Genetic Diversity and Population Structure of *Saccharomyces cerevisiae* Strains Isolated from Different Grape Varieties and Winemaking Regions

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**Abstract**

We herein evaluate intraspecific genetic diversity of fermentative vineyard-associated *S. cerevisiae* strains and evaluate relationships between grape varieties and geographical location on populational structures. From the musts obtained from 288 grape samples, collected from two wine regions (16 vineyards, nine grape varieties), 94 spontaneous fermentations were concluded and 2820 yeast isolates were obtained that belonged mainly (92%) to the species *S. cerevisiae*. Isolates were classified in 321 strains by the use of ten microsatellite markers. A high strain diversity (8–43 strains per fermentation) was associated with high percentage (60–100%) of fermenting samples per vineyard, whereas a lower percentage of spontaneous fermentations (0–40%) corresponded to a rather low strain diversity (1–10 strains per fermentation). For the majority of the populations, observed heterozygosity (\(H_o\)) was about two to five times lower than the expected heterozygosity (\(H_e\)). The inferred ancestry showed a very high degree of admixture and divergence was observed between both grape variety and geographical region. Analysis of molecular variance showed that 81–93% of the total genetic variation existed within populations, while significant differentiation within the groups could be detected. Results from AMOVA analysis and clustering of allelic frequencies agree in the distinction of genetically more dispersed populations from the larger wine region compared to the less extended region. Our data show that grape variety is a driver of populational structures, because vineyards with distinct varieties harbor genetically more differentiated *S. cerevisiae* populations. Conversely, *S. cerevisiae* strains from vineyards in close proximity (5–10 km) that contain the same grape variety tend to be less divergent. Populational similarities did not correlate with the distance between vineyards of the two wine regions. Globally, our results show that populations of *S. cerevisiae* in vineyards may occur locally due to multi-factorial influences, one of them being the grape variety.

**Introduction**

Recent phylogenetic analyses of *Saccharomyces cerevisiae* strains have found that the species as a whole consists of both “domesticated” and “wild” populations. Although the genomes of most *S. cerevisiae* strains with disparate ecological and geographic sources are mosaics, genealogical relationships from DNA sequence diversity showed that domesticated strains derived from two independent clades, corresponding to strains from winemaking and sake (Japanese rice wine). “Wild” populations are mostly associated with oak trees, nectars or insects (e.g. *Drosophila* spp., honey bees and wasps) \([1,2,3,4,5]\).

As reviewed by Martiny et al., \([6]\), a growing body of evidence supports the idea that free-living microbial taxa exhibit biogeographic patterns. Bacterial species vary in abundance, distribution and diversity over various taxonomic and spatial scales, whereas genetic distance is correlated with geographic distance or environmental characteristics such as salinity, depth and latitude. To study the ecology and population dynamics of *S. cerevisiae* strains in both vineyards and wineries, numerous molecular methods were developed recently. Microsatellite analysis can be considered the method of choice for *S. cerevisiae* strain delimitation, allowing high-throughput and precise data generation. Besides, due to the high level of discrimination and unequivocal results, expressed as base pair number (or as repeat number), the generated data are suitable for computational population genetic analysis \([7,8,9,10]\). Twelve highly polymorphic microsatellite loci were used to assess the genetic diversity among 651 *S. cerevisiae* strains from 56 worldwide geographical origins. The genotypes clustered in subgroups, according to the strain’s technological use (i.e. bread, beer, wine, sake). Macrogeographical differentiation of strains from Asia, Europe and Africa accounted for 28% of the observed genetic variation, which suggests clonal reproduction and local domestication of natural strains originating from the same...
geographic area. [5]. Similar phylogenetic relationships related to technological applications were observed when clustering of *S. cerevisiae* strain was based on 32 single-nucleotide polymorphism markers [11] or amplified fragment length polymorphism (AFLP) analysis [12]. Recent studies with winemaking strains showed that populations are strongly structured [13] and that clonal reproduction is likely the main mating system with rare meiotic cycles, which is in agreement with a high percentage of inbreeding (80%). The transition between ‘domesticated’ and ‘natural’ isolates seems to be floating, and gene flow between subpopulations can be considered as significant [5,13,14,15]. However, the forces shaping *S. cerevisiae* population structure are still poorly understood.

In our previous studies, we showed that microsatellites are informative markers for distinguishing populations from vineyards in very close geographical locations (50–100 km). Genetic differences and populational structures among *S. cerevisiae* strains derived from cumulative small microsatellite allele-frequency differences. Within a vineyard the strain's genetic divergence correlated with the distance between sampling points, suggesting a pattern of isolation-by-distance. However, geographical distance was not correlated with genetic proximity, pointing towards the involvement of other factors, for the differentiation of *S. cerevisiae* populations [8].

In this study we test the hypothesis that both geographical region and grape varieties are drivers of population structures of fermentative vineyard-associated *S. cerevisiae* strains. Grape samples of the nine most representative grape varieties were collected at the harvest time of two consecutive years in 16 vineyards from the Bairrada and Vinho Verde appellations of origin in Portugal (BAO and VAO, respectively). For populational analysis 238 samples were obtained, that concluded 94 spontaneous fermentations and 2820 yeast isolates were obtained that belonged mainly (94%) to *S. cerevisiae,* being classified in 321 strains using 10 polymorphic microsatellite markers.

