria Gomes varieties, in order to understand the effect of temperature on the strains diversity. Here we observe strains from Bical fermentation where some strains that grow at 30ºC are also able to grow at 12ºC and both strains ferment at 12ºC (Figure 22). Several strains from this fermentation are closely related at the phylogenic level, and only some of the 12ºC fermentations are clustering apart.

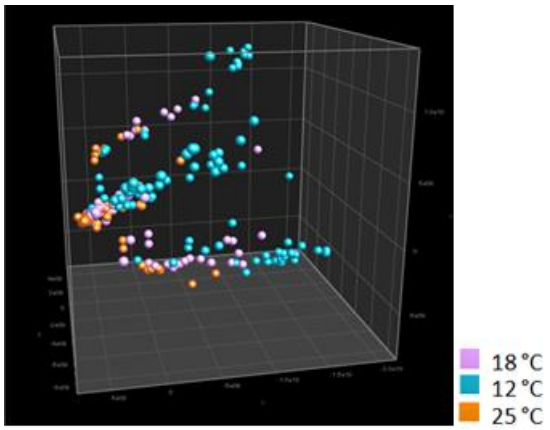


Figure 22. PCA of *S. cerevisiae* strains present at Bical fermentations from different fermentation temperatures.

Figure 23 shows no clustering differentiation for the two isolation temperatures (30º and 12ºC). This result may be due to the fact that the fermentation where they come from is the same and the selective pressure of temperature have already been made before the isolation, since the fermentation occurred at 12ºC.

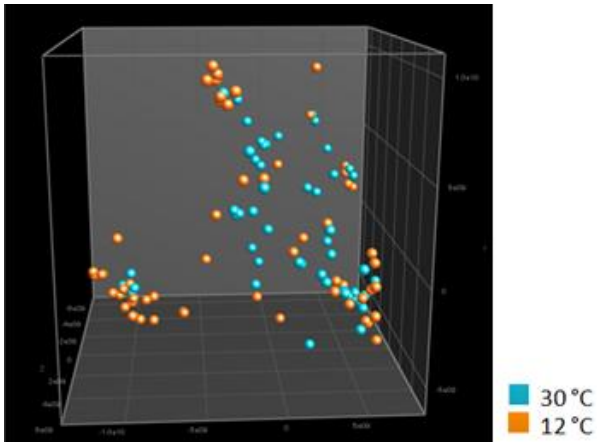


Figure 23. PCA of *S. cerevisiae* strains present at Bical from 12ºC fermentations from different temperatures of isolation (12ºC and 30ºC).

At Figure 24 we observe *S. cerevisiae* strains from different temperatures of Maria Gomes fermentation. The strains from 12ºC fermentation seem to have a characteristic endogenous population but some of the strains cluster together whit the other fermentations strains. The strains from the fermentations at 18º and 25ºC are more related than to the 12ºC fermentation. This could be because 12ºC is a more stressful environment than 18º or 25ºC.

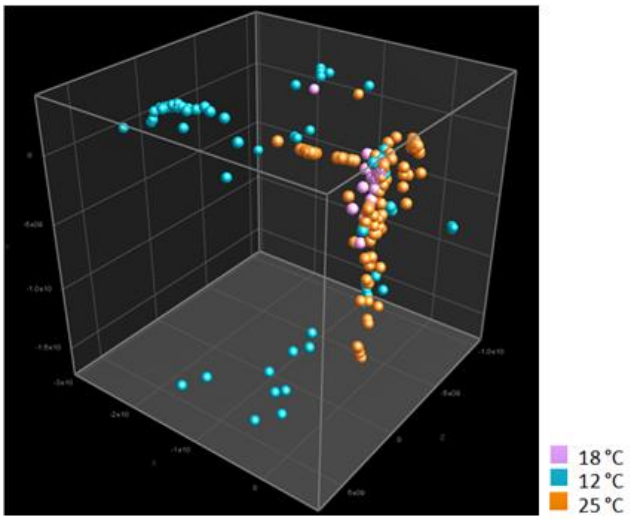


Figure 24. PCA of *S. cerevisiae* strains present at Maria Gomes fermentations from different fermentation temperatures (12°, 18° and 25°C).

