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Microbial Communities of Flor Velums and the Genetic Stability of Flor Yeasts Used for a Long Time for the Industrial Production of Sherry-like Wines

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Abstract: Flor yeast strains represent a specialized group of *Saccharomyces cerevisiae* yeasts used for the production of sherry-like wines by biological wine aging. We sequenced the genome of the industrial flor yeast strain I-329 from a collection of microorganisms for winemaking "Magarach" and the metagenomes of two flor velums based on this strain and continuously maintained for several decades. The winery uses two processes for the production of sherry-like wine: batch aging and a continuous process similar to the criaderas–solera system. The 18S rRNA gene profiling and sequencing of metagenomes of flor velums revealed the presence of the yeasts *Pichia membranifaciens* and *Malassezia restricta* in minor amounts along with the dominant *S. cerevisiae* I-329 flor yeast. Bacteria *Oenococcus oeni* and *Lentilactobacillus hilgardii* together accounted for approximately 20% of the velum microbiota in the case of a batch process, but less than 1% in the velum used in the continuous process. Collection strain I-329 was triploid for all chromosomes except diploid chromosomes I and III, while the copy numbers of all chromosomes were equal in industrial velums. A comparative analysis of the genome of strain I-329 maintained in the collection and metagenomes of industrial velums revealed only several dozens of single nucleotide polymorphisms, which indicates a long-term genetic stability of this flor yeast strain under the harsh conditions of biological wine aging.

Keywords: *Saccharomyces cerevisiae*; flor yeast; velum; metagenome; genetic diversity; SNP

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1. Introduction

The biological aging of wine is the most important process in the formation of the organoleptic characteristics of sherry and sherry-like wines produced in several European countries. The production of such wines is impossible without the use of flor-forming *Saccharomyces cerevisiae* yeasts, which are directly involved in the process of the biological aging of wine. Unlike wine strains, which die at the end of fermentation, flor yeast form a floating biofilm (velum) and switches from alcoholic fermentation to oxidative metabolism. During oxidative metabolism, the biochemical oxidation of ethanol and the remaining carbohydrates occurs, with the formation of acetaldehyde and its derivatives which give a characteristic aroma to sherry-like wines [1–3].

Tolerance to high concentrations of ethanol (>15%) is primarily associated with the ability to form a velum as a result of changes in the hydrophobic characteristics of the yeast cell wall, which leads to cell aggregation. The ascent of formed cell aggregates is ensured by the ability to retain carbon dioxide bubbles formed during metabolism on the cell surface [4]. After surfacing, the cells gain access to oxygen, which allows the biogenic oxidation of ethanol. Using comparative genomic methods, it has been shown that flor yeast strains form a separate lineage departing from the wine yeast [5–7].

Wine yeast is a diverse group of microorganisms with high karyotypic variability in terms of chromosome number and size [8]. As a rule, most yeast strains (~87%) are diploid; haploid and polyploid strains are less common. The share of polyploids (3–5 N) is ~11.5% of strains. In 19.1% of strains, aneuploidy is observed in various chromosomes, but most often in chromosomes I, III, and IX [9]. Flor yeast strains are predominantly diploid, but some, such as F25, Fino 1.28 and GBFlor-C, have a triploid set of chromosomes, while in the strain GBFlor-C isolated from González Byass Sherry wine, aneuploidy for chromosome III was observed [10,11]. In yeast, aneuploidy can have an adaptive character. Thus, strains resistant to high concentrations of ethanol usually have aneuploidy for chromosome III, which is most likely associated with an increased expression of genes associated with ethanol resistance [11,12]. The presence of an additional chromosome III is also associated with higher resistance to elevated temperatures [13]. Aneuploidy for a number of other chromosomes is associated with resistance to various chemically and physically stressful environmental conditions [14].

The comparison of genomes of wine and flor yeast strains revealed a number of genetic variation specific for flor yeast including hundreds of single-nucleotide polymorphisms (SNPs), short insertions and deletions, chromosomal rearrangements, and gene content alterations [5,7,15]. First of all, the difference between flor and wine yeast lies in the variations associated with the genes that provide the hydrophobicity of cells, flocculins. The elevated expression of the *FLO11* gene, determined by the 111 nt deletion in the non-coding RNA (ncRNA) ICR1, results in higher cell-surface hydrophobicity and multicellular aggregate formation [6,10,16,17]. Another feature of flor yeast Flo11 protein is the overgrowth of 12 a.a. long tandem repeats encoding a hydrophobic protein core rich in serine and threonine [18,19].

In contrast to the genomic studies of flor yeast strains, there are only a limited number of studies devoted to the analysis of velum microbiota using molecular genetic methods. The presence of non-*Saccharomyces* yeasts in Sherry wine is known to be scarce, usually less than 1% [20], since the non-*Saccharomyces* yeasts present in grape must fermentation are replaced by *S. cerevisiae* when ethanol concentrations rise above 4% [21]. The analysis of yeast diversity by the internal transcribed spacer profiling of the velum samples in the criaderas–solera system revealed the presence of *Pichia manshurica*, *Pichia membranifaciens*, *Wickerhamomyces anomalus*, *Candida guillermondii*, and *Trichosporon asahii* in addition to the dominant *S. cerevisiae* [22]. However, non-*Saccharomyces* yeasts present in the velums have low influence in the Sherry wine metabolome [22]. Ruiz-Muñoz et al. reported the occurrence of four non-*Saccharomyces* species, namely *W. anomalus, P. membranaefaciens, P. manshurica*, and *Pichia kudriavzevi*, in some flor velum samples [20]. The analysis of the intraspecies variability of *S. cerevisiae* using microsatellite typing revealed nine genotypes [20]. However, to the best of our knowledge, there are currently no published data on the metagenomic studies of flor velums or the presence of bacteria in their microbiota.

