Abstract

Since the beginning of the 1980's, the use of active dried Saccharomyces cerevisiae yeast starters has been extensively generalised. Today, the majority of wine production is based on the use of active dried yeast, which ensures rapid and reliable fermentations. The behaviour of these yeasts in the ecosystem of the vineyard is totally unknown as is their potential impact on the natural microflora. The aim of the present study was to evaluate populational structures among fermenting S. cerevisiae populations and to assess the impact of active dry yeast usage on the genetic structures of the vineyard microflora.

S. cerevisiae isolates were obtained from fermentations with grapes from three vineyards of the Vinho Verde Region where commercial yeast strains were used continuously during the last years. Populational genetic analysis was based on six polymorphic microsatellite loci in 361 isolates. Accumulation of small allele-frequency differences across six loci in groups of strains allowed the identification of populational structures. The continuous use of active dry yeast has a very limited impact on the genetic structure of the vineyard microflora. Correlation of genetic differentiation with the distance between sampling points suggested a pattern of isolation-by-distance, where genetic divergence in a vineyard increased with size.

Commercial yeast utilization and genetic structure of vineyard-associated Saccharomyces cerevisiae populations revealed by microsatellite analysis

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Introduction

The grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, the geographic localization of the vineyard, antifungal applications, the harvest technique, grape variety, the vineyard's age as well as the soil type. Several ecological surveys report a large diversity of Saccharomyces sp. strains among the enological fermentative flora. Some strains seem to be widely distributed in a given viticultural region, can be found in several consecutive years and are also predominant in the fermenting flora hypothesizing the occurrence of specific native strains that can be associated to a terroir [1-3].

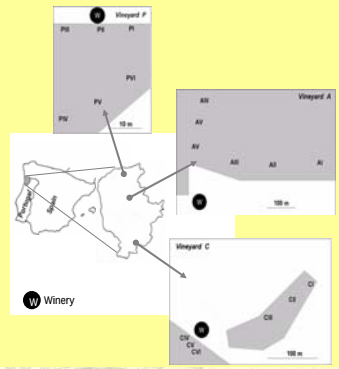
As a result of modern winemaking practices and diversification of wine products, there is an increasing quest for specialised wine yeast strains. At present, leading winemakers demand for autochthonous fermenting strains that are able to enhance the expression of typical sensorial characteristics of wine and ensure the control of the fermentation process, concerning the motto "special yeasts for special traits" [4]. The detailed biogeographical evaluation of fermentative strains is essential for the establishment of adequate selection and improvement programes.

The aim of the present study was to gain insight in the populational structure of vineyard-associated S. cerevisiae populations and to assess whether the continuous use of commercial yeast strains may lead to a shift in the yeast populations found in vines surrounding the wineries where commercial strains are regularly used. This is the first systematical, 3-years biogeographical survey of fermentative S. cerevisiae strains by microsatellite genotyping, aiming at the analysis of populational structures and genetic variability in three vineyards of the Vinho Verde Wine Region of Portugal.

Materials and Methods

Samples

The sampling plan included 3 vineyards with different grape varieties, located in the North of Portugal (Região Demarcada dos Vinhos Verdes), as shown. In each vineyard, six sampling points were defined. The sampling campaigns were performed at the time of the harvest. This experiment was repeated in three consecutive years (2001-2003), resulting in a total of 54 grape samples.



Fermentation

The yeast flora from fermenting grape juice (500 ml) was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Fermenting must samples were diluted and spread on plates with YPD medium. Thirty randomly selected colonies were collected from each spontaneous fermentation and subjected to further analysis.

DNA isolation

Yeast cells were cultivated in 5 ml of YPD medium (24 h, 28ºC, 160 rpm) and DNA isolation was performed using the method described by López et al. [5].

Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 were amplified [6]. Samples were separated in the ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed with the corresponding GENESCAN software. The equivalence of this typing method to previously described ones has been previously shown [7].