Here we observed that the fermentation temperature is a key factor for the *S. cerevisiae* strains diversity, which may also influence the metabolic profile of the wine and consequently their organoleptic characteristics.

4. Allelic frequency and heterozygosity of the Portuguese *S. cerevisiae* population

For what concerns the allelic frequency of each one of the locus in study are presented in Table 10 and Table 11 where we can observe that the allelic frequency differs from allele to allele. There are some alleles that are present in a very high frequency, where we can highlight the allele 219 bp of the ScAAT5 locus and the allele 94 bp of the C9 locus. The most common alleles can be considered the most representative of the *S. cerevisiae* population.

Table 10. Allelic frequency in 911 strains *S. cerevisiae* of the multiplex A microsatellite.

ScAAT1		ScAAT2		ScAAT3		ScAAT4		ScAAT5	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
123	0,001	345	0,011	217	0,006	269	0,063	213	0,002
150	0,002	354	0,001	220	0,001	272	0,026	216	0,104
153	0,011	357	0,132	223	0,020	275	0,024	219	0,462
156	0,018	360	0,062	229	0,001	278	0,017	222	0,264
162	0,018	363	0,005	235	0,004	281	0,003	225	0,004
165	0,001	369	0,165	241	0,051	287	0,002	234	0,003
168	0,240	372	0,042	244	0,006	290	0,032	246	0,039
171	0,067	375	0,192	247	0,164	296	0,017	249	0,008
174	0,007	378	0,188	250	0,109	299	0,003	252	0,009
177	0,006	381	0,184	253	0,004	305	0,075	255	0,007
183	0,001	384	0,009	256	0,117	308	0,015	258	0,013
186	0,001	390	0,001	259	0,025	311	0,003	261	0,004
189	0,061	393	0,007	262	0,141	314	0,002	264	0,022
192	0,024			265	0,133	317	0,003	267	0,027
195	0,211			268	0,028	320	0,002	270	0,004
198	0,009			271	0,080	329	0,480	273	0,014
201	0,119			274	0,017	332	0,024	276	0,010
204	0,061			277	0,033	335	0,005	279	0,004
207	0,003			283	0,001	338	0,002		
210	0,001			307	0,003	341	0,003		
216	0,007			310	0,001	344	0,002		
219	0,056			346	0,045	347	0,002		
225	0,005			358	0,006	356	0,002		
228	0,001			361	0,001	359	0,075		
231	0,005			376	0,001	362	0,005		

ScAAT1		ScAAT2	ScAAT3	ScAAT4		ScAAT5
237	0,019			365	0,007	
243	0,005			368	0,002	
246	0,006			371	0,034	
249	0,013			374	0,003	
255	0,001			377	0,015	
258	0,001			380	0,009	
264	0,001			383	0,014	
267	0,001			389	0,002	
273	0,001			395	0,002	
354	0,001			401	0,003	
357	0,014			404	0,002	
				410	0,002	
				413	0,003	
				419	0,002	
				422	0,003	
				425	0,005	
				431	0,002	
				434	0,002	
				443	0,002	

Table 11. Allelic frequency in 911 strains *S. cerevisiae* of the multiplex B microsatellite.