### Results

#### Wine regions, spontaneous fermentations and *S. cerevisiae* strain diversity

During the harvest time of two consecutive years, grape samples of representative grape varieties were collected in 16 vineyards (1–10 in the VAO and 11–16 in the BAO, Figure 1). The VAO is located in the north west of the country and constitutes the largest wine region in Portugal. The predominating white wine varieties are Alvarinho, Loureiro, Trajadura, Aveso, Arinto and Azal. The BAO region’s most important red grape variety is Baga, producing full-coloured and acidic wines that are well-balanced and of great longevity. Maria Gomes is the predominant white grape variety, Touriga Nacional, Tinta Roriz, Arinto and Rabo de Ovelha are produced in smaller quantities.

In each wine region, five most representative grape varieties were collected (VAO: Alvarinho (A), Arinto (C), Aveso (D), Loureiro (G), Touriga Nacional (I); BAO: Aragonez (B), Baga (E), Bical (F) Maria Gomes (H), Touriga Nacional (I)), being Touriga Nacional shared by both wine regions. In vineyards 2–10 and 11–16, one single predominating grape variety was cultivated and collected. Vineyards 1 (VAO) and 11 (BAO) were chosen as reference vineyards, containing all five grape varieties of each region. With this approach, a total of 238 grape samples were collected for spontaneous fermentations. As detailed in Table 1, from 136 samples that were collected in the VAO region, 45 samples (28%) initiated a spontaneous fermentation and a total of 165 *S. cerevisiae* strains were obtained (average: 3.6 strains per fermentation). In the vineyards of BAO, 132 grape samples were collected and 50 (38%) of spontaneous fermentations occurred, providing 156 *S. cerevisiae* strains (average: 3.1 strains per fermentation). The total yeast count (cfu in YPD medium, determined at the end of spontaneous fermentations) ranged between 1.0×10⁶ and 8.0×10⁶ cfu/ml of must. With a few exceptions, all isolates belonged to the species *S. cerevisiae* due to their inability to grow in a medium containing lysine as sole nitrogen source (data not shown), the amplification of *S. cerevisiae* specific PCR-based interdelta patterns and by the amplification of *S. cerevisiae* specific microsatellite loci (Table 2). No amplification was observed for the non-*Saccharomyces* species mentioned in Table 1 (not shown).

Figure 2 shows the main results regarding spontaneous fermentations and the isolated *S. cerevisiae* strains. The number of strains obtained from one vineyard in one sampling year was between 0–43 and 0–23 in the Vinho Verde and Bairrada region, respectively. Non-*Saccharomyces* species that are well-known for their occurrence in vineyards, were found in vineyard 11 in the samples from final stages of fermentations that were carried out using musts from the grape varieties Aragonez (B; year 2), Maria Gomes (H; year 1) and Touriga Nacional (I, year 1 and 2). The average duration until the beginning of fermentations (lag time, corresponding to a weight loss of 2 gl⁻¹) was between 3.5 and 15.7 days (grape varieties H and C, respectively). The average fermentation period (corresponding to the weight loss from 2 gl⁻¹ to 70 gl⁻¹) was between 6.3 and 18.8 days (grape varieties F and H, respectively). Fermentations with grapes from the Bairrada region started within 6 days, whereas fermentation onset of musts collected in the Vinho Verde Region was much slower (14 days). However, the average fermentation duration was identical for the musts from both regions (12 days). Grape varieties that started fermentations most rapidly (E, H and B; 3.5, 5.7 and 6.9 days, respectively) correlated, by trend, with a higher number of *S. cerevisiae* strains (22 to 27, 25 to 3, 17 to 17 strains in year 1+2 of samples collected from grape varieties E, H and B, respectively). When the percentage of spontaneous fermentations among the six samples collected from each vineyard was compared with the number of isolated *S. cerevisiae* strains (Figure 3), it became evident that a high percentage (60–100%) of fermenting samples per vineyard was associated with higher strain diversity (between 8 and 43 strains per vineyard), whereas low percentage of spontaneous fermentations (0–40%) was associated with rather low strain diversity (between 1 and 10 strains per vineyard). In vineyards 12, 13 and 11 (grape variety E), the high percentage of spontaneous fermentations and strain diversity was observed in both years.

#### Populational analysis of *S. cerevisiae* strains from different grape varieties in the Bairrada and Vinho Verde appellations of origin

The isolated *S. cerevisiae* strains were unique for each vineyard and were also not re-isolated in consecutive years. In addition, none of the strains corresponded to the commercial strains that were used by the wineries in the last few years. The extent of genetic divergence among *S. cerevisiae* populations from different grape varieties and sampling sites was examined by clustering allelic frequencies. Figure 4 shows the tree obtained by the neighbour-joining method. This analysis included strains from both sampling years and only vineyards were included from where at least five *S. cerevisiae* strains were obtained to provide a more representative quantification of allelic frequencies.

