

## Article

# SSR-Marker Analysis—A Method for *S. cerevisiae* Strain Characterization and Its Application for Wineries

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**Abstract:** Considering that many *Saccharomyces cerevisiae* strains exist and that they have different fermentation capacities, the challenge is to select the yeast strain that generates the most interesting wine character and wine flavor for the winemaker. A method based on simple sequence repeats (SSRs) markers, occurring in the yeast genome, was developed to differentiate the collected *S. cerevisiae* strains. For the amplification of the polymorphic SSR markers performed by polymerase chain reaction (PCR), two primer sets showing different size products for different *S. cerevisiae* strains were designed. The PCR-method with gel electrophoresis was validated using capillary sequencing and then used as a service for winegrowers combined with a sensory analysis via napping. This approach can be used for the preservation of the yeast diversity associated with given terroirs and as an option for an increased safety of fermentations. The application of *S. cerevisiae* strains collected in spontaneous fermentations and used for fermentation sustains the initial character of the wine and ensures a secure fermentation at the same time.

**Keywords:** simple sequence repeats markers; *Saccharomyces cerevisiae*; napping

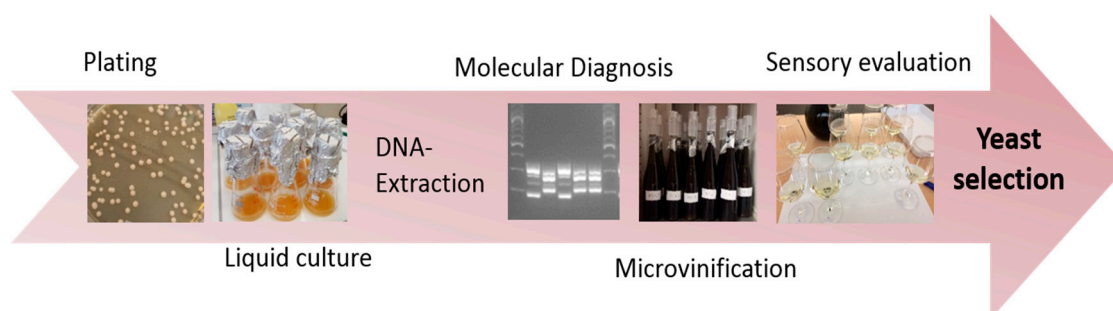
## 1. Introduction

Yeasts, eukaryotic unicellular microorganisms, have been used for thousands of years for the production of alcoholic beverages or food [1]. In the scientific classification, yeasts belong to the kingdom Fungi and are part of the Ascomycota phylum. In wine production, these organisms are essential for the fermentation process and for the character of each specific wine [2,3]. The most abundant yeasts in a vineyard are generally the yeasts of the genre *Candida*, *Hanseniaspora*, *Pichia* and *Metschnikowia* [4,5]. The specie *S. cerevisiae* is present in a smaller proportion. The freshly pressed, sugar-rich grape must offers optimal conditions to these microorganisms for their growth. During fermentation, the diversity of yeasts decreases, due to the production of ethanol, which is toxic to cells and leads to cell death [6,7]. The alcohol-tolerance limit varies depending on the genus and species of the yeast. The species *S. cerevisiae* tolerates the highest ethanol concentration [8]. Non-Saccharomyces yeasts die above an ethanol concentration of 4% (v/v), and *S. cerevisiae* becomes the dominant species [9]. To ensure a successful fermentation, the presence of *S. cerevisiae* is essential for winegrowers. For a reliable and predictable fermentation process, the must fermentation can be carried out by adding a sufficient amount of a pure culture of a selected commercially available *S. cerevisiae* yeast strain [10]. Consequently, this will prevent stuck fermentation and off-flavors that may be generated by other microorganisms naturally present on grapes [11]. In fact, by adding a commercially available wine yeast, other microorganisms present in a negligible quantity in the

must will be suppressed. Contrary to such an induced fermentation, a spontaneous fermentation lets the must ferment exclusively with the yeasts and other microorganisms naturally occurring in the vineyards. These endogenic organisms live on the skin of the grapes or are part of the cellar flora. The implementation of a spontaneous fermentation allows the winegrowers to develop a unique terroir flavor and an individual wine character. In fact, the yeast flora differs from one vineyard to another, indicating that terroir-specific yeasts can contribute to the wine's individuality [12]. The marketing strategy of such wines is to reach a consumer society that is striving for more natural and local products [13]. However, in spontaneous fermentation, risks of fermentation blockage or the presence of off-flavors due to harmful microorganisms exist [14,15]. An alternative strategy for winegrowers consists in propagating yeast that originates from their own vineyard in order to inoculate the must, conjugating unique sensory attributes with safe fermentations [16].

*S. cerevisiae* is widely used as a model organism in cell biology and genetics. In 1996, it was the first eukaryote for which one strain's genome was sequenced [17]. Its nuclear genome, composed of 16 linear chromosomes, contains about 12 million base pairs and 6275 genes. In the genome, Simple Sequence Repeats (SSRs), also called microsatellites, are short DNA motifs (two to six bases) repeated continuously five to 50 times. Weller and Jeffreys first characterized SSR in 1984 [18] as a "polymorphic GGAT repeat" in the human myoglobin gene. The genetic transmission of these noncoding sequences follows Mendel's laws of heredity. Their patterns are very abundant throughout the genome of most eukaryotes and prokaryotes [19]. The sequence lengths of SSRs are intra- and interspecific across species. Because SSRs suffer higher rates of mutation than the rest of the genome [20], polymorphic SSRs are a powerful biological tool for the genetic differentiation of *S. cerevisiae* strains as they can be used as molecular markers. Indeed, depending on the size of the SSR, it is possible to differentiate strains from each other. SSR markers were also used for genotyping the yeast *Brettanomyces bruxellensis* at the strain level [21] and for other organisms such as the potato, whose genotype is highly heterozygous for multiple SSR alleles [22]. SSR markers are highly informative and are useful for fingerprinting and linkage studies.