In this work, we employed a metagenomics approach for the analysis of the composition of the microbial communities of flor velums and the genetic stability of *S. cerevisiae* flor strains used for the industrial production of sherry-type wines in Russia. The Oreanda winery uses two types of production processes. In the first one, fresh fortified wine is periodically introduced into a barrel under the flor velum; towards the end of the biological wine aging, the corresponding volume of the barrel is drained. In the second process, similarly to the criaderas–solera system, wine aging under the velum was carried out continuously in a set of connected barrels, with a gradual overflow of wine, in which young fortified wine material is poured into the first barrel daily, and ready-made sherry-like wine is poured from the last barrel in the same amount. Both processes use the flor strain *S. cerevisiae* I-329 [7,23] and involve continuous operation over several decades without artificial velum renewal or replacement. The high concentrations of acetaldehyde and ethanol could have caused mutations in the genome of *S. cerevisiae* flor strains [24] which may facilitate the adaptation of the strain to the characteristics of a particular production process. The availability of flor velums maintained over several decades in two different

winemaking processes, as well as the original "reference" culture retained in the laboratory collection, provides a unique evolutionary model for studies of the adaptation of flor yeasts to particular environmental conditions.

Here, we report the results of the metagenomic analysis of flor velums used in these two production processes and the composition of microbial communities of yeasts and bacteria. We compare the metagenomes of industrial velums with the reference genome of parental strain I-329 to assess its genetic stability and adaptation to long-term growth under the stressful conditions of the industrial winemaking process. This analysis only revealed several dozens of single-nucleotide polymorphisms, which indicate the long-term genetic stability of flor yeasts under the harsh conditions of biological wine aging.

2. Materials and Methods

2.1. Winemaking Processes and Sampling

In this study, we used the flor yeast strain I-329 (Cherez 96-KM) [7,23], maintained in the collection of microorganisms for winemaking "Magarach" (All-Russian National Research Institute of Viticulture and Winemaking "Magarach", Russian Academy of Sciences) for more than 60 years and used in industrial winemaking by the Oreanda winery (Yalta, Russia).

The first batch process was launched at the Oreanda plant in 1962 and was carried out in oak barrels with a volume of 500 L containing 300 L of liquid (Figure 1). The wine aging process takes approximately 3 months, after which a third of the volume of the finished wine is drained, and the same volume of fresh fortified wine is added to the barrel. Two velum samples, designated M2 and M6, were obtained from the surface of the wine in one barrel after the addition of fresh fortified must and three months later, at the end of the maturation of the wine, before the finished wine was poured. The velum was maintained in a barrel for approximately 60 years and is believed to have originated from the collection strain I-329.

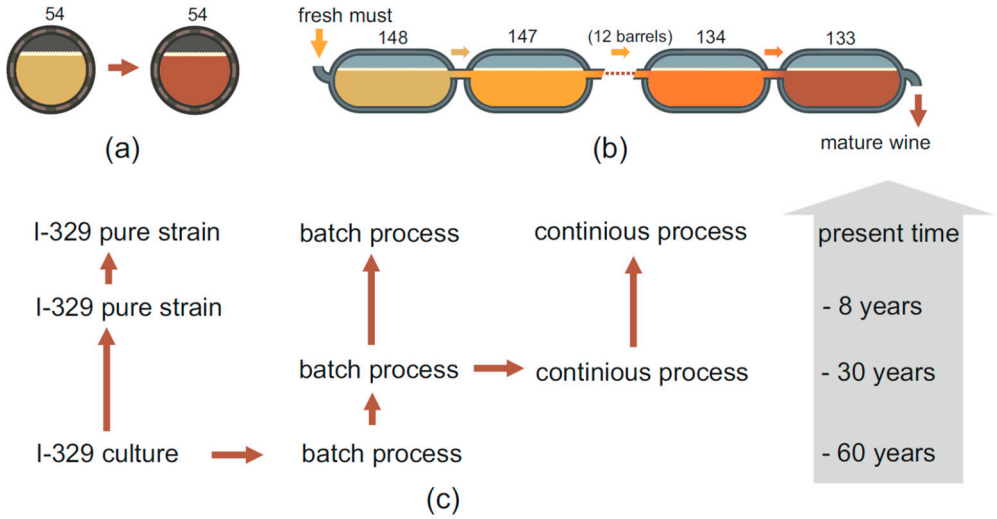

Figure 1. Schemes of batch (**a**) and continuous (**b**) processes used by the Oreanda winery for the production of sherry-like wine. Note that continuous process involves 16 barrels. A diagram showing the sources of velums used in these winemaking processes and a timescale is shown in (**c**).