The highest bootstrap support was found for *S. cerevisiae* populations from grape variety E in the vineyards 11 and 12 (BAO), as well as grape variety D in vineyards 8 and 9 (VAO), which were 5–10 Km apart. These populations were also well
separated from other groups. Conversely, the S. cerevisiae strains from different grape varieties in the same vineyards (11 E and 11I; 1C, 1I and 1G) were much more divergent. The grape variety Touriga Nacional (I) is cultivated in most of Portuguese wine-making regions and was therefore included as a reference in our approach. S. cerevisiae populations from these grapes obtained in vineyards 1, 10, 11 and 13 were unresembling. In summary, we can assert that populational structures prevail according to winemaking regions, whereas S. cerevisiae populations are most similar in vineyards in close vicinity (at least up to 10 km) where the same grape varieties were cultivated.

For the majority of the populations, observed heterozygosity (\(H_o\)) was about two to five times lower than expected heterozygosity (\(H_e\)) for the ten loci analyzed (Table S1). Populations from vineyards 8 and 9 showed a higher heterozygosity than the expected values (\(H_o/H_e>1\)) for six microsatellites. The average of \(F_{ST}\) values over all loci was rather high for most groups (mean of 0.61), pointing towards inbreeding as the predominating reproductive mechanism. The pattern and degree of populational divergence in the ten nuclear microsatellites among subpopulations was estimated by \(F_{ST}\) determination over all loci by AMOVA analysis, as shown in Table 3 and Figure 5. For this analysis, only S. cerevisiae populations were included that consisted of at least 5 isolates (per sampling year, grape variety and vineyard). The contribution of variation within the populations defined was always high, ranging from 81 to 93%. For the analysis between wine regions, vineyards and grape varieties, the assemblage of several populations was considered as a group, indicated by parenthesis in Table 3. For all analyses, differences within groups constituted 7 to 16%, whereas differences among groups constituted only up to 7% of variation. \(F_{ST}\) values ranged between 0.07 and 0.19, corresponding to a moderate (0.05–0.15) to great (0.15–0.25) genetic differentiation [16]. The highest \(F_{ST}\) value was obtained for the comparison of vineyards 1 and 11 that contained multiple grape varieties and that are located at a distance of 180 km. This value decreased to 0.133 and 0.145 when all populations from VAO and BAO were compared, including or not the populations from vineyards 1 and 11, respectively. In

Figure 1. Geographic location of the vineyards 1–16 in the Vinho Verde and Bairrada appellations of origin (VAO and BAO), (1: Ponte da Barca; 2: Alvaredo; 3: Barbeita; 4: Longos Vales; 5: Ponte de Lima; 6: Amares; 7: Sousela; 8: São Tomé de Covelas; 9: Tresouras; 10: Ervedosa do Douro; 11: Quintã; 12: Cantanhede; 13: Mealhada; 14: Antes; 15: Outil; 16: Cerro). Subscript letters refer to the grape varieties that were cultivated in the vineyards and that were sampled within this study (A: Alvarinho; B: Aragoneỳ; C: Arinto; D: Avesso; E: Baga; F: Bical; G: Loureiro; H: Maria Gomes; I: Touriga Nacional).