This work focuses on the development of a fast method for *S. cerevisiae* strain differentiation. Strain differentiation is already possible by sequencing the genome, but this is still a cost-intensive method. Other methods such as karyotyping or the restriction fragment length polymorphism (RFLP) analysis of mtDNA or the SC1/SC2 marker are used for strain differentiation [23–25] but have limits because they are hardly applicable for a routine analysis or insufficiently discriminating at the strain level [26]. On the contrary, SSR analyses were already used for the characterization of *S. cerevisiae* strains [26,27] or for *S. cerevisiae* strain diversity studies [28]. Here, a polymerase chain reaction (PCR)-based SSR-marker method combined with microvinifications and a napping was applied as an alternative method to be used as a quick and low-budget service for winegrowers [26,29,30]. The developed method is based on the analysis of polymorphic SSRs in yeast genome. For the development, 15 primers were tested [28]. These primers were designed in order to amplify specific polymorphic SSR-regions of *S. cerevisiae*. Multiplex sets were realized by combining several primers. The length of the fragment was observed by agarose gel electrophoresis (Figure 1). The aim is a winery's own yeast, which can be provided as a starter culture and applied in a winery in the Netherlands (Flevoland) following a detailed protocol (Figure 1).



**Figure 1.** The progress of the yeast selection starting with the plating and ending with a yeast for a winery in order to ensure its spontaneous fermentation.

## 2. Materials and Methods

### 2.1. Material

#### 2.1.1. Commercially Available *Saccharomyces Cerevisiae* Strains

Nine commercially available *S. cerevisiae* yeasts were used. These dry yeasts, listed in Table 1, were rehydrated and used as indicated in the manufacturer's instructions.

**Table 1.** Commercially available yeast strains.

Commercial Name	Abbreviation	Origin
Lalvin BM4*4	Bm4	Lallemand, Fredericia, Denmark
Oenoferm Color	Oc	Erbslöh, Geisenheim, Germany
Oenoferm Bouquet	Ob	Erbslöh, Geisenheim, Germany
Oenoferm Klosterneuburg	Ok	Erbslöh, Geisenheim, Germany
Oenoferm Freddo	Of	Erbslöh, Geisenheim, Germany
Melody	Mel	Chr Hansen, Hoersholm, Denmark
Siha Activhefe 8	Sa8	Eaton Technologies, Langenlonsheim, Germany
1895c	1895c	Swiss Wineyeast GmbH, Meilen, Switzerland
Lalvin EC 1118	Lec	Lallemand, Fredericia, Denmark

#### 2.1.2. Wine Samples

The samples were collected in 2019 in five different spontaneous fermentations tanks of a winery. The winery was harvested in 2017 for the first time. The vineyard was isolated and located in Lelystad (Province of Flevoland) in the Netherlands. Only spontaneous fermentation were conducted in this winery.

#### 2.1.3. Yeast Isolation and Preservation

The samples were plated on YPD-Agar (Yeast Extract-Peptone-Dextrose, Carl Roth, Karlsruhe, Germany) culture medium. For an optimal isolation, these samples were diluted in 0.9% NaCl, with dilution factors ranging from  $10^3$  to  $10^6$ . After three days, 10 colonies of *S. cerevisiae* from each sample were transferred into liquid YPD-Medium. For yeast conservation, 700  $\mu$ L of glycerol (AppliChem, Darmstadt, Germany) and 300  $\mu$ L of the three days-old liquid culture were put in a cryogenic tube. The tubes were homogenized and immersed in liquid nitrogen in order to shock-freeze the yeasts. 10  $\mu$ L of the culture were also observed under the microscope before preservation in order to verify the absence of contamination. Ten randomly chosen *S. cerevisiae* yeast colonies of each sample, and therefore a total of 50 yeasts, were isolated and preserved.

## 2.2. Methods:

### 2.2.1. DNA Extraction

DNA-extraction was done with the Qiagen DNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Cell lysis was performed by the lyticase from *Arthrobacter luteus* (Sigma-Aldrich, Darmstadt, Germany). DNA extracted from cultures was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., Darmstadt, Germany).

### 2.2.2. SSR-PCR

Fifteen published primer pairs were tested [28] (Table 2). The primers were first checked in order to determine the size range of the PCR product. Primers generating product-length differences between the nine commercially available strains listed in Table 1 were selected for the multiplex combination.

**Table 2.** Primers' origin and sequence with the motif and names of the SSR (simple sequence repeats).

Primer	Motif	ORF Coordinates	Sequences
ScAAT2	TAA	YBL084c	FW:CAGTCTTATTGCCTTGAACGA RV:GTCTCCATCCTCCAAACAGCC
ScAAt3	TAA	YDR160w	FW:TGGGAGGAGGGAAATGGACAG RV:TTCAGTTACCCGCACAATCTA
C5	GT	VI-210250/210414	FW:TGACACAATAGCAATGGCCTTCA RV:GCAAGCGACTAGAACACAATCACA
C3	CAA	YGL139w	FW:CTTTTATTTACGAGCGGGCCAT RV:AAATCTCATGCCTGTGAGGGGTAT
C8	TAA	YGL014w	FW:CAGGTCGTTCTAACGTTGGTAAAATG RV:GCTGTTGCTGTGGTAGCATTACTGT
C11	GT	X-518870/519072	FW:TTCCATCATAACCGTCTGGGATT RV:TGCCTTTTCTTAGATGGGCTTTC
YKR072c	GAC	YKR072c	FW:AGATACAGAAGATAAGAACGAAAA RV:TTATTGATGCTTATCTATTATACC
SCYOR267c	TGT	YOR267c	FW:TACTAACGTCAACACTGCTGCCAA RV:GGATCTACTTGCAGTATACGGG
YKL172w	GAA	YKL172w	FW:CAGGACGCTACCGAAGCTCAAAAG RV:ACTTTTGGCCAATTCTCAAGAT
ScAAT1	TTA	XIII-86902/87140	FW:AAGCGTAAGCAATGGTGTAGATACTT RV:CAAGCCTCTTCAAGCATGACCTTT
C4	TAA + TAG	XV-110701/110935	FW:AGGAGAAAAATGCTGTTTATTCTGACC RV:TTTCTCCCGGGACGTGAAATA
C9	TAA	YOR156c	FW:AAGGGTTCGTAAACATATAACTGGCA RV:TATAAGGGAAAAGAGCAGATGGC
ScAAT5	TAA	XVI-897051/8970210	FW:AGCATAATTGGAGGCAGTAAAGCA RV:TCTCCGCTTTTTTGTACTGCGTG
C6	CA	XVI-485898/485996	FW:GTGGCATCATATCTGTCAATTTTATCAC RV:CAATCAAGCAAAAAGATCGGCT
YPL009c	CTT	YPL009c	FW:AACCCATTGACCTCGTTACTATCGT RV:TTCGATGGCTCTGATAACTCCATTC