ScAAT6		C11		C4		C5		C9		ScYOR267c	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
247	0,029	174	0,004	188	0,003	113	0,119	91	0,125	257	0,005
250	0,055	182	0,012	191	0,001	115	0,035	94	0,634	266	0,024
253	0,029	186	0,010	194	0,001	117	0,026	97	0,209	272	0,001
256	0,474	188	0,148	197	0,001	121	0,009	100	0,019	275	0,187
259	0,412	190	0,168	200	0,002	125	0,078	103	0,003	278	0,109
262	0,001	192	0,001	206	0,002	127	0,036	106	0,010	281	0,008
292	0,001	194	0,006	209	0,001	129	0,022			284	0,065
		196	0,002	212	0,001	131	0,065			287	0,334
		198	0,027	218	0,001	133	0,020			290	0,002
		200	0,052	221	0,002	135	0,041			302	0,035
		202	0,032	227	0,001	137	0,055			308	0,049
		204	0,030	230	0,001	139	0,041			311	0,002
		208	0,020	233	0,003	141	0,007			314	0,001
		210	0,164	236	0,003	143	0,009			317	0,064
		212	0,126	242	0,015	145	0,010			320	0,024
		214	0,122	245	0,192	147	0,019			323	0,010
		216	0,029	248	0,141	149	0,136			326	0,001
		218	0,035	251	0,103	151	0,016			329	0,002
		220	0,012	254	0,238	153	0,022			332	0,041
				257	0,116	155	0,009			338	0,005
				260	0,077	161	0,004			341	0,012
				263	0,009	163	0,006			344	0,009
				266	0,001	179	0,001			350	0,001
				275	0,008	183	0,001			353	0,002

ScAAT6	C11	C4		C5		C9	ScYOR267c	
		278	0,002	185	0,009		356	0,005
		281	0,001	187	0,001		419	0,001
		284	0,005	189	0,001			
		287	0,017	193	0,001			
		293	0,001	195	0,003			
		296	0,001	197	0,003			
		299	0,003	199	0,006			
		302	0,005	211	0,001			
		305	0,004	213	0,004			
		308	0,001	215	0,012			
		314	0,001	217	0,007			
		320	0,001	219	0,071			
		323	0,001	221	0,070			
		329	0,001	223	0,016			
		332	0,002	225	0,006			
		338	0,001					
		341	0,001					
		344	0,004					
		353	0,001					
		362	0,001					
		368	0,001					
		374	0,002					
		383	0,001					
		386	0,001					
		389	0,001					
		395	0,001					
		398	0,001					
		401	0,001					

ScAAT6	C11	C4		C5	C9	ScYOR267c
		416	0,001			
		422	0,001			
		425	0,002			
		431	0,001			
		434	0,002			
		443	0,002			
		446	0,001			
		449	0,001			

For the heterozygosity we observed great variability at different loci, between 11% at the locus C9 and 57% at the locus ScAAT4 (Table 12). From the eleven microsatellites selected, loci C4 and C5 were the most informative since they have great genotype diversity. This result is in accordance with the number of alleles since this are two of the loci with higher alleles number.

Table 12. Characteristics of 11 microsatellite loci for the characterization of *S. cerevisiae* strains.

Locus	Alleles	Genotypes	Heterozygosity (%)
C11	19	66	27
C4	60	125	40
C5	39	86	28
C9	6	11	11
ScAAT1	36	76	20
ScAAT2	13	30	12
ScAAT3	25	52	20
ScAAT4	44	75	57
ScAAT5	18	49	31
ScAAT6	7	9	17
SCYOR267C	26	56	13

When comparing the allelic frequencies of the total Portuguese population studied, to the populations of other studies there are just a few allelic frequencies in common (*37*). This shows that we have gathered a characteristic population. Regarding on the heterozygosity percentage only ScAAT5 locus is approximately the same of the Legras et al. (2005) study (*28*). Since our sampling is higher than the mentioned studies our results are more representative of the *S. cerevisiae* strains diversity.

4.1. Allelic frequency of *S. cerevisiae* population of each wine appellation

To understand the population singularity, the allelic frequency of the different wine appellations was calculated. Comparing the allelic frequency of the population from Alentejo, Bairrada, Dão and Douro we observed that some alleles are present in just one of the wine appellation (Annex B). When comparing the allelic frequency between wine appellations we also observe a different allelic frequency on each wine appellation. These facts reinforce the hypotheses that there is a characteristic population of *S. cerevisiae* in the Portuguese wine appellations.