The second process, similar to the criaderas–solera system, was launched in 1992, and is carried out continuously in a set of 16 connected 5000 L metal barrels 60% filled (Figure 1). Every day, 100 L of wine from a barrel is poured into the downstream barrel, fresh fortified wine is added to the first barrel, and the ready wine is drained from the last barrel. Velum samples were taken from the first barrel (sample M7 (M3)) into which fermented fortified must (16.4–16.7% ethanol content) is poured from the penultimate barrel in the production chain (sample M9 (M4)). This process was initiated using the velum from the older batch

process approximately 30 years ago and was continuously run up to the present time without the artificial replacement of the velum (Figure 1).

Velum samples were taken with a small spoon at three points (pieces approximately 2–5 cm in diameter weighting 100–300 mg). The samples included the entire velum thickness, from the upper layer exposed in air to the lower layer in contact with the liquid. Velum samples were kept at –20 °C until DNA isolation. Total DNA was isolated using the DNeasy PowerSoil Kit (Qiagen, Germany) and quantitated using Qubit Quant-iT dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Chemical Analysis of Wine Samples

At the time of sampling, aliquots of wine were also taken for chemical analyses. The contents of volatile and titratable acids, alcohol, aldehydes and acetals were analyzed using standard methods adopted in winemaking as described earlier [25]. Ethanol content was measured with a hydrometer. The mass concentration of aldehydes was determined by a method based on the ability of aldehydes to bind to sodium hydrosulfite in a complex non-volatile compound. Excess hydrosulfite was oxidized with iodine and then the aldehyde sulfite compound was decomposed with alkali. The liberated sulfur aldehyde was titrated with a 0.005 M iodine solution. The mass concentration of acetals was determined by the method based on the vacuum distillation of aldehydes and volatile acetals of wine with the further acid cleavage of the acetals remaining in the flask and the determination of the released volatile aldehydes by iodometric titration. Sugars were determined by liquid chromatography.

2.3. 18S rRNA-Based Yeast Community Profiling

To determine the taxonomic composition of the yeast community, a fragment of the 18S rRNA gene was obtained by PCR using primers V4_1f (5′ – CCA GCA SCY GCG GTA ATW CC –3′) and TAReukREV (5′– ACT TTC GTT CTT GAT YRA –3′) [26]. The obtained 18S rRNA gene fragments were barcoded using Nextera XT Index Kit v.2 (Illumina, San Diego, CA, USA) and purified using Agencourt AMPure beads (Beckman Coulter, Brea, CA, USA). PCR fragments were sequenced using the Illumina MiSeq instrument in the format of paired-end reads (2 × 300 nt). Paired reads were merged using the FLASH v.1.2.1126 program [27].

Low-quality sequences and singletons were removed and the remaining reads were clustered into operational taxonomic units (OTUs) with a cluster distance of 0.97 using USEARCH v. 11 [28]. To estimate the abundance of OTUs, all reads were mapped to OTU sequences with a threshold of 97%. The taxonomic identification of OTUs was carried out by searches against the NCBI NR database.

2.4. Sequencing of Complete Genome of Strain I-329

Cells of strain I-329 from frozen glycerol stocks were grown on YPD plates at room temperature. A single colony was grown in 50 mL YPD at 20 °C for 24 h, and cells were collected, washed in TE, and freeze-dried. Genomic DNA was isolated with the CTAB extraction method [29] and further purified using the QIAGEN Genomic-tip 500/G kit.

The complete genome of strain I-329 was obtained using a combination of Illumina HiSeq2500 and MinION (Oxford Nanopore) technologies. The sequencing of a TrueSeq DNA library on the Illumina HiSeq2500 instrument generated 13,371,670 paired-end reads (2 × 250 nt). In addition, genomic DNA was sequenced on a MinION device (Oxford Nanopore, Oxford, UK) using the ligation sequencing kit 1D and FLOMIN110 cells. Sequencing resulted in 637,169 reads with a total length of ~3.9 Gb. Contigs were de novo assembled from Illumina and Nanopore reads using Unicycler v. 0.4.8 [30].

Gene search was performed using the PRODIGAL v.2.6.3 program [31]. Annotation was carried out using the Diamond v.2.0 program [32–34] based on the homologs found in the Uniref database, and according to the KEGG database [35] by the program METABOLIC v.4.0 [36].

2.5. Sequencing of Metagenomic DNA, Assembly, and Analysis of Metagenome-Assembled Genomes

The total DNA isolated from the velum samples was sequenced using the Illumina HiSeq2500 platform according to the manufacturer's instructions. The sequencing of a paired-end (2 × 150 bp) TruSeq DNA library generated 82,927,688 read pairs (~24.9 Gb) for the M6 sample and 92,365,384 read pairs (~27.7 Gb) for the M9 sample. The adapter removal and trimming of low-quality sequences (Q < 30) were performed using Cutadapt v.1.17 [37] and Sickle v.1.33 (https://github.com/najoshi/sickle, accessed on 15 March 2023), respectively. The reads were de novo assembled into contigs using the metaSPAdes v.3.15.4 program [38].