For the PCR, the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) was used following the instructions suggested for the amplification of microsatellite loci (Table 2). The PCR settings were the following: Initiation (95 °C, 3 min); Denaturation (95 °C, 30 s); Annealing (60 °C, 30 s); Extension (72 °C, 1 min); and Final Extension (72 °C, 7 min). Thirty-five cycles were performed.

### 2.2.3. Fragment Length Determination by Agarose Gel Electrophoresis

The result of the PCR was visualized by gel electrophoresis using a 2% agarose gel. 100 mL TAE-Buffer (AppliChem GmbH, Darmstadt, Germany) and 2 g agarose (AppliChem GmbH, Darmstadt,

Germany) were taken, as well as 10 µL of the fluorescent dye GelRed (GeneON, Ludwigshafen, Germany). The loaded samples were composed of 5 µL of PCR product added to 1 µL loading dye (Thermo Fisher Scientific, Waltham, MA, USA). An electric field (90 mA, 90 V, 8 W) was applied for 2 h.

#### 2.2.4. Capillary Sequencing and Method Validation

For a better precision in order to validate the method of the agarose gel, the Capillary Sequencer ABI 3130xl Genetic Analyzer (Applied Biosystems, located within Geilweilerhof, Siebeldingen, Germany) was used. The PCR was carried out as described, but labeled primers were used for the detection in the sequencer. The electrophoresis profiles were captured by ABI 3130 xL Data Collection software. The reading of the size of the different amplified SSRs from the sequencer was performed using the GeneMapper v4.0 (Thermo Fisher Scientific, Waltham, MA, USA) software.

#### 2.2.5. Fermentation and Sensory Tests

For each yeast strain, 750 mL Weißburgunder must was inoculated with  $1.0 \times 10^8$  yeast cells. The determination of the yeast concentration and viability was performed by the Oculyze Fermentation Wine (FW, Oculyze GmbH, Wildau Germany). 50 µL of yeast culture were added to 50 µL 0.01% alkaline methylene violet. After 5 min, 50 µL of the reaction were taken for the measurement. After inoculating the must, the fermentation was checked weekly with a density meter DM 35 Basic (Anton Paar, Ostfildern, Germany) for a few weeks, depending on the rate of each individual yeast fermentation. For the sensory perception, a panel of 17 people was taken for a napping sensory test [29,30]. Graphic coordinates were attributed to each wine with the Fizz biosystems software, and the software FactoMineR [31] was used for the data analysis. The script was developed by Vestner 2019. The produced wines were also tasted by the owner of the winery who decided which of the identified yeasts were interesting enough for future wine-making.

### 3. Results

#### 3.1. Primer Sets' Composition and Method Validation

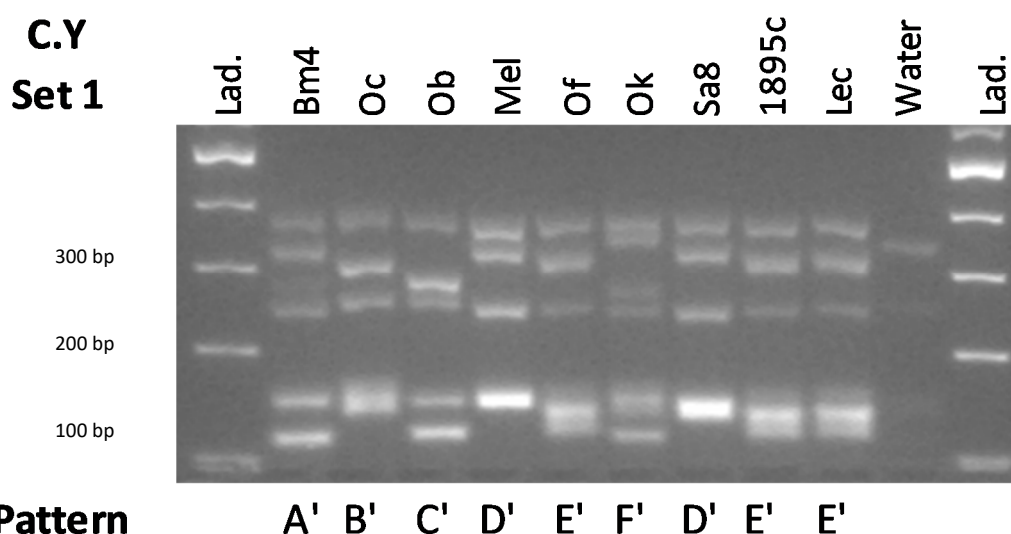
The size ranges of the commercially available yeast's fragment length, obtained by agarose gel electrophoresis or sequencing, are shown in Table 3 for each primer set. Based on these results, two multiplex sets were created by combining primers of different size ranges. The first one combines the primers ScAAT2, ScAAT3, C5, SCYOR267c, C8 and C11. The second combines the primers YKL172w, C4, C9, ScAAT5, C6 and YPL009c. The molecular markers were combined while making sure to avoid overlapping fragment sizes and matching PCR conditions. The primers C3, ScAAT1 and YKR072c were not used for the primer set composition because they did not fulfill these preconditions.

**Table 3.** The table shows the fragment lengths of the marker in base pairs (bp) resulting from the DNA of commercially available yeasts and the arrangement of the primer sets.