Conclusion

The identification of the Portuguese endogenous wine yeasts is essential to uncover the natural richness of fermentative yeasts, which can have a positive impact on the global sensorial properties of wine. In this study our objective was to examine the endogenous biodiversity of fermentative yeasts from four Portuguese wine appellations. For this we studied:

i) Wine cellar fermentations' microflora;
ii) The microflora of white wine spontaneous fermentations at low temperature.

In this study, *S. cerevisiae* represented all the endogenous populations from the wine cellar fermentations. Here we have built a diversified *S. cerevisiae* strains collection from the wine cellar fermentations from Alentejo, Bairrada, Dão and Douro. The *S. cerevisiae* strains populations were different between wine appellations and between grape varieties. This shows a characteristic population at each wine cellar from each wine appellation.

Concerning white grapes fermentations, we have concluded that the temperature is a critical factor. We also observed that the fermentations kinetics were slower at 12°C than at 18° and 25°C fermentations. The endogenous population of Bairrada white fermentations was able to ferment white grape musts at 12°C, thus demonstrating their potential application in white wines fermentation. Non-*Saccharomyces* isolated herein also had the ability to carry out fermentations at 12°C, and we believe this is of great potential for winemaking.

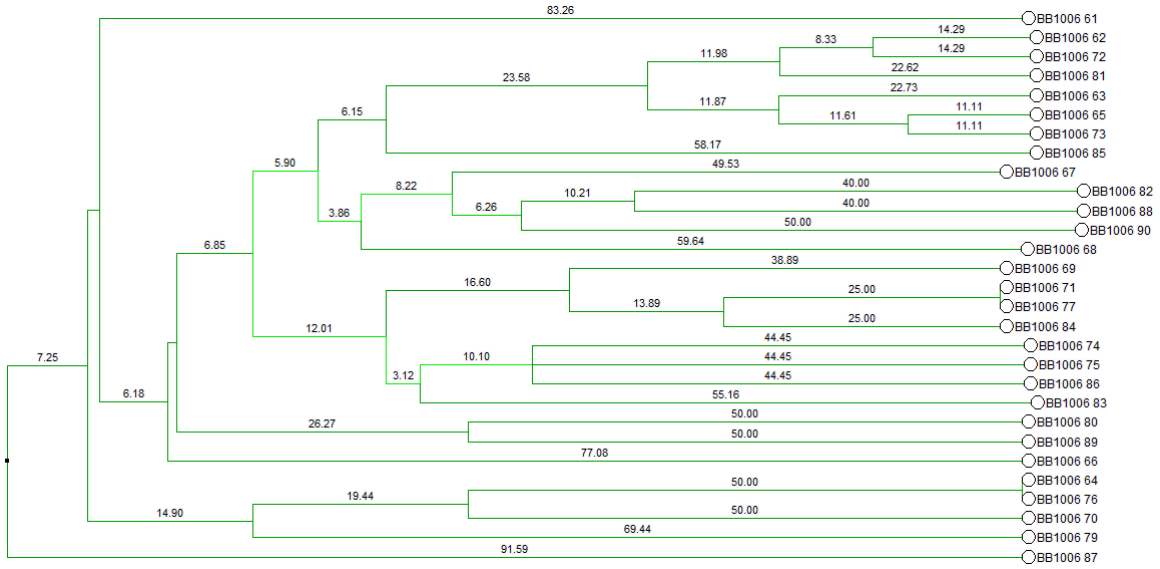
Altogether, we have observed that at the studied fermentations the *S. cerevisiae* strains presented different strains population for different wine appellations, for different grape variety, and for different fermentation temperatures (*19*).