The obtained contigs were binned into metagenome-assembled genomes (MAGs) using programs MetaBAT v.2.15 [39], MaxBin v.2.2.7 [40], and Concoct v.1.1.0 [41]. Based on the completeness and contamination of the bins obtained by each of the three programs, an improved binning scheme was obtained by the DAS Tool 1.1.4 [42]. CheckM 1.1.3 [43] was used to evaluate the completeness and contamination values of obtained MAGs. The MAGs were taxonomically classified using the Genome Taxonomy Database Toolkit (GTDB-Tk) v.1.5.0 [44] and Genome Taxonomy Database [45].

To identify single-nucleotide polymorphisms (SNPs) between *S. cerevisiae* from the velum samples and the collection I-329 strain, Illumina reads obtained for M6 and M9 metagenomes were mapped to the strain I-329 reference genome using Bowtie2 v.2.3.5.1 [46]. Program Freebayes v.1.2.0. [47] was used to find SNPs.

3. Results

3.1. Features of Two Winemaking Processes and Wine Characteristics

In the production of sherry-like wines at the Oreanda winery, two technological processes are used. The batch process involves aging the wine in a wooden barrel under a flor velum. After its completion, part of the finished wine is drained and the barrel is filled with fresh fortified wine material. The characteristics of wine materials during aging are presented in Table 1. The duration of the aging period was 105 days, during which the amount of aldehydes increased from 98 mg/L to 412 mg/L and the amount of acetals reached 112 mg/L. The ethanol content decreased by 0.3%. The mass concentrations of sugars, titratable, and volatile acids did not change.

Table 1. Chemical composition of wine samples.

Sample	Barrel	Ethanol (% *v*/*v*)	Redusing Sugars (g/L)	Total Acidity (g/L)	Volatile Acidity (g/L)	Aldehydes (mg/L)	Acetals (mg/L)
Batch process							
M2	54	16.2	1.9	5.0	0.52	98	-
	54	16.1	1.9	5.0	0.52	132	64
	54	16.1	1.9	5.0	0.51	259	67
	54	15.9	1.9	5.0	0.51	328	89
M6	54	15.7	1.9	5.0	0.5	412	112
Continuous process							
M7	148	16.4	2.0	4.7	0.52	86	32
M9	134	16.1	1.4	4.6	0.46	288	100

The second continuous process is similar to the criaderas–solera system. The characteristics of wine were determined for the first barrel (No 148), into which fresh wine material is loaded, and the penultimate barrel in the chain (No 134), from which samples of velums were taken for molecular genetic studies. With this technology, a near-constant chemical composition of wine is maintained in each barrel, which is facilitated by the daily

renewal of wine by 3–5%. The analysis of the chemical composition shows that the wine materials from barrel 148 contain higher amounts of alcohol and volatile acids and a lower concentrations of aldehydes and acetals than the wine from barrel 134 (Table 1). At the same time, in barrel 134, the wine accumulates on average approximately 280 mg/L of aldehydes and 100 mg/L of acetals. According to technological conditions, at the end of the continuous process, sherry-like wine must contain at least 350 mg/L of aldehydes and at least 100 mg/L of acetals.

3.2. Diversity of Yeasts in Industrial Velums Revealed by 18S rRNA Genes Profiling

To identify the eukaryotic microorganisms in the industrial velums thriving under different technological conditions, the community was profiled by the 18S rRNA gene fragments. Four velum samples were analyzed—M2, M6, M7, and M9.

For each sample, 50–60 thousand sequences of 18S rRNA gene fragments were obtained, which were clustered into only three OTUs (Table S1). The yeast community in all studied samples was the same. As expected, *S. cerevisiae* dominated, accounting for more than 99% of 18S rRNA gene sequences. *P. membranifaciens* (99.5% identity with *P. membranifaciens* IFO 10215) and *Malassezia restricta* (99.27% identity with *M. restricta* CBS 7877) were found in minor amounts (<0.1%). The relative abundances of both non-*Saccharomycete* yeasts were lower in velums covering mature wine (M2 vs. M6 and M7 vs. M9).

P. membranifaciens is widely distributed in nature on the surface of grapes and in wine-related environments, and it has an oxidative metabolism and is resistant to high alcohol content [48,49]. *P. membranifaciens* also competes with *S. cerevisiae* and can inhibit their development by secreting killer toxins (PMKT and PMKT2) into the environment [50]. *Malassezia* was also found in wine aging in oak barrels [51]. However, it is still unknown whether these yeasts have wine environments as their natural niche and have a specific role during winemaking or whether they are simply contaminants [51].

3.3. Sequencing of Metagenomes and Assembly of MAGs

In order to obtain the genomes of the dominant microorganisms in the flor velums, we sequenced the metagenomes of samples M6 and M9 using Illumina technology. The obtained reads were assembled into contigs, which were binned into metagenome-assembled genomes (MAGs).

Both velums were dominated by a single phylotype of *S. cerevisiae*, accounting for more than 90% of metagenomes. The average sequence coverage of the corresponding MAGs in samples M6 and M9 was 1088 and 702, respectively (Table 2). The genomes of *P. membranifaciens* and *M. restricta*, minor components of the velum microbiota, could not be assembled due to insufficient sequencing coverage.

