Abstract

The analysis of six polymorphic microsatellite loci was performed in 361 Saccharomyces cerevisiae isolates obtained from spontaneous fermentations. This population derived from a previous screening (using mtDNA RFLP) of 1620 isolates of grapes collected in three vineyards of the Vinho Verde Region, in northwest Portugal, during the 2001 – 2003 harvest seasons. Among the 93 alleles obtained, 52 new alleles were identified. For all loci analyzed, observed heterozygosity was three to four times lower than the expected value, probably due to a strong populational substructuring. Populational structures were identified based on the accumulation of small allele-frequency differences across six loci in groups of strains. Genetic differentiation in the same vineyard in consecutive years was of the same order of magnitude as the differences verified among sampling sites within each vineyard. 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. The present work is the first large-scale approach showing that microsatellite typing reveals a very fine population resolution of indigenous S. cerevisiae strains isolated from vineyards.

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].

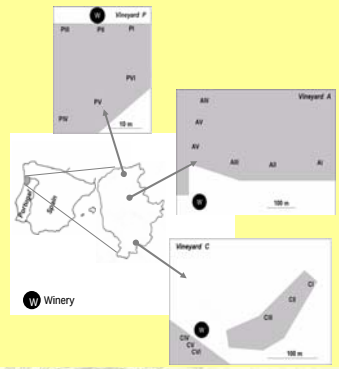
As a result of modern winemaking practices and diversification of wine products, there is an increasing quest for specialised wine yeast strains. At present, leading winemakers demand for autochthonous fermenting strains that are able to enhance the expression of typical sensorial characteristics of 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 programes.

This is the first systematical, 3-years biogeographical survey of fermentative S. cerevisiae strains using microsatellites for 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. 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 shown for a set of 23 commercial S. cerevisiae strains [7].

Microsatellite	Chromosome	Repeat (number)
ScAAT1	XIII	$(ATT)_{26}$
ScAAT2	II	$(ATT)_{30}$
ScAAT3	IV	$(ATT)_{23}$
ScAAT4	VII	$(ATT)_{32}$
ScAAT5	XVI	$(AATT)_{13}$
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	Samples (A1 - A6)	Number of Isolates (*)	Number of genotypes
A	2001	A1 - A6	90	11 ①
	2002	C1 - C6	180	34 ● ① ②
	2003	C1 - C6	180	41 ● ①
C	2001	P1 - P6	240	26
	2002	P1 - P6	30	1
	2003	P1 - P6	210	35 ● ● ①
P	2001	P1 - P6	240	64 ● ② ●
	2002		150	12 ● ②
	2003		300	59 ● ② ①

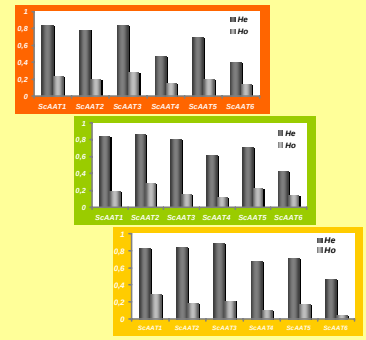
- The strain collection obtained from this survey comprises 1620 isolates, that were classified in 283 genetic patterns according their allelic distribution.
  (*) Several samples could not be collected due to a very bad sanitation state of the grapes after heavy rainfalls
- The highest biodiversity 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

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

Allelic frequencies

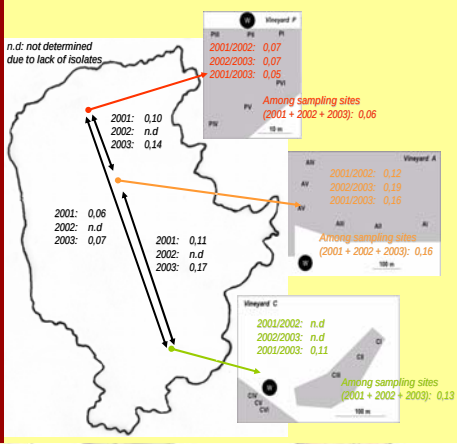
- The six markers revealed a high degree of genetic variability, being ScAAT1 and ScAAT3 the most polymorphic markers with 29 and 19 alleles, respectively.
- Besides the 41 alleles (51 strains) previously described for ScAAT1-ScAAT6 [3], 52 new alleles ● were identified in the present study.
- Some newly described alleles ○ occur with relative high frequency and can 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.