In the future, we aim at doing a deeper analysis of the allelic frequency, comparing each population from different grape varieties, in order to compare the allelic frequency of these populations. With this study we have gathered a significant sampling of endogenous diversity of *S. cerevisiae* leading to vast potential to be explored that will allow a deeper study on the genetic diversity. Therefore, this study could be the basis for future work in which we intend to exploit the oenological potential of these strains by doing a phenotypic characterisation of the distinguished strains, especially with tests of enological interest. With this work we believe we have gathered valuable information to apply in winemaking.

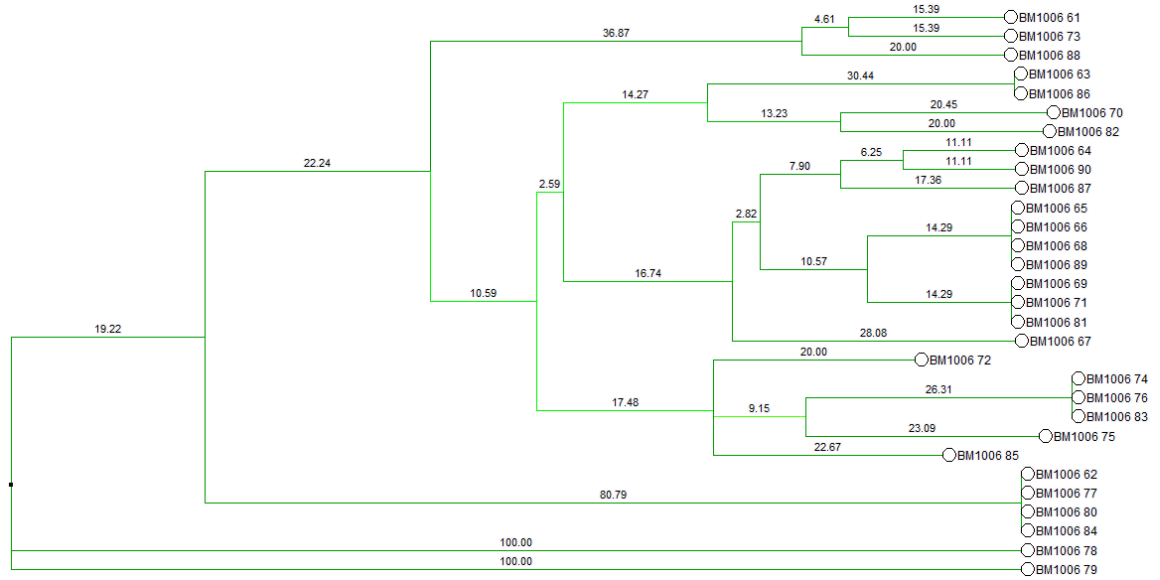
Annexes

Annex A – Clusters of *S. cerevisiae* strains isolated from Bairrada wine fermentations

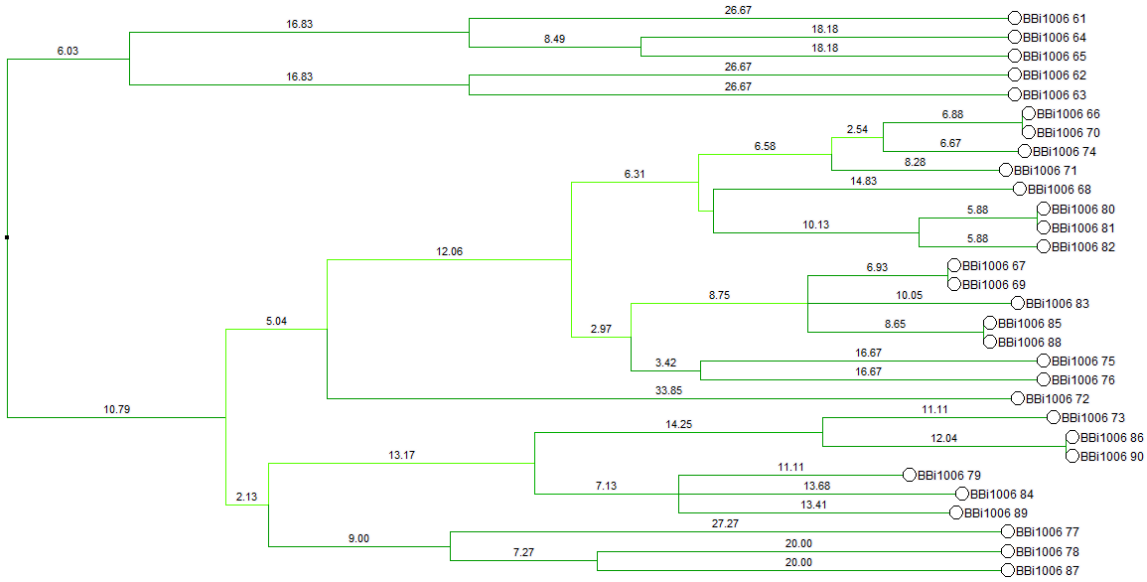
Annex A 1. Cluster of *S. cerevisiae* strains present at Baga fermentation from Bairrada wine appellation.