Table 2. MAGs obtained from metagenomes of flor velums.

MAG	Average Sequencing Coverage *	
	velum M6	velum M9
S. cerevisiae	1088	702
O. oeni	226	5.3
L. hilgardii	75	4.4

* excluding mitochondrial genome.

In addition to the dominant flor yeast strain, two bacterial MAGs were assembled from the metagenomes and assigned to *Oenococcus oeni* and *Lentilactobacillus hilgardii* based on the average nucleotide sequence identity of >99% with the known genomes of these species. Taking into account the average sequence coverage of *O. oeni* and *L. hilgardii* MAGs (Table 2), the shares of these bacteria in the velum microbiota in the M6 sample were 21% and 7%, respectively. In the metagenome of the M9 sample, the coverage of *O. oeni*

and *L. hilgardii* MAGs was much lower, which indicates their significantly lower relative abundance in this velum, which is less than 1%.

O. oeni and *L. hilgardii* are often found in wine-related environments [52]. *O. oeni* can carry out malolactic fermentation in wine, thereby reducing its acidity and improving its aroma [53]. The second species, *L. hilgardii*, is a wine spoilage lactic acid bacterium. For both species, strains that can grow at ethanol concentrations of 17–19% have been described [54].

3.4. Complete Genome of Strain I-329 from the Culture Collection: Ploidy and Large-Scale Chromosomal Rearrangements

The draft genome of the flor yeast strain I-329 was previously sequenced and analyzed; the gains and losses of genes as well as the single-nucleotide polymorphisms (SNPs) that distinguish this strain from the reference genome of *S. cerevisiae* strain S288c were identified [7]. To determine the ploidy and identify structural rearrangements at the chromosomal level, we assembled the complete genome of strain I-329 using nanopore sequencing data. Strain I-329 genome consisted of 16 chromosomes ranging from 181.8 kb to 1406 kb, with a total size of 11.37 Mb (Table 3).

Table 3. Strain I-329 genome: chromosome sizes and average sequencing coverage.

Chromosome	Length in Strain I-329 (nt)	Average Sequencing Coverage		
		strain I-329	velum M6	velum M9
I	181,889	147	1116	707
II	780,859	203	1059	688
III	289,399	132	1044	678
IV	949,947	207	1070	700
V	530,121	204	1067	695
VI	234,038	205	1080	695
VII	1,039,546	203	1079	708
VIII	515,069	204	1051	681
IX	406,561	207	1048	673
X	716,704	204	1072	694
XI	663,196	203	1100	708
XII	984,655	226	1359	824
XIII	1,406,980	202	1071	704
XIV	757,406	204	1057	681
XV	1,019,606	206	1077	706
XVI	897,340	202	1062	696

Two approaches were used to determine the ploidy at the chromosomal level: the calculation of the distribution of SNP and the comparison of average sequencing coverage. According to the SNP analysis, all chromosomes, with the exception of I and III, have a peak in the dominant allele frequency at approximately ~66%. This distribution is typical for a copy number of chromosomes that is a multiple of three, and most likely, these chromosomes are represented by three copies. For chromosome III, the dominant allele frequency has a peak at ~50%, which is expected for chromosomes whose copy number is a multiple of two. The I chromosome has the smallest number of SMPs per unit length compared to the rest of the chromosomes without a pronounced allele frequency peak, which indicates the presence of two or more copies in the near-homozygous state (Figure 2).

The determination of the ploidy by sequencing coverage showed that the average sequencing depth of chromosomes I and III was 147 and 132, respectively (Table 3). These values correspond to approximately 2/3 of the average sequencing coverage of the other chromosomes (206, ranging from 202 to 226). The most likely explanation is that all chromosomes are triploid except for chromosomes I and III, which are present in two copies.

The genome of strain I-329 relative to the reference strain S288c has a high level of collinearity on a chromosomal level. Intrachromosomal rearrangements are less than 5 kb

in size and are mainly associated with the presence/absence of mobile elements. The number of interchromosomal translocations is also low. The largest translocation was the transfer of approximately 500 kb fragments located on chromosome IV in strain S288c to chromosome XIII in strain I-329 (Figure S1).