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| Appellation of origin | Vineyard | Grape variety | Sampling year | Number of grape samples | Number of spontaneous fermentations | Number of S. cerevisiae strains | Non-Saccharomyces species (number of isolates) |
|----------------------|----------|---------------|---------------|-------------------------|----------------------------------|-----------------------------|---------------------------------------------|
|                      |          |               |               |                         |                                  |                             | Candida glabrata   Candida zemplinina Hanseniaspora uvarum Hanseniaspora oomphila Issatchenkia orientalis Issatchenkia terricola Kluyveromyces thermod tolerantans Zygosaccharomyces bailii |
| Vinho Verde          | 1        | A             | 1             | 6                       | 1                                | 1                           | -                                           |
|                      | 1        | A             | 2             | 6                       | 0                                | -                           | -                                           |
|                      | 1        | C             | 1             | 6                       | 1                                | 2                           | -                                           |
|                      | 1        | C             | 2             | 6                       | 1                                | 8                           | -                                           |
|                      | 1        | D             | 1             | 6                       | 2                                | 3                           | -                                           |
|                      | 1        | D             | 2             | 6                       | 1                                | 5                           | -                                           |
|                      | 1        | G             | 1             | 6                       | 0                                | -                           | -                                           |
|                      | 1        | I             | 1             | 6                       | 2                                | 8                           | -                                           |
|                      | 1        | I             | 2             | 6                       | 2                                | 7                           | -                                           |
|                      | 1        | A             | 1             | 6                       | 3                                | 5                           | -                                           |
|                      | 2        | A             | 2             | 6                       | 1                                | 6                           | -                                           |
|                      | 3        | A             | 1             | 6                       | 6                                | 8                           | -                                           |
|                      | 3        | A             | 2             | 6                       | 2                                | 8                           | -                                           |
|                      | 4        | A             | 1             | 6                       | 2                                | 4                           | -                                           |
|                      | 4        | A             | 2             | 6                       | 0                                | -                           | -                                           |
|                      | 5        | G             | 1             | 6                       | 2                                | 5                           | -                                           |
|                      | 5        | G             | 2             | 6                       | 1                                | 3                           | -                                           |
|                      | 6        | G             | 1             | 6                       | 2                                | 3                           | -                                           |
|                      | 6        | G             | 2             | 6                       | 0                                | -                           | -                                           |
|                      | 7        | C             | 1             | 6                       | 3                                | 10                          | -                                           |
|                      | 7        | C             | 2             | 6                       | 2                                | 16                          | -                                           |
|                      | 8        | D             | 1             | 6                       | 2                                | 4                           | -                                           |
|                      | 8        | D             | 2             | 6                       | 6                                | 46                          | -                                           |
|                      | 9        | D             | 1             | 6                       | 0                                | -                           | -                                           |
|                      | 9        | D             | 2             | 6                       | 1                                | 10                          | -                                           |
|                      | 10       | I             | 1             | 6                       | 2                                | 5                           | -                                           |
|                      | 10       | I             | 2             | 6                       | 0                                | -                           | -                                           |
| Bairrada             | 11       | B             | 1             | 6                       | 0                                | -                           | -                                           |
|                      | 11       | B             | 2             | 6                       | 2                                | 3                           | -                                           |
|                      | 11       | E             | 1             | 6                       | 3                                | 14                          | -                                           |
| Appellation of origin | Vineyard | Grape variety | Sampling year | Number of grape samples | Number of spontaneous fermentations | Number of S. cerevisiae strains | Non-Saccharomyces species (number of isolates) |
|-----------------------|----------|---------------|---------------|------------------------|-------------------------------------|---------------------------------|-----------------------------------------------|
|                       |          |               |               |                        |                                     |                                 | Candida glabrata                      |
|                       |          |               |               |                        |                                     |                                 | Candida zemplinina                    |
|                       |          |               |               |                        |                                     |                                 | Hanseniaspora uvarum                  |
|                       |          |               |               |                        |                                     |                                 | Hanseniaspora osmophila               |
|                       |          |               |               |                        |                                     |                                 | Issatchenkia orientalis               |
|                       |          |               |               |                        |                                     |                                 | Issatchenkia terricola                |
|                       |          |               |               |                        |                                     |                                 | Kluyveromyces thermotolerans          |
|                       |          |               |               |                        |                                     |                                 | Zygosaccharomyces bailii              |
| Bairrada 11 E         | 2        | 6             | 4             | 8                      | -                                   | 22                              | -                               |
| Bairrada 11 F         | 1        | 6             | 1             | 1                      | -                                   | -                               | -                               |
| Bairrada 11 F         | 2        | 6             | 0             | -                      | -                                   | -                               | -                               |
| Bairrada 11 H         | 1        | 6             | 3             | 0                      | 90                                  | -                               | -                               |
| Bairrada 11 H         | 2        | 6             | 0             | -                      | -                                   | -                               | -                               |
| Bairrada 11 I         | 1        | 6             | 2             | 1                      | -                                   | -                               | 30                              |
| Bairrada 11 I         | 2        | 6             | 3             | 9                      | 2                                   | 2                               | -                               |
| Bairrada 12 E         | 1        | 6             | 4             | 17                     | -                                   | -                               | -                               |
| Bairrada 12 E         | 2        | 6             | 5             | 20                     | -                                   | 7                               | -                               |
| Bairrada 13 I         | 1        | 6             | 6             | 20                     | -                                   | -                               | -                               |
| Bairrada 13 I         | 2        | 6             | 6             | 14                     | -                                   | -                               | -                               |
| Bairrada 14 H         | 1        | 6             | 2             | 2                      | -                                   | -                               | -                               |
| Bairrada 14 H         | 2        | 6             | 1             | 23                     | -                                   | -                               | -                               |
| Bairrada 15 B         | 1        | 6             | 2             | 14                     | -                                   | -                               | -                               |
| Bairrada 15 B         | 2        | 6             | 3             | 3                      | -                                   | -                               | -                               |
| Bairrada 16 F         | 1        | 6             | 1             | 1                      | -                                   | -                               | -                               |
| Bairrada 16 F         | 2        | 6             | 0             | -                      | -                                   | -                               | -                               |
agreement with the data presented in the previous section, S. cerevisiae populations were less divergent in the smaller BAO, where $F_{ST}$ values ranged between 0.067 and 0.120, whereas the corresponding values for the larger VAO region were between 0.157 and 0.173.