Annex A 2. Cluster of *S. cerevisiae* strains present at Maria Gomes fermentation from Bairrada wine appellation.



Annex A 3. Cluster of *S. cerevisiae* strains present at Bical fermentation from Bairrada wine appellation.



Annex B – Allelic frequency from the four wine appellations

Annex B 1. Table of the allelic frequency of Alentejo *S. cerevisiae* strains of the multiplex A microsatellites.

ScAAT1		ScAAT2		ScAAT3		ScAAT4		ScAAT5	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
162	0,060	360	0,029	220	0,143	305	0,209	216	0,021
189	0,024	369	0,145	223	0,143	308	0,045	219	0,468
192	0,012	375	0,014	241	0,143	329	0,687	222	0,511
195	0,214	378	0,710	265	0,286	332	0,015		
201	0,024	381	0,072	283	0,143	335	0,030		
204	0,048	384	0,029	361	0,143	371	0,015		
219	0,476								
225	0,036								
228	0,012								
231	0,024								
237	0,036								
243	0,036								

Annex B 2. Table of the allelic frequency of Alentejo *S. cerevisiae* strains of the multiplex B microsatellites.

ScAAT6		C11		C4		C5		C9		ScYOR267c	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
256	0,953	182	0,011	191	0,017	113	0,603	91	0,056	275	0,840
259	0,047	188	0,091	233	0,017	115	0,159	94	0,865	278	0,012
		190	0,045	236	0,017	117	0,032	97	0,079	308	0,086
		198	0,170	245	0,100	129	0,032			341	0,037
		208	0,091	248	0,100	131	0,048			344	0,025
		210	0,330	251	0,333	133	0,032				
		212	0,068	254	0,100	139	0,016				
		214	0,023	257	0,100	141	0,016				
		216	0,136	260	0,083	153	0,032				
		220	0,034	263	0,017	163	0,032				
				368	0,017						
				374	0,017						
				389	0,017						
				395	0,017						
				416	0,017						
				425	0,017						
				434	0,017						

Annex B 3. Table of the allelic frequency of Bairrada *S. cerevisiae* strains of the multiplex A microsatellites.