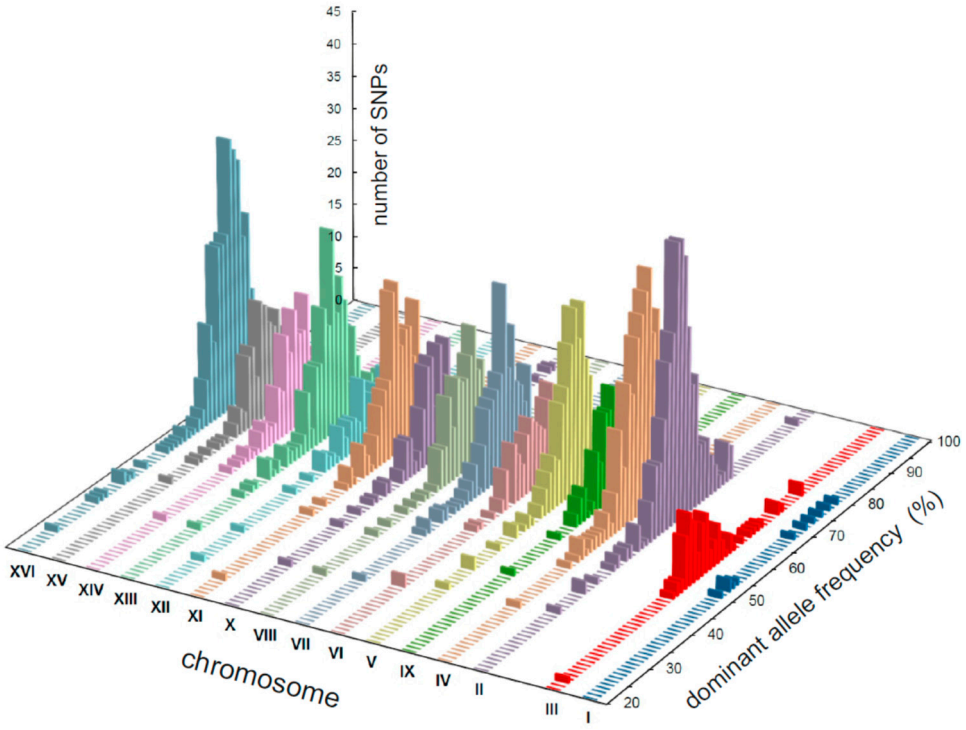

Figure 2. Frequencies of the dominant alleles at the SNP sites in the chromosomes.

3.5. Genetic Differences between Yeast from Industrial Velums and Strain I-329 Maintained in the Culture Collection

The studied industrial velums were obtained on the basis of the selected strain I-329 and maintained for several decades, while the parental strain was maintained in the culture collection. Taking into account the harsh and stressful winemaking conditions, we analyzed how the MAGs of flor yeasts in velum samples differ from the genome of strain I-329, which was maintained in the collection. These Illumina reads, obtained by metagenome sequencing of samples M6 and M9, were mapped to the reference genome of strain I-329.

This comparison revealed only 54 SNPs (Table S2). Among them, 22 SNPs were found in intergenic regions, 10 were synonymous substitutions, and 22 were missense mutations. SNPs occur on chromosomes I, III, IV, VII, VIII, IX, XII, XIII, and XIV. The distribution of SNPs across chromosomes is also uneven. All 19 SNPs found on chromosome III are localized in one region that is approximately 40 kb long, 4 SNPs on chromosome IX are located in a region that is approximately 12 kb long, and 4 SNPs on chromosome I are clustered in a region of length less than 3 kb.

The majority of SNPs (36), including all 19 SNPs on chromosome III and 4 SNPs on chromosome IX, were present in both velum samples and supported by more than 95% of reads, while a few reads with another allele may represent sequencing errors. Probably, these polymorphisms accumulated in the genome of strain I-329 during its maintenance in the collection after being used as a source for industrial velum 60 years ago. Nonsynonymous substitutions were found in the genes for bifunctional AP-4-A phosphorylase/ADP sulfurylase, uncharacterized protein YCL049C, sporulation-specific protein Sps22p, glucose-induced degradation complex subunit GID7, hypothetical protein Gfd2p, transcription factor Fzf1p, chromatin-remodeling histone chaperone SPT6, phosphatase

YCH1, N,N-dimethylaniline monooxygenase, zinc-coordinating transcription factor SFP1, and glyoxylate reductase GOR1.

The remaining 18 SNPs were detected in one or both of the velums at lower frequencies, which, however, are most likely not the result of sequencing errors. The frequencies of most of these SNPs in both velums ranged from 5 to 95%, indicating the presence in the velums of multiple flor yeast strains whose genomes differ in the corresponding position. Only in three cases can it be assumed that one of the velums is genetically homogeneous: at position 1,141,395 nt of chromosome XIII (synonymous substitution in the E1 ubiquitin-activating protein UBA2 gene, frequency 88.9% in sample M9, and 0% in sample M6), at position 861,117 nt in chromosome XII (intergenic region, frequency 100% in sample M9, and 88.5% in sample M6), and at position 847,052 nt on chromosome XII (nonsynonymous substitution in the gene encoding tethering complex ATP-binding subunit VPS33, frequency 98.4% in sample M9 and 21.0% in sample M6). We also note a nonsynonymous substitution in the glucokinase gene (position 926,887 nt of chromosome IV), the frequency of which is 86.2% in the M9 sample and 6.7% in the M6 sample.

To compare the number of copies of individual chromosomes in the genomes of flor yeasts from industrial velums, we determined the average coverage of chromosomes by mapping reads obtained from metagenome sequencing. In contrast to the reference strain I-329, all yeast chromosomes from the velums had approximately the same average sequence coverage, including chromosomes I and III (Table 3).

4. Discussion

4.1. Genetics of Flor Yeasts of Industrial Velums

Flor yeast velum can be continuously used for the biological aging of wine for many years. Under such conditions, flor yeasts are constantly exposed to the mutagenic factors of elevated concentrations of ethanol and aldehydes. At the same time, yeast retained its physiological properties and ensures the aging of wine to the required technological characteristics. Here, for the first time, we examined how the cultivation of yeast velums under harsh industrial conditions affects the stability of the yeast genome and the accumulation of mutations.