Observed (Ho) and expected (He) heterozygosity

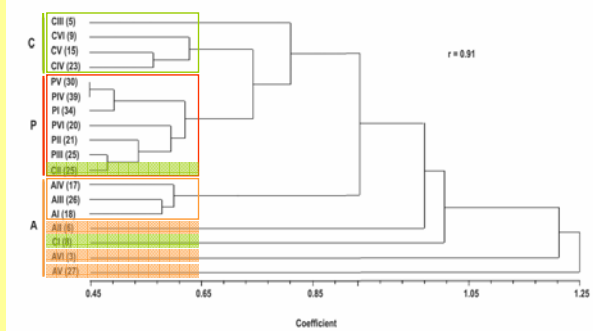


- For all loci, observed heterozygosity (Ho) was three to four times lower than the estimated value (He). Heterozygous genotypes reduction relative to that expected under random mating can be consequence of population substructure.
- Population substructuring can be due to a previously described mechanism called "genome renewal" [10] that has been proposed for natural wine yeast strains, which are mostly diploid and homozygous for the homothallism gene (HO/HO). They can undergo mating among their progeny cells and thereby change multiple heterozygote into completely homozygous cells, leading to gradual replacement of heterozygous diploids.
- Heterozygous deficiencies can also be explained by the presence of null alleles that arise when mutations prevent primers from binding, so that many of the apparent homozygotes can be, in reality, heterozygotes between a visible and a null allele.

FST values based on microsatellite data



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.



Poplational analysis: Genetic variation of S. cerevisiae populations from vineyards A, C and P.

- AMOVA analysis showed that the S. cerevisiae populations from A, C and P were significantly different (P(random value< observed value) < 0.001) in three consecutive years, when populations from different vineyards were pair wise associated (A/C, A/P and P/C). FST values were higher for A/C and A/P (0.11-0.17 and 0.10-0.14) when compared to P/C (0.06-0.08) corresponding to a moderate (0.05 – 0.15) to great (0.15 – 0.25) genetic differentiation.
- Populations within a vineyard varied in consecutive years, being more variable in A (FST = 0.12 – 0.19) compared to C (FST = 0.05 – 0.12) and P (FST = 0.05 – 0.12).
- When samples were pooled across year-classes within the sampling sites of each vinery, the highest FST value was again obtained for A (0.16) compared to C (0.13) and P (0.06).

- The existence of a populational substructure, characteristic for each vineyard is shown by three defined clusters, comprising sampling sites of vineyards C, P and A. Populations within groups C and P are in general 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.
- The cophenetic correlation factor r was 0.91, indicating that the genetic relationships were not distorted by hierarchic clustering.
- Population from CII lies within the P-cluster, indicating that genetic differences do not delimit specific populations with fixed geographic boundaries.
- Further 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).

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Conclusions

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

The present results clearly show the existence of general, region-wide strains, but at the same time specialized populations, typical for a specific winery. Both high degrees of homozygosity and the appearance of rare alleles point to the existence of genetically isolated subpopulations of yeast strains with distinct genetic constitution, possibly due to locally limited specific evolutionary processes. As most alleles were widespread, 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. 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).

Genetic distances and geographical localization of populations did not correlate, and data clearly agree in the distinction of the more similar populations belonging to the more distant vineyards C - P (~100 km) compared to the opposite situation for the closer vineyards A - C (~60 km) and A - P (~40 km). Whether the different grape varieties contribute to the genetic differentiation between the strains is subject of current studies.

Within a vineyard, values of genetic differentiation are correlated with the distance between sampling points and consequently the size of the vineyards. S. cerevisiae strains may become 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 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.

This poster is available at:

http://repositorium.sdum.uminho.pt

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