To further investigate associations between genetic differentiation and geographic distance, pair wise vineyard comparisons were performed according to their geographic distance (Figure 5). The genetic divergence was again highest when population 1CGI was compared with other populations. In this case, the highest $F_{ST}$ values (0.20–0.23) were obtained when 1 CGI was compared with 11 E, 12 E and 13 I, suggesting a strict correlation between grape variety and口味. The corresponding values for the larger VAO region were between 0.13–0.16, independent of the geographic distance. The lowest $F_{ST}$ values (0.067–0.120) were obtained when 1 CGI was compared with the closer VAO populations 2 A, 3 A and 7 C, suggesting a less divergent (F<sub>ST</sub>) between 0.13–0.16, independent of the geographic distance. The lowest $F_{ST}$ values (0.067–0.120) were obtained when 1 CGI was compared with the closer VAO populations 2 A, 3 A and 7 C, suggesting a less divergent (F<sub>ST</sub>) of the remaining comparisons, where F<sub>ST</sub> was performed using the software Instruct that determined the optimal number of 13 clusters. Each run used a burn-in period of 200,000, followed by 100,000 iterations. Ten replicate runs were performed and the CLUMPP software was used for finding optimal alignments of replicate cluster analyses of the same data, using the greedy algorithm that computed a symmetric similarity coefficient of 0.89. The inferred ancestry of populations is given in Figure 6. S. cerevisiae populations were distinguished by a considerable degree of admixture. Deeper divergence was observed between both geographic regions and grape varieties, being more evident in the VAO region which is in agreement with AMOVA analysis. Populations from vineyards 8 and 9, that shared a heterozygous excess ($H_o/H_e$>1) for six microsatellites loci, can be clearly distinguished. Clusters 1 and 3 were more represented in VAO populations, whereas clusters 5 and 10 were more predominant in BAO populations. Populations from multiple grape varieties in vineyards 1 and 11 (black bars in Figure 6) were more diverse in vineyard 1 compared to vineyard 11, in agreement with previously presented data.

### Discussion

Vineyards are an important yeast ecosystem. S. cerevisiae occurs in extremely low number on healthy undamaged grape berries (<0.1%) or soils [17,18,19], while damaged grapes provide inocula of 10<sup>5</sup>–10<sup>7</sup>cells/ml must [20]. A plethora of studies documented the occurrence and dynamics of S. cerevisiae in many wine regions in France [17,21,22,23,24] Spain [25,26,27,28,29,30], Portugal [8,31], Germany, Switzerland and Austria [32,33], Italy [34,35] Hungary, Bosnia and Herzegovina [36,37,38], Greece [39], South Africa [40,41,42], New Zealand [13,14], Chile and Peru [15], Argentina [13], India [44] and China [45]. While most of these studies are rather descriptive in terms of yeast diversity, recent ecological studies show relationships between yeast communities...
and agricultural practices such as the farming (organic versus conventional) and floor management systems [46,47].

In our previous studies we showed that within a vineyard the genetic divergence of S. cerevisiae strains correlated with the distance between sampling points, suggesting a pattern of isolation-by-distance. However, this relationship was not found for larger geographical distances, pointing towards the involvement of other factors such as the grape variety, which we evaluate within the present study. We highlight that S. cerevisiae isolates were obtained after enrichment through must fermentation and therefore may not accurately represent vineyard populations. Our experimental approach is therefore an acceptable compromise that allows for estimation of population composition, but does not provide a precise description in terms of relative strain abundance in nature.

Fermented musts from the grape varieties C, D and E (but also others such as varieties B, H and I in vineyards 15, 14 and 13, respectively) showed a notable strain diversity, which seems to be correlated with the percentage of spontaneous fermentations in a vineyard. Contrarily to the view that strains compete for nutrients under stressful fermentative conditions, it was surprising to find numerous strains at the end of the fermentations, suggesting a cooperative effect that may guarantee efficient fermentation when strain diversity is rather high. The faster fermentation onset observed for several grape varieties might also be related with a more favorable nutritional composition of the grapes. Spontaneous fermentations can be considered as complex multifactorial process, where strain diversity is one variable for a rapid onset, while the grape variety appears to be also relevant.

The occurrence and survival of S. cerevisiae in vineyards depend on climatic factors [19,48] or viticulture practices [46,47,49,50]. These were very similar in almost all vineyards studied (data not shown) with the exception of vineyard 8, where biodynamic organic farming is being practiced for several years according to the anthroposophy of Rudolf Steiner (1861–1925). This vineyard had a very high S. cerevisiae strain abundance, elevated percentage of spontaneous fermentations and low fermentative lag time compared to the closely located (10 km) vineyard 9, where the same soil, microclimatic conditions grape variety occurred. This result is in agreement with recent research showing that phytosanitary treatment has an impact on grape associated biodiversity [46]. Further studies on this topic are required, considering in particular that the production of organic wines relies solely on the yeast communities on the grape surfaces and winery environments.

### Figure 2. Summary of spontaneous fermentations.

Bars indicate the average fermentation duration of must samples that underwent a spontaneous fermentation in each of the vineyards (1–16) and for all grape varieties (A–H) in sampling year 1 (open circles) and 2 (closed circles). The light grey part of the bars indicates the average number of days until the beginning of fermentation (lag time, corresponding to a weight loss of 2 g l⁻¹), whereas the dark grey part indicates the average days of fermentation (corresponding to a weight loss from 2 gl⁻¹ to 70 gl⁻¹). The average number of S. cerevisiae strains from sampling years 1 and 2 is also indicated, as well as average lag and fermentation times for samples from all grape varieties. The number of spontaneous fermentations for each variety/vineyard combination is given in Table 1.