Microsatellite	Chromosome	Repeat (number)
ScAAT1	XIII	$(ATT)_{35}$
ScAAT2	II	$(ATT)_{30}$
ScAAT3	IV	$(ATT)_{23}$
ScAAT4	VII	$(ATT)_{32}$
ScAAT5	XVI	$(AAT)_{13}$
ScAAT6	IX	$(AAT)_{29}$

Computer assisted data analysis

A group of strains with unique microsatellite profiles (obtained from 30 isolates per fermentation) was considered the population corresponding to each sampling site. The pattern and degree of temporal and spatial divergence in the nuclear microsatellites ScAAT1 to ScAAT6 among subpopulations was estimated by Fst determination over all loci by AMOVA analysis (computed by the Arlequin software [8]). A similarity matrix of allelic frequencies was computed by the program NTSYSpc 2.0 [9], based on the Euclidean distance and average linkage (UPGMA).

RESULTS

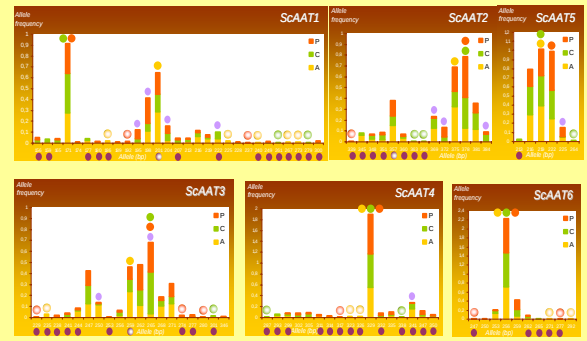
Strains collected

Winery	Year	Number of Isolates	Number of genotypes	Commercial strains recovered: Zymaflore F15	Uvaferm BDX	Zymaflore VL1	Zymaflore F10	ICV D254	Lalvin D423
A	2001	90	11	①					
	2002	180	34	❶ ① ❷					
	2003	180	41	❶ ①	+	+	+	+	+
C	2001	240	26		+	+	+	+	+
	2002	30	1						
	2003	210	35	❶ ❶ ❷	+	+	+	+	+
P	2001	240	64	❶ ❷ ❸			+		+
	2002	150	12	❶ ❷			+		+
	2003	300	59	❶ ❷ ④	+		+		+

❶ Number of perennial genotypes (regional distribution)
① Number of perennial genotypes (limited to one vinayard)
❸ Number of annual genotypes (multiple sites of one vineyard)
④ Number of annual genotypes (in multiple sites of two vineyards)

- The strain collection obtained from this survey comprises 1620 isolates, that were classified in 283 genetic patterns according their allelic distribution.
  (*) Several samples could not be collected due to a very bad sanitation state of the grapes after heavy rainfalls
- The highest biodiversity was observed in winery P (690 isolates, 135 patterns), followed by winery A (450 isolates, 86 patterns) and C (480 isolates, 62 patterns).
  Several genotypes showing a wider temporal and geographical generalized pattern of sporadic presence, absence and reappearance across sampling sites, vineyards or years, as mentioned below

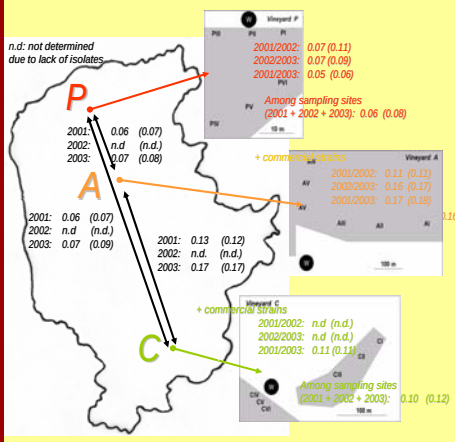
Allelic frequencies for all isolates obtained from the Vinho Verde Region



- The six markers revealed a high degree of genetic variability, being ScAAT1 and ScAAT3 the most polymorphic markers with 29 and 19 alleles, respectively.
- Besides the 41 alleles (51 strains) previously described for ScAAT1-ScAAT6 [3], 52 new alleles ● were identified in the present study.
- Some newly described alleles ○ occur with relative high frequency and may be used as indicative alleles for this wine region.
- The vast majority of alleles were evenly distributed among S. cerevisiae populations belonging to vineyard A, C and P, but differences are notorious for few alleles, which can be considered as vineyard(s) – indicative ●.
- Distinct most frequent ● ● ● and unique ○ ○ ○ alleles were found in each of the three populations.

