

Microsatellite analysis reveals populational structures of *Saccharomyces cerevisiae* strains from different appellations of origin and grape varieties

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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 populational 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 6-10S *Saccharomyces* specific microsatellite loci [5, 6].

Vinho Verde

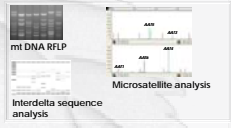
Isolated strains were analysed by mitochondrial DNA restriction patterns (mtDNA RFLP) [7]. Strains with identical mtDNA RFLP patterns were grouped and one representative strain was further characterised by microsatellite analysis.

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).

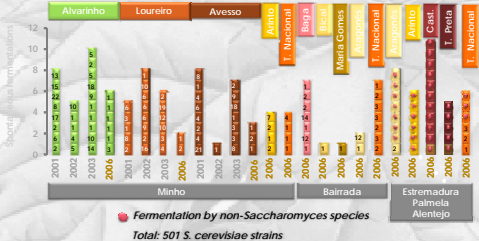


RESULTS

Strains collected

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

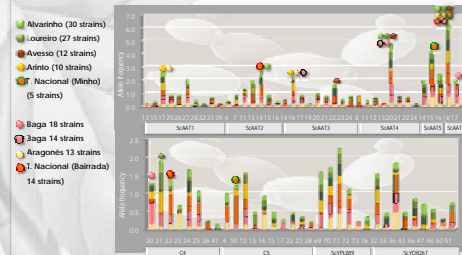
Allelic frequencies

Distribution of the most frequent (■) microsatellite alleles

Locus	Nº of alleles	Allele (nº of repeats)
ScAAT 1	43	1315161718192022232425262728293031323334353639404143474951535455
ScAAT 2	13	3 4 5 6 7 8 10111213141516
ScAAT 3	21	9 1012131415161718192021222324252627344955
ScAAT 4	19	6 8 9 10111213141516181920212223242627
ScAAT 5	5	141451617
ScAAT 6	11	1819151617181920212328
C4	11	2021222324252627282931
C5	26	4 5 6 8 10 12 14145 1617181920222324252628303134
YPL009 24	4	435158555758596162636566697071727376777880818889
ScYOR267	26	1922253031323335363739414344454647484950515253455558

- The ten markers revealed a high degree of genetic variability, being ScAAT1, 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



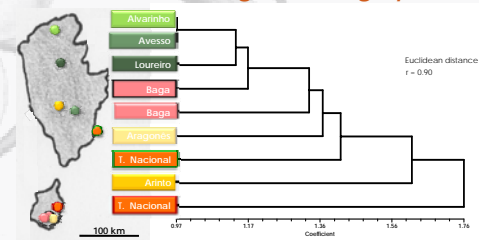
- The colored circles mark the three most frequent alleles for *S. cerevisiae* isolates from different grape varieties in the Vinho Verde and Bairrada regions.
- Five alleles were shared by more *S. cerevisiae* populations, whereas alleles 20(ScAAT4), 20(C4), 21(C4), 22(C4) and 10(C5) can be considered 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 populational 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 populational 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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