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Microsatellite typing of the chosen loci (Table 2) followed by allelic analysis permitted a fine populational screen, and revealed deeper insight into the biogeography of \textit{S. cerevisiae} strains, even within geographically close regions. The isolated \textit{S. cerevisiae} strains were unique for each vineyard and were also not re-isolated in consecutive years. However, in our previous research we found strains with a wider temporal and spatial distribution [8]. This difference could be explained by the fact that ten microsatellite loci were included in the present study, whereas our previous work relied on the analysis of six loci. Isolates with identical alleles for six loci might not share alleles of the remaining four loci and might therefore be considered as different strains with increasing number of analyzed loci. Genetic differences among \textit{S. cerevisiae} populations derived mainly from gradations in allele frequencies rather than from distinctive “diagnostic” genotypes. These markers are useful for unambiguous populational analysis, but it needs to be considered that sub-strain level discrimination may occur due to their relative high mutation rate. Clonal expansion with some cycles of homothallic self-mating is considered to be the most likely reproduction in yeasts, generating the high observed homozygosity and very structured populations due to inbreeding or genome renewal [51]. The determined $F_{IS}$ values suggest that Portuguese yeast populations are inbred, which is in agreement with previous results obtained with strains from Chile and New Zealand, where $F_{IS}$ values ranged between 0.4–0.75 [13,14,15]. Heterozygote reduction can be explained by mitotic recombination, gene conversion during asexual reproduction or by the presence of null alleles that arise when mutations prevent primer annealing. Genetic differentiation may result from natural selection favoring different genotypes in different subpopulations, but also from random processes in the transmission of alleles from one generation to the next or from stochastic differences in allele frequency among the initial founders of the subpopulations.

Populations from vineyard 8 and 9 showed a low genetic variation and seemed to evolve towards increased heterozygosity at multiple loci such as a clonal population evolving only under mutation. The
allelic combinations in these groups of strains were very similar and varied frequently by just one microsatellite repeat among alleles for all loci. These populations might have lost genetic variability after a bottleneck. In such a case, variability starts to increase due to new mutations as soon as the population size becomes larger, whereas the average number of alleles per locus might increase faster than the average heterozygosity after the bottleneck. In alternative, strains of these populations could be affected by microsatellite instability associated with defective DNA mismatch repair as described for human malignancies.

Various approaches were used to determine the genetic structure of *S. cerevisiae* populations from different vineyards and grape varieties. Results from *F*~ST~ analysis and clustering of allelic frequencies agree in the distinction of genetically more dispersed populations from the larger VAO compared to the BAO region. Our data indicate that the grape variety can be a driver of populational structures because populations associated with different grape varieties from vineyards 1 and 11 were genetically more divergent than populations obtained from the same grape variety in vineyards in close locations (11E-12E (5 km), 12E-11D (10 km)). Comparison of strains from variety A in the close vineyards 2 and 3 (10 km) revealed a higher *F*~ST~ value, but these populations were still less differentiated than the ones obtained from vineyard 1. A correlation between genetic and geographical distances was only evident when the more divergent populations from vineyard 1 were compared to other groups of strains. The higher genetic differentiation of yeasts from the experimental vineyard 1 may be attributed to the fact that it contains ten different grape varieties in larger quantities and 152 varieties of a clonal ampelographic collection, that were introduced three to four years before our study was initiated. The inferred ancestry of populations support strong admixture whereas divergence was again observed between both geographic regions and grape varieties. Interestingly population 10I from VAO seemed more related with populations from BAO when the inferred ancestry was analyzed, This is one of the few varieties that is used in all Portuguese winemaking regions and might have been introduced in the VAO together with the yeast strains as a commensal member of grapevine flora, as previously suggested by Legras [5].

Recent studies showed that *S. cerevisiae* strains have been globally dispersed by humans, supporting the importance of geography in shaping *S. cerevisiae*’s population structure [3,14]. However, for close geographical locations this association is not evident. Globally, our results show that a correlation between genetic distance and grape variety can arise. Local populations of *S. cerevisiae* in vineyards occur due to multi-factorial influences, being the grape variety one of them. These findings are in agreement with a recent report about distinctive non-*Saccharomyces* yeast populations occurring on different grape varieties in the same vineyard [46]. It is desirable to extend these studies to table-3-
Environmental genomics approaches regarding the abundance, distribution and diversity of yeasts in natural environments. Such data may also contribute to improved vineyard management and the elucidation of the role of yeast communities from specific grapevines to the outcome of spontaneous or industrial fermentations.