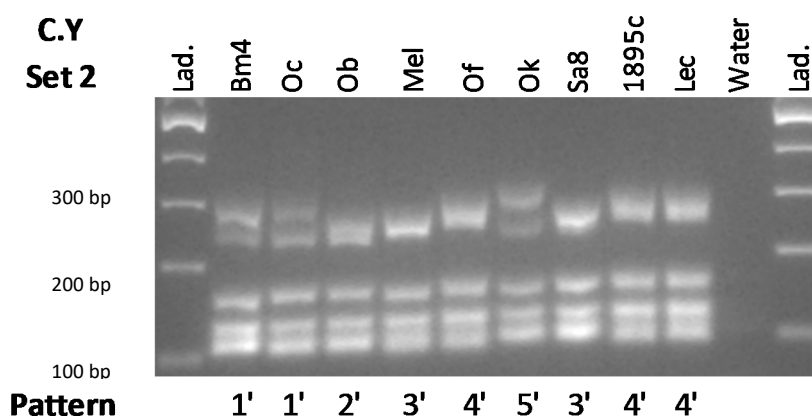
Primer	Size Range by Agarose gel (bp)	Size Range by Capillary Sequencing (bp)	Primer Set
ScAAT2	310–390	376–385	
ScAAT3	260	244–274	
C5	130–170	/	
SCYOR267c	305–390	305–325	
C8	150	133–146	
C11	200–250	/	
YKL172w	135	120–125	
C4	240–320	248–260	
C9	100	90–100	
ScAAT5	160–180	156–162	
C6	100–120	100–102	
YPL009c	250–290	276–311	

The results of the gel electrophoresis for the commercially available yeasts after multiplex PCR are shown in Figures 2 and 3. Six different fragment length patterns were obtained for Set 1 (A' to

F') and five for Set 2 (1' to 5'). Six pattern combinations for nine commercially available yeasts were obtained (A'1', B'1', C'2', D'3', E'4' and F'5').



**Figure 2.** Results of the gel electrophoresis for the commercially available yeasts named above after multiplex PCR using Multiplex Set 1 with the nomenclature of the patterns A–F.



**Figure 3.** Results of the gel electrophoresis for the commercially available yeasts named above after multiplex PCR using Multiplex Set 2 with the patterns numbered from 1–5.

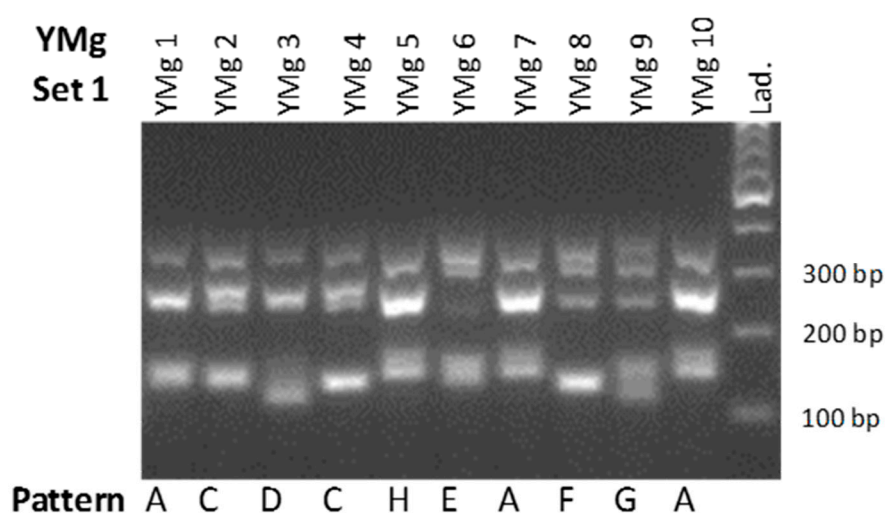
### 3.2. Yeast Differentiation

The results of the different patterns obtained by gel electrophoresis for the winery's own yeast after two multiplex SSR PCRs using sets 1 and 2 are summarized in Table 4. Ten different patterns (A to J) were present for the multiplex set 1, and eight different patterns (1 to 8) were present for the multiplex set 2. When combining the two sets, 21 different patterns could thus be obtained for the 50 yeasts. Examples of the patterns A and C–H are shown in Figure 4. The combination 1A shows the most recurrent combination, since it contains 18 of the 50 yeasts. Sixteen yeasts were single in their pattern category.



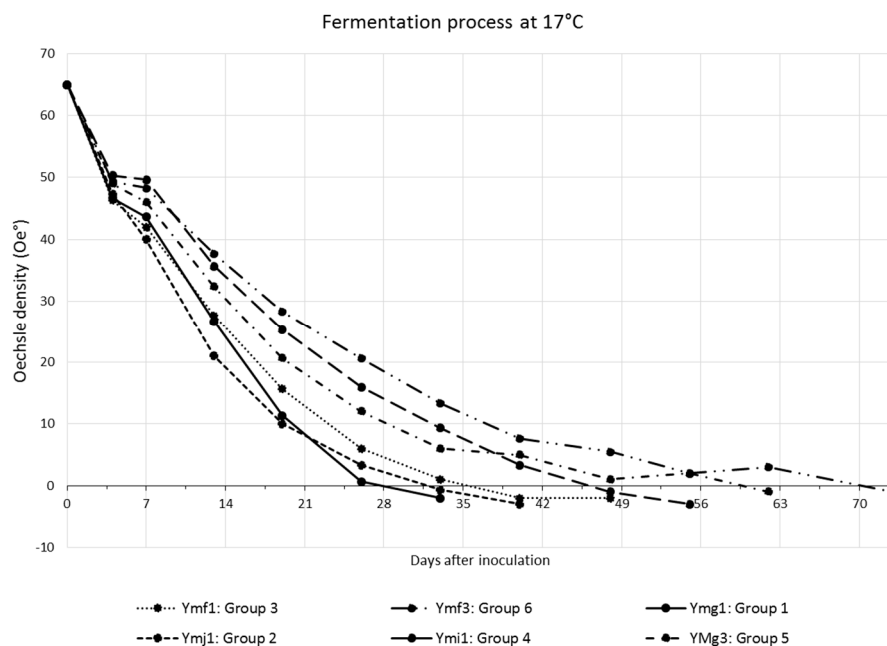
**Table 4.** Number of patterns obtained by gel electrophoresis for the winery's own yeast after SSR PCR using multiplex set 1 (1 to 8) and multiplex set 2 (A to J).

