

Populational analysis of *Saccharomyces cerevisiae* strains from different appellations of origin and grape varieties by microsatellite analysis

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Introduction

Fermented beverages and foods have a longstanding cultural and historical tradition in most societies and the economical interest of fermentation technologies is constantly increasing. *Saccharomyces cerevisiae* is one of the world's premier fermenting microorganisms. This yeast is predominantly found in association with human activities, particularly the production of alcoholic beverages. Whether the vineyard is the natural environment of *S. cerevisiae*, is still a matter of controversy, but recent studies have shown that specialized strains for the production of alcoholic beverages have been derived from natural populations unassociated with alcoholic beverage production, rather than the opposite [1]. 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 [2-3]. The objective of the present study was to evaluate population relationships among *S. cerevisiae* strains isolated from some of the Portuguese most important grapevine varieties in different appellations of origin, using polymorphic microsatellites.

Materials and Methods

Samples

The sampling plan included 20 vineyards and 9 grape varieties in several Portuguese appellations of origin. In each region, appellation-specific recommended grape varieties were collected in two vineyards comprising several grape varieties or single grape varieties, as shown below. Grape samples were obtained from six sampling points in each vineyard, and the yeast flora (30 randomly selected isolates) from spontaneously 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.



Molecular identification

Estremadura, Palmela and Alentejo

Preliminary discrimination between *Saccharomyces* and non-*Saccharomyces* yeast was based on the inability to grow in YNB medium containing L-lysine [4]. *S. cerevisiae* strains were further characterised by analysis of 6-10 *S. cerevisiae* specific microsatellite loci [5, 6].

Vinho Verde

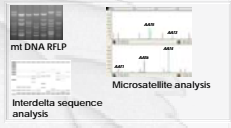
Isolated strains were analysed by mitochondrial DNA restriction patterns (mtDNA RFLP) [7]. Strains with identical mtDNA RFLP patterns were grouped and one representative strain was further characterised by microsatellite analysis.

Bairrada

Isolated strains were analysed by PCR-amplification of interdelta sequences [8] as preliminary screen, followed by microsatellite analysis for in-depth characterization. The equivalent discriminatory power of mtDNA RFLP and interdelta analysis has been previously reported [9].

Computer assisted data analysis

Groups of strains with unique microsatellite profiles were considered as populations corresponding to each vineyard. The pattern and degree of temporal and spatial divergence in the nuclear microsatellites among subpopulations was estimated by F_{ST} determination over all loci by AMOVA analysis (Ariquin software [9]). A similarity matrix of allelic frequencies was computed by the program NTSyspc 2.0 [10], based on Euclidean distance and average linkage (UPGMA).

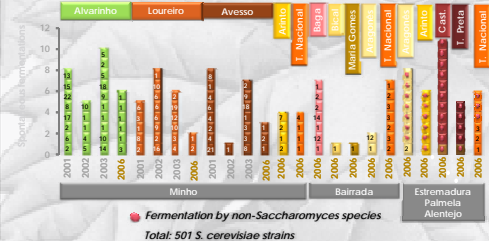


RESULTS

Strains collected

Nº of samples	Minho		Bairrada		Estremadura Palmela Alentejo		Total
	2001-2003	2006	2006	2006	2006	2006	
Spontaneous fermentations	54	30	29	36	36	36	149
Number of isolates	1620	900	870	1080	1080	1080	4470
<i>Saccharomyces</i> sp.	100 %	100 %	100 %	9 %	9 %	9 %	

S. cerevisiae strains involved in spontaneous fermentations



- From a total of 300 grape samples 4470 isolates were obtained; 192 samples (2850 isolates) were collected in the five wine regions during the grape harvest of 2006. The fermentative yeast flora from the Vinho Verde and Bairrada Regions were composed by *S. cerevisiae* strains, whereas spontaneous fermentation of grapes from the other regions were mainly conducted by non-*Saccharomyces* species. A total of 501 *S. cerevisiae* strains was obtained.
- The number of *S. cerevisiae* strains is indicated for each spontaneous fermentation, that corresponds to a square in each column related to the sampling year and grape variety. The composition of *S. cerevisiae* strains was very variable, ranging from 1 – 22 among 30 randomly collected isolates.