Industrial velums obtained on the basis of the flor yeast strain *S. cerevisiae* I-329 were analyzed using molecular methods. The determination of the complete genome sequence of this strain showed that its karyotype, consisting of 16 chromosomes, is similar to other wine and flor yeasts. The number of chromosomes is the same as in the laboratory strain *S. cerevisiae* S288c, but the sizes of each of the assembled chromosome sequences, except for chromosome XIII, are smaller than that of the strain S288c. This is mainly due to the presence/absence of mobile elements and shorter assemblies of the telomere-proximal regions. Chromosome XIII in strain I-329 is significantly larger than in S288c due to the translocation of a region approximately 500 kb from chromosome IV.

In the laboratory strain I-329, all chromosomes except for I and III are present in tree copies, while these two chromosomes are diploid. Unlike the natural strains of *S. cerevisiae*, which has a diploid set of chromosomes, industrial strains are usually characterized by an increase in ploidy and aneuploidy, most often in the cases of chromosomes I, III, and XI [9]. For flor yeast strains, the aneuploidy of chromosome III is most typical. It is associated with resistance to stressful conditions, such as high ethanol concentrations and elevated temperature. However, in the laboratory strain I-329, the number of copies of chromosome III, on the contrary, is less than the others. At the same time, in yeast strains from both industrial velums, the copy number of all 16 chromosomes is the same, although we cannot determine whether they are diploid or triploid. Chromosome III contains regions responsible for resistance to ethanol and yeast strains tolerant to high concentrations of ethanol often have three or more copies of this chromosome [11]. For example, the strain 2-200-2, which has three copies of chromosome III, was able to grow even in 14% ethanol concentration, while a derivative of this strain, in which the extra copy of the chromosome III was removed, was not able to grow on even a 10%-ethanol medium [11].

The laboratory culture I-329 was maintained in the collection on a standard medium without high concentrations of ethanol. Therefore, it can be assumed that, in the laboratory strain, after the initiation of industrial velums, a decrease in the copy number of two chromosomes (I and III) relative to others occurred, while under the harsh conditions of biological wine aging, the original karyotype was preserved. An alternative explanation may be that the I-329 strain has other efficient mechanisms of resistance to high ethanol concentrations, which makes it independent of the chromosome III copy number variations. In any case, our data show that the long-term maintenance of flor under conditions of high ethanol concentration did not lead to the selection of yeast strains with an elevated copy number of chromosome III.

Some yeast strains have additional copies of chromosome XIII that contain certain genes related to the oxidative conversion of ethanol into acetaldehyde during biological ageing [55]. An elevated copy number of this chromosome was not observed in both the laboratory strain and the velums at the winery.

High concentrations of ethanol and aldehydes during sherry production are an inevitable mutagenic factor affecting the stability genomes of flor yeast strains. However, strain I-329 shows high genetic stability both under the batch process of production of sherry-like wines and under a continuous criaderas–solera-like production system, as evidenced by the identification of only 54 SNPs.

When analyzing the identified genetic differences between the flor yeasts from the velums and the collection strain I-329, it must be taken into account that, 60 years ago, at the time when this strain was used to initiate the velum for a batch process, it was not a pure culture, but was a population resulting from selection work. This population was maintained by cultivation in a liquid medium. Only recently was a pure culture of I-329 strain obtained for genomic sequencing and other molecular genetic studies. Therefore, both industrial velums initially represented the populations of genetically non-identical cells, in which the selection of strains best adapted to the winemaking technologies used as well as the accumulation of new mutations could take place. A velum for a continuous process (sample M9) was obtained after approximately 30 years from a velum used in a batch process (sample M6). Thus, 36 SNPs that distinguish the sequenced genome of strain I-329 from both velum samples where the alternative alleles occur with frequencies close to 100% can either be the result of the accumulation of mutations in the course of maintenance of strain I-329 in the culture collection, or already be present in the I-329 population used for the inoculation of industrial films. The latter seems more likely for the 23 clustered SNPs on chromosomes III and IX (Table S2).

The 18 remaining polymorphisms occur in industrial velums at various frequencies. The difference in the frequencies of non-reference alleles probably reflects the adaptation of the consortium to particular conditions of winemaking and the corresponding change in the relative abundances of particular strains in the population. It should be noted that, under the conditions of a continuous process (sample M9), the chemical composition of wine is relatively constant, while in a batch process (sample M6), flor yeasts are periodically stressed when fresh fortified must is added, with a higher content of ethanol and sugars, but lower in aldehydes. However, the frequencies of alleles in two samples differed by more than three times only for seven polymorphisms. Of these, four SNPs were located in intergenic regions, and one SNP caused a synonymous substitution. Only two SNPs were missense variants. A substitution in the *VPS33* gene occurred with a frequency of 98.4% in the M9 sample and with a frequency of 21.0% in the M6 sample. This gene encodes an ATP-binding protein that is a subunit of the HOPS and CORVET complexes essential for protein sorting, vesicle docking, and fusion at the vacuole [56]. The significance of this protein under the conditions of biological wine aging is unclear. The C548A substitution was found in the *YDR248C* gene coding for gluconokinase (chromosome IV), its frequency was 86.1% in the M9 sample and 6.7% in the M6 sample. Glucokinase is the first enzyme in the glycolysis pathway that is downregulated in flor yeast when they switch to oxidative metabolism, but it is possible that the higher frequency of this allele in a continuous

culture growing in a constantly low sugar environment is somehow related to the efficient utilization of the remaining glucose.