### Materials and Methods

**Sampling**

The sampling plan included a total of 16 vineyards (ten in the Vinho Verde and six in the Bairrada region) as shown in Figure 1. The grape varieties cultivated in each vineyard correspond to the

| Wine region | Source of variation | Combination of groups of vineyards and grape varieties | Percentage of variation (AG) | Percentage of variation (AGWP) | Percentage of variation (WP) | F_{ST} | P (r<o) |
|-------------|---------------------|--------------------------------------------------------|-------------------------------|--------------------------------|-----------------------------|--------|---------|
| VAO and BAO | All vineyards       | (1c, 1o, 1i, 2a, 3a, 5a, 7c, 8o, 9o, 10o) => (1c, 11, 12a, 13, 14a, 15a) | 3.52                          | 10.94                          | 85.54                       | 0.145  | <0.000001 |
| VAO and BAO | Vineyards with single grape varieties | (2a, 3a, 5c, 7c, 8o, 9o, 10o) => (12t, 13, 14a, 15a) | 3.20                          | 10.08                          | 86.72                       | 0.133  | <0.000001 |
| VAO and BAO | Vineyards 1 and 11 | (1c, 1o, 1i) => (11c, 11i) | 6.32                          | 12.62                          | 81.06                       | 0.189  | <0.000001 |
| VAO and BAO | Grape variety I     | (1c, 10o) => (11c, 13) | -0.80                         | 16.09                          | 84.71                       | 0.153  | <0.000001 |
| VAO          | Grape varieties     | (2a, 3a) => (1c, 7c) => (8o, 9o) => (10o, 5c) => (1, 10c) | 2.77                          | 12.94                          | 84.29                       | 0.157  | <0.000001 |
| VAO          | Vineyard 1 and other vineyards/grape varieties | (1c, 1o, 1i, 2a, 3a, 5c, 7c, 8o, 9o, 10o) | 3.59                          | 13.71                          | 82.69                       | 0.173  | <0.000001 |
| VAO          | Vineyard 1 and other vineyards/grape varieties | (1c, 1o, 1i) => (12c, 13, 14a, 15a) | 6.07                          | 9.69                           | 84.25                       | 0.157  | <0.000001 |
| VAO          | Vineyard 1 and other vineyards/grape varieties | (1c, 1o, 1i) => (11c, 11c, 12c, 13, 14a, 15a) | 7.14                          | 8.28                           | 84–58                       | 0.154  | <0.000001 |
| BAO          | Grape varieties     | (11c, 12c) => (11c, 13c) | 1.95                          | 10.19                          | 87.85                       | 0.120  | <0.000001 |
| BAO          | Vineyard 11 and other vineyards/grape varieties | (11c, 11c) => (2a, 3a, 5c, 7c, 8o, 9o, 10o) | 3.73                          | 11.18                          | 85.09                       | 0.150  | <0.000001 |
| BAO          | Vineyard 11 and other vineyards/grape varieties | (11c, 11c) => (12c, 13, 14a, 15a) | -0.54                         | 7.24                           | 93.30                       | 0.067  | <0.000001 |
| BAO          | Vineyard 11 and other vineyards/grape varieties | (11c, 11c) => (1c, 1o, 1i, 2a, 3a, 5c, 7c, 8o, 9o, 10o) | 2.82                          | 13.84                          | 83.33                       | 0.167  | <0.000001 |

All comparisons are statistically significant (P(random value<observed value)<0.000001). doi:10.1371/journal.pone.0032507.t003

**Figure 5.** Correspondence analysis between geographic distance and population differentiation (F_{ST}) for pair wise comparisons of *S. cerevisiae* populations from vineyards 1, 2, 3, 7, 11, 12, 13 and grape varieties A, C, D, E and I. Comparisons between vineyards with the same grape variety are shown in black ovals. All comparisons are statistically significant (P(random value<observed value)<0.000001). doi:10.1371/journal.pone.0032507.g005
recommended varieties for both winemaking regions (Vinho Verde: Alvarinho, Aveesso, Arinto, Loureiro; Bairrada: Aragones, Baga, Bical and Maria Gomes). Besides, grapes of the Touriga Nacional variety were sampled, which is common to most of the Portuguese winemaking regions. In each vineyard, six sampling points were defined according to the size of the vineyard. Healthy and undamaged grape samples were collected a few days before the harvest, in two consecutive years. Grapes were not always collected from the same rootstock, but from the same area (6–2 m). Vineyards 2–10 (VAO) and 12–16 (BAO) contained mainly one predominant grape variety. In addition, one vineyard was chosen in each region, where multiple grape varieties were cultivated (vineyard 1: Alvarinho, Aveesso, Arinto, Loureiro, Touriga Nacional; vineyard 11: Aragones, Baga, Bical, Maria Gomes and Touriga Nacional) to evaluate associations between the \textit{S. cerevisiae} populations and the grape variety. All necessary permits were obtained for the described field studies, the owners of the vineyards agreed with the collection of grape samples and the sampling plan.

**Fermentation and strain isolation**

From each sampling point, approximately 2 kg of undamaged and healthy grapes were aseptically collected and the extracted grape juice was fermented at 20°C in small volumes (500 ml). Fermentation progress was monitored by daily weight determinations. When must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10⁻² and 10⁻³) were spread on YPD plates (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2%, w/v), and 30 randomly chosen colonies were collected after incubation (2 days, 28°C). The isolates obtained throughout this work were stored in glycerol (30%, v/v) at −80°C.