ScAAT1		ScAAT2		ScAAT3		ScAAT4		ScAAT5	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
123	0,0016	345	0,016	217	0,009	269	0,071	213	0,003
150	0,0031	354	0,002	223	0,028	272	0,014	216	0,058
153	0,0156	357	0,183	235	0,002	275	0,009	219	0,534
156	0,0172	360	0,087	241	0,060	278	0,024	222	0,255
162	0,0078	369	0,217	244	0,002	281	0,005	225	0,004
168	0,2761	372	0,056	247	0,161	287	0,002	234	0,004
171	0,0655	375	0,203	250	0,152	290	0,033	246	0,043
174	0,0031	378	0,064	253	0,002	296	0,024	249	0,012
183	0,0016	381	0,157	256	0,127	299	0,005	252	0,013
186	0,0016	384	0,006	259	0,021	305	0,052	255	0,008
189	0,0780	393	0,010	262	0,187	308	0,012	258	0,017
192	0,0109			265	0,062	311	0,005	261	0,005
195	0,222			268	0,006	314	0,002	264	0,031
198	0,012			271	0,054	317	0,005	267	0,005
201	0,142			277	0,049	320	0,002	273	0,005
204	0,069			346	0,067	329	0,480	279	0,005
207	0,005			358	0,009	332	0,016		
210	0,002			376	0,002	335	0,002		
216	0,003					341	0,005		
219	0,006					344	0,002		
231	0,002					347	0,002		
237	0,009					356	0,002		
246	0,008					359	0,089		
249	0,017					362	0,007		
258	0,002					368	0,002		

ScAAT1		ScAAT2	ScAAT3	ScAAT4		ScAAT5
264	0,002			371	0,042	
267	0,002			374	0,005	
354	0,002			377	0,014	
357	0,016			380	0,012	
				383	0,019	
				389	0,002	
				395	0,002	
				401	0,005	
				404	0,002	
				410	0,002	
				413	0,005	
				419	0,002	
				422	0,005	
				425	0,005	
				431	0,002	
				443	0,002	

Annex B 4. Table of the allelic frequency of Bairrada *S. cerevisiae* strains of the multiplex B microsatellites.

ScAAT6		C11		C4		C5		C9		ScYOR267c	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
247	0,039	182	0,001	188	0,003	113	0,037	91	0,133	266	0,034
250	0,074	186	0,013	194	0,001	115	0,014	94	0,559	272	0,002
253	0,039	188	0,190	197	0,001	117	0,031	97	0,265	275	0,139
256	0,350	190	0,168	200	0,003	121	0,012	100	0,025	278	0,083
259	0,496	194	0,001	206	0,003	125	0,091	103	0,005	281	0,011
262	0,001	196	0,001	209	0,001	127	0,047	106	0,014	284	0,055
292	0,001	200	0,038	212	0,001	129	0,026			287	0,398
		202	0,044	218	0,001	131	0,043			290	0,003
		204	0,041	221	0,003	133	0,004			302	0,051
		208	0,016	227	0,001	135	0,051			308	0,054
		210	0,177	230	0,001	137	0,053			311	0,003
		212	0,112	233	0,001	139	0,033			314	0,002
		214	0,158	242	0,019	141	0,008			317	0,070
		216	0,022	245	0,228	145	0,010			320	0,005
		218	0,013	248	0,154	147	0,016			323	0,010
		220	0,004	251	0,061	149	0,183			326	0,002
				254	0,262	151	0,004			329	0,003
				257	0,085	153	0,026			332	0,055
				260	0,074	155	0,012			338	0,002
				263	0,004	161	0,006			341	0,007
				266	0,001	163	0,002			344	0,008
				275	0,011	179	0,002			350	0,002
				278	0,003	183	0,002			353	0,002
				284	0,004	185	0,012				
				287	0,023	187	0,002				

ScAAT6	C11	C4		C5		C9	ScYOR267c
		299	0,004	189	0,002		
		302	0,005	193	0,002		
		305	0,005	195	0,004		
		308	0,001	197	0,004		
		314	0,001	199	0,008		
		320	0,001	211	0,002		
		323	0,001	213	0,006		
		329	0,001	215	0,016		
		332	0,003	217	0,010		
		338	0,001	219	0,096		
		341	0,001	221	0,094		
		344	0,005	223	0,022		
		353	0,001	225	0,008		
		362	0,001				
		383	0,001				
		386	0,001				
		401	0,001				
		422	0,001				
		425	0,001				
		431	0,001				
		434	0,001				
		443	0,003				
		446	0,001				
		449	0,001				

Annex B 5. Table of the allelic frequency of Dão *S. cerevisiae* strains of the multiplex A microsatellites.