4.2. Non-Saccharomyces Yeasts and Bacteria in Industrial Velums

The analysis of the composition of the eukaryotic community by 18S rRNA genes profiling revealed the presence of two other yeasts along with *S. cerevisiae*,—*P. membranifaciens* and *M. restricta.* The presence of *P. membranifaciens* in flor velums was previously reported [20,22] while *Malassezia* was discovered for the first time. The shares of these yeast species in the pool of 18S rRNA gene sequences did not exceed 0.1% and were several times lower in the velum samples at the end of wine maturation in both winemaking processes. Therefore, it can be assumed that these species were delivered to the velums with fresh fermented must but were not able to adapt to the conditions of biological wine aging to the same extent as the *S. cerevisiae* flor yeasts.

The metagenomic analysis of velums revealed the presence of two species of lactic acid bacteria of the *Lactobacillaceae* family—*O. oeni* and *L. hilgardii*. *O. oeni* is a characteristic representative of lactic acid bacteria involved in processes of wine maturation [53,57]. It is responsible for the occurrence of desirable forms of malolactic fermentation in which bacteria decarboxylate L-malic acid to L-lactic acid with the release of carbon dioxide gas [58]. Malolactic fermentation generally improves the organoleptic characteristics of a wine, making it softer and is an important stage in the maturation of many wine varieties [59]. Strains of *O. oeni* are a major source of malolactic starter cultures used in the wine industry. However, the excessive activity of *O. oeni* can negatively affect the quality of wine, including its spoilage [60]. It is possible that the carbon dioxide released during malolactic fermentation can contribute to the floating of the flor yeast biofilm.

Lentilactobacillus species are involved in the production of some fermented drinks, including wine [61–63]. The *L. hilgardii* we found, previously known as *Lactobacillus hilgardii* [64], is typical of wine-related environments, but more often this species has been identified as a major cause of spoilage, especially in the case of sweet fortified wine [65,66]. In the co-culture of *O. oeni* and *L. hilgardii*, competition for nitrogenous nutrients, mainly peptides, produces a negative growth response, decreasing the growth yield of *O. oeni*, and hence adversely affecting the malolactic fermentation conducted by *O. oeni* and the quality of the final product [67].

Interestingly, although *O. oeni* and *L. hilgardii* were found in the metagenomes of flor velums used in both batch and continuous processes, their share in the velum microbiomes differed drastically. Under the conditions of a batch process, these bacteria accounted for approximately 20% of the microbial community of the velum at the end of wine aging (assessed by the average sequencing coverage of the corresponding MAGs), and *O. oeni* was three times more abundant than *L. hilgardii*. However, in the velum at the end of the continuous process, their shares in the communities were only approximately 0.3% each. Probably, the periodic supply of fresh immature wine provides more beneficial conditions for the development of lactobacilli, while at the end of the continuous process, all the available substrates already became exhausted in the upstream barrels. A metagenomic analysis of a successive series of barrels in a continuous process could allow this hypothesis to be tested.

It is possible that, under the conditions of a batch process, abundant *O. oeni* carry out malolactic fermentation, which helps reduce the acidity of wine. The same can happen in the upstream barrels in a continuous process if *O. oeni* is present in significant quantities. The evidence that *O. oeni* can grow at high concentrations of ethanol [54] suggests that these bacteria may be metabolically active under the conditions of wine aging. We suppose that this issue is of interest for further research, since the presence of lactic acid bacteria in flor velums and their possible role in the technological process of sherry-like wine fermentation has not been previously considered.

5. Conclusions

As a result of sequencing the complete genome of *S. cerevisiae* flor strain I-329, it was found that the strain is triploid for all chromosomes, except for chromosomes I and III, which are diploid. The largest structural difference between the genomes of strain I-329 and the reference strain S288c is the transfer of ~ 500 kb long region from chromosome IV to chromosome XIII.

The metagenomic analysis of flor velums used in the production of sherry-like wines in a batch process and in a continuous process similar to the criaderas–solera system revealed the presence of *P. membranifaciens* and *M. restricta* yeasts in minor amounts along with dominant *S. cerevisiae* flor yeasts. The bacteria *O. oeni* and *L. hilgardii* were also found, which, together, accounted for approximately 20% of the velum microbial community in the case of batch process, but less than 1% in the velum used in continuous process.

The comparative analysis of the genome of parental strain I-329 maintained in the collection and metagenomes of industrial velums continuously maintained for several decades revealed only a few dozen single-nucleotide polymorphisms, indicating the high genetic stability of the strain under the harsh conditions of biological wine aging.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040367/s1, Figure S1: Location of homologous regions in the chromosomes of strain I-329 (vertical axis) and reference strain S288c (horizontal axis); Table S1: Diversity of yeasts in industrial velums revealed by 18S rRNA genes profiling; Table S2: SNPs distinguishing strain I-329 genome and metagenomes of velum samples.

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