Pattern	1	2	3	4	5	6	7	8
A	18	0	0	0	1	0	0	0
B	0	1	0	0	0	0	0	1
C	4	0	1	1	0	7	0	3
D	1	0	1	0	0	0	0	0
E	0	0	0	0	1	0	0	0
F	0	1	0	0	0	1	0	0
G	2	0	0	0	0	0	0	0
H	1	0	0	0	1	1	0	0
I	0	0	0	0	0	0	1	1
J	1	0	0	0	0	0	0	0

**Figure 4.** Example of an agarose gel showing the patterns A and C–H. The fragment lengths in base pairs (bp) for the yeast strains Ymg 1–10, amplified with the primer set 1, are shown.

### 3.3. Fermentative Tests

Representative yeast strains were chosen for microvinification. Seventeen yeasts identified by SSR PCR were chosen: three yeasts of group 1A, one of 5A, one of 2B, one of 3C, one of 4C, two of 6C, one of 8C, one of 3D, one of 5E, one of 2F, one of 1G, one of 5H, one of 6H and one of 7I. These yeasts were chosen since they showed a different pattern in the SSR PCR and thus seemed to be different strains. Three yeasts from group 1A were taken in order to see if similarities could be observed as they were identical yeasts in terms of the pattern of the SSR PCR. After inoculation of sterile must with each yeast, the process of fermentation was monitored by a density meter. The fermentation process of one yeast of each group is presented in Figure 5, and the fermentation time is listed in Table 5. The initial must had a density of 65° Oe. The end of fermentation is characterized by a negative Oechsle degree. The fermentation time ranges from 33 to 73 days after inoculation, forming six groups with the same fermentation time.



**Figure 5.** Fermentation process for the microvinifications. One density measurement per group is presented here from 33 to 73 days.

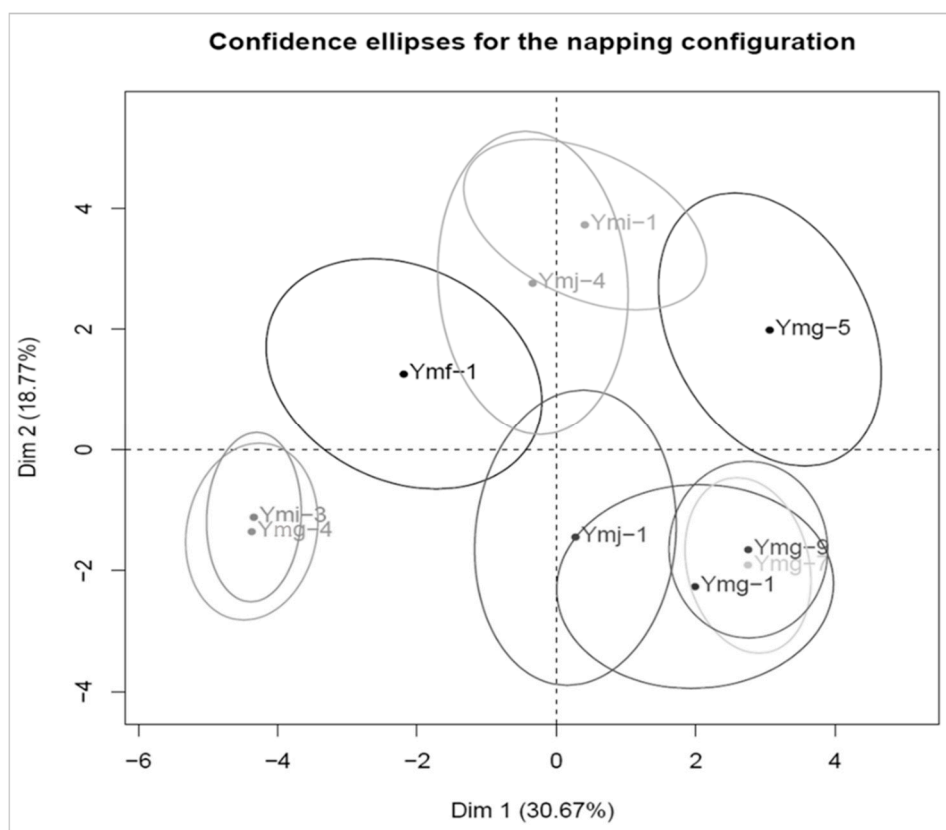
**Table 5.** Fermentation time in days for the yeast strains. The strains were arranged in six groups according to their fermentation time.

Group	Fermentation Time (days)	Yeasts	Total Number of Yeast
Group 1	33	Ymg1	1
Group 2	40	Ymj1, Ymg4, Ymg7, Ymg9, Ymj4	5
Group 3	48	Ymf1, Ymg5, Ymi3	3
Group 4	55	Ymi1, Ymg2, Ymg8, Ymj2	4
Group 5	62	Ymg3, Ymh4	2
Group 6	73	Ymg6, Ymf3	2