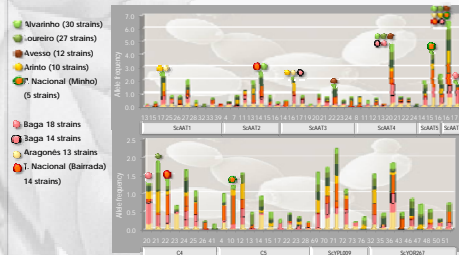
Allelic frequencies

Distribution of the most frequent (■) microsatellite alleles

Locus	Nº of alleles	Allele (nº of repeats)
ScAA1	43	1315161718192022232425262728293031323334353639404143474951535455
ScAA2	13	3 4 5 6 7 8 10 11 12 13 14 15 16
ScAA3	21	9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 34 49 55
ScAA4	19	6 8 9 10 11 12 13 14 15 16 18 19 20 21 22 23 24 26 27
ScAA5	5	14 15 16 17
ScAA6	11	13 15 16 17 18 19 20 21 22 23
C4	11	20 22 23 24 25 26 27 28 29 31
C5	26	4 5 6 8 10 11 12 13 14 15 16 17 18 19 20 22 23 24 25 26 28 30 31 34
YPL009	24	4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
ScYOR267	26	19 22 25 30 31 32 33 35 36 37 39 41 43 44 46 47 48 49 50 51 52 53 54 55 58

- The ten markers revealed a high degree of genetic variability, being ScAA1, C5 and YOR267 the most polymorphic markers with 43, 26 and 26 alleles, respectively.

Most frequent alleles among *S. cerevisiae* strains from grape varieties of the Vinho Verde and Bairrada Regions



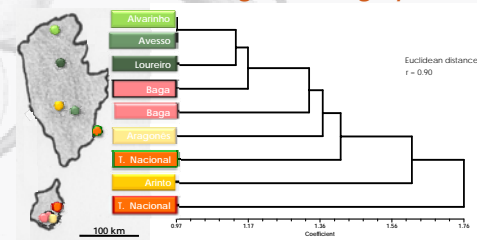
- The colored circles mark the three most frequent alleles for *S. cerevisiae* isolates from different grape varieties in the Vinho Verde and Bairrada regions.
- Five alleles were shared by more *S. cerevisiae* populations, whereas alleles 20(ScAA4), 20(C4), 21(C4), 22(C4) and 10(C5) can be considered as characteristic of the vineyards from where these isolates were obtained.
- The allelic distribution of *S. cerevisiae* strains from Baga and Touriga Nacional grapes shows that the genetic constitution of the strains is rather vineyard-specific than grape variety-specific.

Distribution of the most frequent alleles among vineyards of the Vinho Verde Region in consecutive years



- S. cerevisiae* populations isolated from the Alvarinho, Avesso and Loureiro grape varieties share the most frequent alleles, that are equally distributed among populations isolated from consecutive sampling years.
- Less frequent alleles that were found in *S. cerevisiae* populations from the three vineyards showed a differential distribution.
- Vineyard-specific alleles that occurred in one or two locations were also detected.
- Less frequent alleles showed more variations in their distribution in consecutive years.

Similarity of *S. cerevisiae* populations from distinct wine regions and grape varieties



- The degree of spatial divergence in ten nuclear microsatellite markers was determined by cluster analysis (UPGMA) based on a Euclidean distance dissimilarity matrix of allelic frequencies.
- Vineyard-specific population structures show that genetic proximity is not related with the geographic distance. The most similar populations were obtained from vineyards in the Vinho Verde Region (Alvarinho and Avesso, located at a distance of ca. 150 km). Contrarily, yeast populations of vineyards in closer locations (e.g. Avesso and Arinto, at a distance of ca. 20 km) were more distinctive.
- Genetic proximity is not related with the grape variety. Populations isolated from Baga and Touriga Nacional grapes in different locations are not closely related.

Conclusions

The fermenting yeast flora of grapes from the Vinho Verde and Bairrada Regions is mainly composed by *S. cerevisiae*, contrarily to the prevailing non-*Saccharomyces* yeast flora in southern Portuguese wine regions. These differences may be assigned to climatic or other ecological factors.

The finding of 501 *S. cerevisiae* strains over four years reveals a fascinating genetic diversity of *S. cerevisiae* strains in vineyard environments.

Mathematical analysis of the distribution of 192 alleles from 10 microsatellite loci permitted a high resolution population screen and showed that:

- genetic differences among *S. cerevisiae* populations derived from both "diagnostic" vineyard-specific alleles and the accumulation of small allele-frequency differences across ten microsatellite loci allowed the identification of population structures.
- each vineyard contains differentiated *S. cerevisiae* populations. This is supported by the finding of identical or similar *S. cerevisiae* strains in consecutive years, hypothesizing the occurrence of specific native strains that can be associated with a terroir.

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