ScAAT1		ScAAT2		ScAAT3		ScAAT4		ScAAT5	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
162	0,037	357	0,057	229	0,009	269	0,045	216	0,333
165	0,009	363	0,038	235	0,018	272	0,030	219	0,343
168	0,009	369	0,038	241	0,054	290	0,061	222	0,204
171	0,148	372	0,029	244	0,027	305	0,121	225	0,009
174	0,037	375	0,295	247	0,279	308	0,015	246	0,046
177	0,046	378	0,419	250	0,036	329	0,394	264	0,009
189	0,009	381	0,095	253	0,018	332	0,091	267	0,046
192	0,120	384	0,019	256	0,036	338	0,015	273	0,009
195	0,231	390	0,010	259	0,054	359	0,091		
201	0,093			262	0,081	365	0,061		
204	0,037			265	0,225	371	0,015		
216	0,037			268	0,081	377	0,045		
219	0,046			271	0,054	425	0,015		
225	0,009			307	0,018				
231	0,009			310	0,009				
237	0,074								
243	0,009								
255	0,009								
273	0,009								
357	0,019								

Annex B 6. Table of the allelic frequency of Dão *S. cerevisiae* strains of the multiplex B microsatellites.

ScAAT6		C11		C4		C5		C9		ScYOR267c	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
256	0,867	174	0,037	188	0,007	113	0,227	91	0,216	257	0,038
259	0,133	182	0,083	236	0,013	115	0,064	94	0,676	275	0,086
		190	0,315	245	0,027	125	0,036	97	0,108	278	0,248
		192	0,009	248	0,114	127	0,009			284	0,019
		194	0,046	251	0,235	131	0,182			287	0,200
		196	0,009	254	0,201	133	0,091			308	0,019
		198	0,093	257	0,268	135	0,018			317	0,133
		200	0,019	260	0,081	137	0,100			320	0,124
		210	0,019	263	0,034	139	0,091			323	0,019
		212	0,120	281	0,007	143	0,018			332	0,010
		214	0,028	293	0,007	145	0,018			338	0,010
		218	0,204	374	0,007	147	0,045			341	0,038
		220	0,019			149	0,009			353	0,010
						151	0,082			356	0,038
						163	0,009			419	0,010

Annex B 7. Table of the allelic frequency of Douro *S. cerevisiae* strains of the multiplex A microsatellites.

ScAAT1		ScAAT2		ScAAT3		ScAAT4		ScAAT5	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
156	0,119	375	0,134	247	0,066	269	0,138	216	0,238
162	0,048	378	0,224	256	0,170	272	0,241	219	0,161
168	0,762	381	0,642	259	0,009	275	0,345	222	0,196
171	0,024			262	0,009	290	0,034	246	0,042
201	0,024			265	0,340	329	0,207	255	0,014
204	0,024			268	0,066	434	0,034	258	0,014
				271	0,226			261	0,007
				274	0,113			267	0,147
								270	0,028
								273	0,077
								276	0,077

Annex B 8. Table of the allelic frequency of Douro *S. cerevisiae* strains of the multiplex B microsatellites.

ScAAT6		C11		C4		C5		C9		ScYOR267c	
Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies	Alleles	Frequencies
256	0,667	190	0,058	233	0,038	125	0,500	91	0,014	275	0,046
259	0,333	200	0,404	242	0,038	143	0,500	94	0,971	278	0,218
		210	0,019	245	0,308			100	0,014	284	0,253
		212	0,423	248	0,038					287	0,356
		218	0,038	251	0,038					308	0,011
		220	0,058	254	0,077					320	0,057
				257	0,154					323	0,011
				260	0,115					332	0,011
				284	0,077					338	0,023
				296	0,038					344	0,011
				302	0,038						
				398	0,038						

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