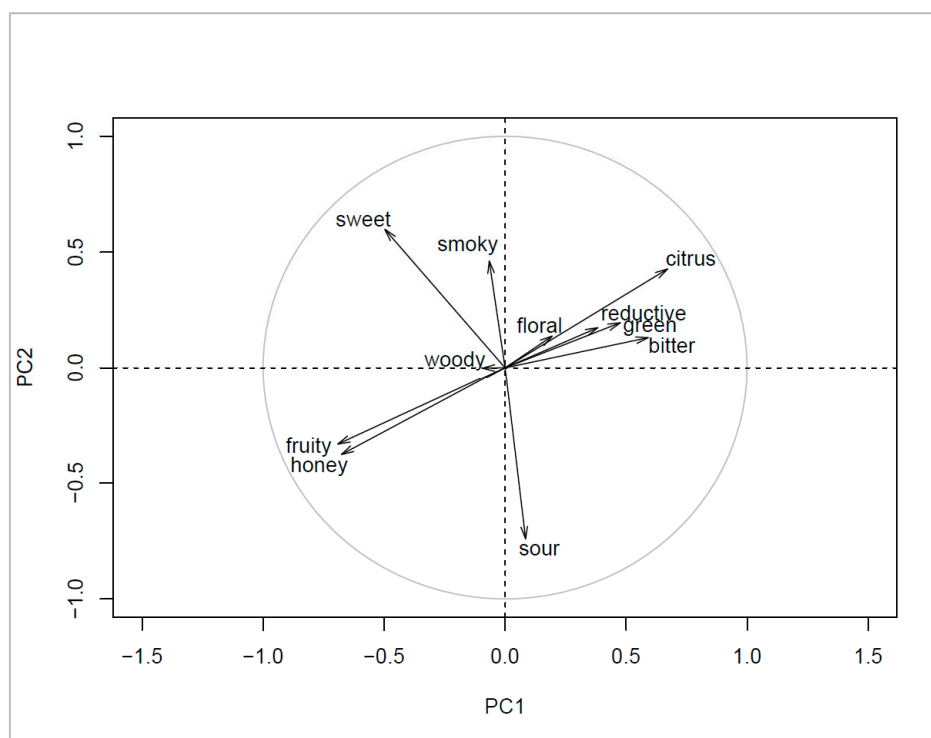
### 3.4. Sensory Differences

The ten yeasts showing the shortest fermentation time (Table 5) were taken for the sensory test. The results of the napping test are shown in Figure 6. This representation shows the sensory distance between the wines, as well as the 95% confidence ellipses. Figure 7 shows the descriptor correlations. Sensory differences as well as similarities can be observed. The wines Ymi-3 and Ymg-4 are close in taste, both exhibiting the descriptors fruity and honey, whereas the wines Ymj-1, Ymg-9, Ymg-7 and Ymg-1 all share the descriptor sour. The taste of wine Ymg-5 is quite different from other wines and corresponds to the citrus descriptor. Ymi-1 and Ymj-4 are linked as well and have a smoky taste. The wine Ymf-1 has its own sensory taste by being sweeter. The final choice was made by the vineyard owner who selected the yeast Ymf-1 because of its great harmonized taste of sweetness, fruitiness and sourness.





**Figure 6.** The results of the napping procedure. The wines are named after the yeast strain used for the fermentation, and the wines with the brighter ellipses were grouped together by the panelists.



**Figure 7.** The figure shows the descriptors the panelists used for the wines and the correlations resulting from the arrangement of the wines.

## 4. Discussion

### 4.1. Method Validation and Primer Set Composition

The fragment lengths obtained by the capillary sequencer and by the traditional agarose gel electrophoresis were compared. It can be stated that the results correlate, so that the SSR-PCR method with the agarose gel electrophoresis is validated. However, the capillary sequencer has a higher precision than the traditional agarose gel technique (1 bp versus 20 bp). Small differences could thus have been overseen using the agarose gel: a more precise instrumentation is required in this case. The use of a capillary sequencer allowed a differentiation between all nine commercially available yeasts. However, even if the traditional agarose gel method has a lower precision, it remains a good and useful tool for routine and low-budget equipment.

The fragment lengths obtained by the primers are in the same range as the values reported in the literature [10,26,27,32,33].

Concerning the PCR, the recommended primer annealing temperature was 57 °C [28]. However, after testing this PCR-condition, the results on the agarose gel were unreadable. When increasing the annealing temperature to 60 °C, the end products were optimal for all primers except for ScaAT1 and YKR072c. Since YKR072c was described as “slightly variable” in contrast to other primers [33], and since only two out of 15 primers did not work at 60 °C, this annealing temperature was kept for the subsequent PCR experiments.

Primers were combined depending on the product size obtained after PCR, according to the literature [28]. Primers showing products with different sizes were combined, while avoiding superpositions that could mask out the results. However, these primers were designed specifically for the amplification of polymorphic SSRs of *S. cerevisiae* strains [28]. Ultimately, it was important to determine different patterns of PCR products for the subsequent differentiation of *S. cerevisiae* strains. Six different combinations of patterns could be obtained for the nine commercially available yeasts.

### 4.2. Winery's Own Yeast Differentiation

By combining the results of both multiplex sets, 21 different patterns could be obtained, which means that 21 different yeasts were isolated and differentiated by the developed method. However, it could happen that two different yeasts showing small (< 10 bp) fragment length differences were present in the same group due to the low precision of the agarose gel.

There are two possible reasons to explain why fewer than six bands were sometimes detected for the six primers of a set: on the one hand, some SSRs could have been absent in some yeasts or could have malfunctioned. On the other hand, the fragment length of a primer pair might have been too close to another primer pair, making them undistinguishable.

As the aim of the project was to provide winegrowers with a particular yeast from their own vineyard or cellar to affirm their own terroir character, it was important to verify that the isolated yeasts were not commercially available yeasts. In fact, the samples were collected during spontaneous fermentation, but the fermentation tank may have had leftovers from yeasts used for previous fermentation with commercially available yeasts or there may have been a nearby tank with commercially available yeast strains fermenting nearby. A comparison of the biomolecular results of the winery's own yeast to the commercially available yeasts showed that only Set 2 presented some similarities between the two yeast categories. Therefore, by combining both sets, none of the Flevo yeasts showed a similar pattern to the nine commercially available yeasts. If the comparison would have been done with Set 2 only, similarities could have been found with commercially available yeasts. It was predictable that no commercially available yeasts would be identified, as the vineyard in Flevoland never employed such yeast strains, which confirms our results.

However, there are more than 200 commercially available *S. cerevisiae* yeast strains present on the market, making it a challenge to ensure that the yeast strains found by this method are not commercial

ones, especially for wineries that use or have used commercially available yeasts, unlike the winery in Flevoland. It is possible that the patterns found are similar to other commercially available yeasts.