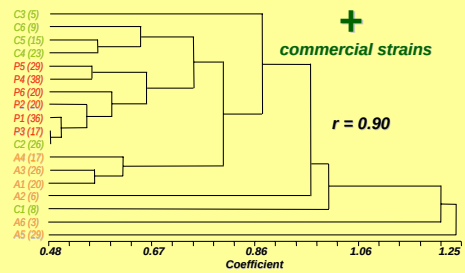
AMOVA analysis - Fst values based on microsatellite data

Numbers in parenthesis indicate the Fst values obtained when the genotypes of commercial yeast strains were excluded



Relationships among the populations belonging to six sampling points in three vineyards, determined by cluster analysis (UPGMA) based on a Euclidean distance dissimilarity matrix of allelic frequencies.

Numbers in parenthesis indicate the number of strains corresponding to unique patterns

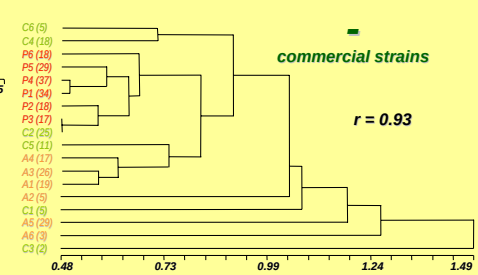


+ commercial strains

r = 0.90

Cluster analysis

- The existence of a populational substructure, characteristic for each vineyard is shown by several clusters, comprising sampling sites of vineyards C, P and A. Populations within groups C and P are more closely related, while S. cerevisiae populations belonging to vineyard A are much more heterogeneous and also more distinct from C and P, which is in accordance with data from Fst analysis.
- Population from CII lies within the P-cluster, indicating that genetic differences do not delimit specific populations with fixed geographic boundaries.
- Exceptions from the vineyard - specific population structure (sampling sites CIII, AII, and AVI) may be due to a low number of analyzed strains or to the presence of rare alleles (AV).
- When genotypes of commercial yeast strains were excluded, a similar populational structure could be observed.
- The cophenetic correlation factor r was between 0.90 – 0.93, indicating that the genetic relationships were not distorted by hierarchic clustering.



- commercial strains

r = 0.93

AMOVA analysis

- The S. cerevisiae populations from A, C and P showed moderate (0.05-0.15) genetic differentiation in three consecutive years, when populations from different vineyards were pairwise associated (A/C, A/P and P/C). Fst values were not correlated with the distance between the vineyards.
- Populations within a vineyard varied in consecutive years, being more variable in A (Fst = 0.11 – 0.17) compared to C (Fst = 0.11) and P (Fst = 0.05 – 0.07).
- When samples were pooled across year-classes within the sampling sites of each vinery, the highest Fst value was again obtained for A (0.17) compared to C (0.12) and P (0.06).
- When the genotypes of commercial yeast strains were excluded, similar Fst values were obtained for the above mentioned populational comparisons.

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Conclusions

Microsatellite typing with loci ScAAT1-ScAAT6, followed by statistical analysis permitted a high resolution populational screen, and is therefore the appropriate method to obtain a deeper insight in the ecology and biogeography of S. cerevisiae strains, even among geographically close regions.

Within a vineyard, genetic differentiation was correlated with the distance between sampling points and consequently the size of the vineyards. S. cerevisiae strains were more distinctive in a larger vineyard that constitutes a bigger "evolutionary playground", hypothesizing that local populations may evolve due to multi-factorial influences being the size of the vineyard one of them. Genetic differences among S. cerevisiae populations derived mainly from gradations in allele frequencies rather than from distinctive "diagnostic" genotypes, and the accumulation of small allele-frequency differences across six loci allowed the identification of a population structure. Genetic heterogeneity in a vine could follow a pattern of isolation-by-distance, where genetic divergence increases with vineyard size. However, the forces causing a global shift in a vineyard's S.cerevisiae populations still remain to be clarified. The extension of the current approach to strains isolated from other viticultural regions is desirable, since a preliminary comparison revealed major differences in both allelic combinations and frequencies (our unpublished data).

The continuous use of commercial yeast strains over more than 5 years does not significantly change the S.cerevisiae populations isolated from vineyards surrounding a winery.

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