#### 4.3. Fermentative Tests

A correlation was observed between yeast strains of the same group, which showed the same pattern after SSR-PCR and fermentation time. Groups 1A and 6C represent the categories with the largest amount of yeast strains. The three yeast strains of group 1A have a fermentation time of 33 to 48 days and belong to the fastest ten yeasts that completely fermented the must. Both yeast strains of group 6C have a fermentation time of 55 days, which shows a good correlation between the group and fermentation time. The density discrepancy between the three replicates can be explained by the fact that these experiments were realized with living organisms. Indeed, each yeast, even if it is the same strain, can evolve differently.

Fermentation tests were realized at 17 °C in order to create a stressful environment for the yeast strains and because they approximated the conditions found in a winery. The capability and the velocity rate of the fermentation in a stressful environment is indeed a significant factor [34]. Therefore, the choice of wines used for the sensory test was made on the basis of the fermentation rate. The first 10 finished wines were sensorily tested, since a fast fermentation is a prerequisite for strain selection.

#### 4.4. Sensory Differences

The aim of the sensory test was to show organoleptic differences between the wines fermented with different yeast strains. The ten first yeast strains that fermented the must into wine were used, given that the winegrower's interest focused on yeast strains that did not show overly high fermentation rates in order to avoid sluggish fermentations.

A good correlation was found between the placement of the wines chosen by the panelists during the napping procedure and the descriptors. Different descriptors were given for each wine, proving the differences occurring with the yeast strains. In a previous study [32], the evaluation of sensory characteristics between the wines produced using different *Saccharomyces* strains also identified differences resulting from the use of different strains [35]. However, some wines fermented with yeasts like Ymi-3 and Ymg-4 were close to each other and therefore had similar fermentation skills (both fermented the must with an interval of only eight days), even if they originated from different groups (8C and 4C, respectively). Wines fermented with the yeasts Ymg-9 and Ymg-7 were also sensorily similar: both fermented the must in 40 days, but their molecular patterns differed. Although the sensory results showed differences, the confidence ellipses were quite important, which rendered some differences insignificant.

### 5. Conclusions

Simple Sequence Repeats Markers are powerful biomolecular tools for the differentiation of yeast strains. *S. cerevisiae* strain differences were shown, confirming the relevance of the method for the wine industry. Since yeast sequencing is still an expensive technique, this PCR- and agarose-gel-based method is a good low-budget alternative, even if the precision is lower. Based on the comparison of SSR fragment lengths obtained after PCR, an effective differentiation could be obtained for the vineyard in Flevoland, as there were 21 different yeasts for a total of 50 isolated yeast strains. After fermentation and sensory tests, it was possible to provide a yeast strain with specific properties for the winery in Flevoland. The choice of a specific yeast to be used for further fermentations was based on the fermentation velocity and the sensory perception of the produced wine. Since the number of tested commercially available yeasts was low compared to the existing number of products (approx. 200), an improvement to the method would be to include more commercially available yeast strains. This method could also be useful for diversity and phylogenetic studies of worldwide *S. cerevisiae* strains.

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## References

- Chambers, A.H.; Evans, S.A.; Foltá, K.M. Methyl Anthranilate and Gamma-Decalactone Inhibit Strawberry Pathogen Growth and Achene Germination. *J. Agric. Food Chem.* **2013**, *61*, 12625–12633. [[CrossRef](#)] [[PubMed](#)]
- Nykänen, L. Formation and Occurrence of Flavor Compounds in Wine and Distilled Alcoholic Beverages. *Am. J. Enol. Vitic.* **1986**, *37*, 84–96.
- Lambrechts, M.G.; Pretorius, I.S. Yeast and its Importance to Wine Aroma—A Review. *S. Afr. J. Enol. Vitic.* **2019**, *21*, 97–129. [[CrossRef](#)]
- Fleet, G.H. Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **2003**, *86*, 11–22. [[CrossRef](#)]
- Clemente-Jimenez, J.M.; Mingorance-Cazorla, L.; Martínez-Rodríguez, S.; Las Heras-Vázquez, F.J.; Rodríguez-Vico, F. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiol.* **2004**, *21*, 149–155. [[CrossRef](#)]
- van Uden, N. Chapter 2—Ethanol Toxicity and Ethanol Tolerance in Yeasts. In *Annual Reports on Fermentation Processes*; Tsao, G.T., Ed.; Elsevier: Munich, Germany, 1985; pp. 11–58.
- D’Amore, T.; Panchal, C.J.; Russell, I.; Stewart, G.G. A study of ethanol tolerance in yeast. *Crit. Rev. Biotechnol.* **1989**, *9*, 287–304. [[CrossRef](#)] [[PubMed](#)]
- Stanley, D.; Bandara, A.; Fraser, S.; Chambers, P.J.; Stanley, G.A. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **2010**, *109*, 13–24. [[CrossRef](#)] [[PubMed](#)]
- Romano, P. Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* **2003**, *86*, 169–180. [[CrossRef](#)]
- Techera, A.G.; Jubany, S.; Carrau, F.; Gaggero, C. Differentiation of industrial wine yeast strains using microsatellite markers. *Lett. Appl. Microbiol.* **2001**, *33*, 71–75. [[CrossRef](#)]
- Bartowsky, E.J.; Pretorius, I.S. Microbial formation and modification of flavor and off-flavor compounds in wine. In *Biology of Microorganisms on Grapes, in Must and in Wine*; Springer: Heidelberg, Germany, 2009; pp. 209–231.
- Alexandre, H. Wine Yeast Terroir: Separating the Wheat from the Chaff—For an Open Debate. *Microorganisms* **2020**, *8*, 787. [[CrossRef](#)]
- Merle, A.; Piotrowski, M. Consommer des produits alimentaires locaux—Comment et pourquoi? *Décisions Mark.* **2012**, *67*, 37–48. [[CrossRef](#)]
- Capozzi, V.; Fragasso, M.; Romaniello, R.; Berbegal, C.; Russo, P.; Spano, G. Spontaneous Food Fermentations and Potential Risks for Human Health. *Fermentation* **2017**, *3*, 49. [[CrossRef](#)]
- Erusso, P.; Ecapozzi, V.; Espano, G.; Ecorbo, M.R.; Esinigaglia, M.; Bevilacqua, A. Metabolites of Microbial Origin with an Impact on Health: Ochratoxin A and Biogenic Amines. *Front. Microbiol.* **2016**, *7*, 482. [[CrossRef](#)]
- Capozzi, V.; Fragasso, M.; Russo, P. Microbiological Safety and the Management of Microbial Resources in Artisanal Foods and Beverages: The Need for a Transdisciplinary Assessment to Conciliate Actual Trends and Risks Avoidance. *Microorganisms* **2020**, *8*, 306. [[CrossRef](#)]
- Goffeau, A.; Barrell, B.G.; Bussey, H.; Davis, R.W.; Dujon, B.; Feldmann, H.; Galibert, F.; Hoheisel, J.D.; Jacq, C.; Johnston, M.; et al. Life with 6000 Genes. *Science* **1996**, *274*, 546–567. [[CrossRef](#)] [[PubMed](#)]

18. Weller, P.; Jeffreys, A.; Wilson, V.; Blanchetot, A. Organization of the human myoglobin gene. *EMBO J.* **1984**, *3*, 439–446. [[CrossRef](#)] [[PubMed](#)]
19. Field, D.; Wills, C. Abundant microsatellite polymorphism in *Saccharomyces cerevisiae*, and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae*, result from strong mutation pressures and a variety of selective forces. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1647–1652. [[CrossRef](#)]
20. Jarne, P.; Lagoda, P.J. Microsatellites, from molecules to populations and back. *Trends Ecol. Evol.* **1996**, *11*, 424–429. [[CrossRef](#)]
21. Albertin, W.; Panfili, A.; Miot-Sertier, C.; Goulielmakis, A.; Delcamp, A.; Salin, F.; Lonvaud-Funel, A.; Curtin, C.; Masneuf-Pomarede, I. Development of microsatellite markers for the rapid and reliable genotyping of *Brettanomyces bruxellensis* at strain level. *Food Microbiol.* **2014**, *42*, 188–195. [[CrossRef](#)]
22. Ghislain, M.; Spooner, D.M.; Rodríguez, F.; Villamón, F.; Núñez, J.; Vásquez, C.; Waugh, R.; Bonierbale, M. Selection of highly informative and user-friendly microsatellites (SSRs) for genotyping of cultivated potato. *Theor. Appl. Genet.* **2003**, *108*, 881–890. [[CrossRef](#)]
23. Garofalo, C.; Berbegal, C.; Grieco, F.; Tufariello, M.; Spano, G.; Capozzi, V. Selection of indigenous yeast strains for the production of sparkling wines from native Apulian grape varieties. *Int. J. Food Microbiol.* **2018**, *285*, 7–17. [[CrossRef](#)]
24. Granchi, L.; Ganucci, D.; Buscioni, G.; Mangani, S.; Guerrini, S. The Biodiversity of *Saccharomyces cerevisiae* in Spontaneous Wine Fermentation: The Occurrence and Persistence of Winery-Strains. *Fermentation* **2019**, *5*, 86. [[CrossRef](#)]
25. Tufariello, M.; Maiorano, G.; Rampino, P.; Spano, G.; Grieco, F.; Perrotta, C.; Capozzi, V. Selection of an autochthonous yeast starter culture for industrial production of Primitivo “Gioia del Colle” PDO/DOC in Apulia (Southern Italy). *LWT* **2019**, *99*, 188–196. [[CrossRef](#)]
26. Vaudano, E.; Garcia-Moruno, E. Discrimination of *Saccharomyces cerevisiae* wine strains using microsatellite multiplex PCR and band pattern analysis. *Food Microbiol.* **2008**, *25*, 56–64. [[CrossRef](#)] [[PubMed](#)]
27. Legras, J.-L.; Ruh, O.; Merdinoglu, D.; Karst, F. Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **2005**, *102*, 73–83. [[CrossRef](#)] [[PubMed](#)]
28. Börlin, M.; Venet, P.; Claisse, O.; Salin, F.; Legras, J.L.; Masneuf-Pomarede, I. Cellar-Associated *Saccharomyces cerevisiae* Population Structure Revealed High-Level Diversity and Perennial Persistence at Sauternes Wine Estates. *Appl. Environ. Microbiol.* **2016**, *82*, 2909–2918. [[CrossRef](#)]
29. Pages, J. Recueil direct de distances sensorielles: Application à l'évaluation de dix vins blancs du Val-de-Loire. *Sci. Aliment.* **2003**, *23*, 679–688. [[CrossRef](#)]
30. Pagès, J. Collection and analysis of perceived product inter-distances using multiple factor analysis: Application to the study of 10 white wines from the Loire Valley. *Food Qual. Prefer.* **2005**, *16*, 642–649. [[CrossRef](#)]
31. Lê, S.; Josse, J.; Husson, F. FactoMineR: AnRPackage for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [[CrossRef](#)]
32. Hennequin, C.; Thierry, A.; Richard, G.F.; Lecointre, G.; Nguyen, H.V.; Gaillardin, C.; Dujon, B. Microsatellite Typing as a New Tool for Identification of *Saccharomyces cerevisiae* Strains. *J. Clin. Microbiol.* **2001**, *39*, 551–559. [[CrossRef](#)]
33. Sihlhavy, K.; Berger, S.U.; Mandl, K.A.; Hack, R.O.; Regner, F.E. Microsatellite analysis of commercial wine yeast strains. *Mitt. Klosterneubg.* **2006**, *56*, 140–146.
34. Torija, M.J. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2003**, *80*, 47–53. [[CrossRef](#)]
35. Iranzo, J.U.; Magaña, F.G.; Viñas, M.G. Evaluation of the formation of volatiles and sensory characteristics in the industrial production of white wines using different commercial strains of the genus *Saccharomyces*. *Food Control* **2000**, *11*, 143–147. [[CrossRef](#)]

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