**Molecular analysis**

Yeast cells were cultivated in 1 ml YPD medium (36 h, 28°C, 160 rpm). DNA isolation was performed as previously described [9].

In a preliminary approach, all isolates were analysed by interdelta sequence typing [9,52]. One representative strain from each group of isolates with identical interdelta amplification patterns was further analysed by the microsatellite loci summarized in Table 2, using previously described PCR mixtures and amplification conditions [7,8,9,10]. Isolates that showed no interdelta pattern and failed to amplify ten microsatellite loci were considered to belong to non-\textit{Saccharomyces} species. These species were identified by restriction analysis of the ribosomal internal transcribed spacer (ITS) region as previously described [53]. The ITS region of representative isolates from each restriction pattern was sequenced for further confirmation. According to our (unpublished) results, the primer pairs used for amplification of microsatellite loci are predominantly specific for \textit{S. cerevisiae} and fail to amplify all of the corresponding homologous loci in sibling species such as \textit{S. bayanus} and \textit{S. paradoxus} that can be found occasionally in winemaking environments. We therefore consider that these sibling species did not occur in our spontaneous fermentations.

**Data analysis**

Based on the genome sequence for strain S288C (SGD database, http://genome-www.stanford.edu.saccharomyces) and the results obtained for the size of microsatellite amplicons of this strain, the number of repeats for all alleles was calculated. Allelic frequencies, observed and expected heterozygosity, \(F_{IS}\) determinations as well as AMOVA analysis was performed by the software Arlequin 3.11 [54]. An allelic frequencies matrix, based on Euclidean distance was computed and clustered by the neighbour joining algorithm using the program PowerMarker [55]. The validity of nodes was obtained with the Consens program.

**Figure 6. Results of InStruct analysis.** Optimal alignments of replicate clusters were determined by the CLUMPP software. Each population is represented by a vertical bar partitioned into colored segments according to the probability of belonging to one of the 13 color-coded genetic clusters. Numbers in parenthesis correspond to the numbers of strains. Grey and black bars label \textit{S. cerevisiae} populations from the same grape varieties and vineyards, respectively.

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(Phylip3.69 package). Bayesian individual clustering of 16 populations was performed with the software INSTRUCT [56], which infers population structure and selling rates at the population level. Assuming a number of clusters from K = 1–20, the most likely number of clusters was 13. Following, the program was run with 10 chains (200,000 iterations with 100,000 burn-in steps) and the optimal alignments of 10 replicate cluster analyses was determined by the CLUMPP software [57] using the greedy algorithm.

Supporting Information

Table S1 Observed (Ho) and expected (He) heterozygosity for S. cerevisiae populations from vineyards 1–15 and grape varieties A–I. The ratios between observed (Ho) and expected (He) heterozygosity are indicated by underlined bold letters (Ho/He>1) and underlined letters (0.5>Ho/He>1). For the remaining fields the ratio was <0.5. (TIF)

References

1. Greig D, Leu JY (2009) Natural history of budding yeast. Current Biology 19: R896–R900.
2. Liti G, Carter DM, Moses AM, Warringer J, Parts L, et al. (2009) Population genomics of domestic and wild yeasts. Nature 458: 337–341.
3. Schacierer J, Shapiro JA, Raiferder DM, Kryglik L (2009) Comprehensive polymorphism survey elucidates population structure of Saccharomyces cerevisiae. Nature 458: 342–345.
4. Fay JC, Benavides JA (2005) Evidence for domesticated and wild populations of Saccharomyces cerevisiae. PLoS Genetics 1: 66–71.
5. Legras JL, Merdinoglu D, Cournet JM, Karst F (2007) Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history. Molecular Ecology 16: 2091–2102.
6. Martiny JB, Bohannan BJ, Brown JH, Colwell RA, Brown JH, et al. (2006) Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol 4: 102–112.
7. Schuller D, Pereira I, Alves H, Cambon B, Dequin S, et al. (2007) Genetic characterization of commercial Saccharomyces cerevisiae isolates recovered from vineyard environments. Yeast 24: 625–636.
8. Schuller D, Casal M (2007) The genetic structure of fermentative vineyard-associated Saccharomyces cerevisiae populations revealed by microsatellite analysis. Antonie van Leeuwenhoek 91: 137–150.
9. Schuller D, Valero E, Dequin S, Casal M (2004) Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiology Letters 231: 19–26.
10. Legras JL, Ruh O, Merdinoglu D, Cournet JM, Karst F (2007) Bayesian individual clustering of 16 populations from vineyards 1–15 and grape varieties A–I. The ratios between observed (Ho) and expected (He) heterozygosity are indicated by underlined bold letters (Ho/He>1) and underlined letters (0.5>Ho/He>1). For the remaining fields the ratio was <0.5. (TIF)

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Conceived and designed the experiments: DS PG. Performed the experiments: DS FC SS PG AGC. Analyzed the data: DS. Contributed reagents/materials/analysis tools: DS MASS MC. Wrote the paper: DS.

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38. Orlic S, Vojvoda T, Babic KH, Arroyo-Lopez FN, Jeromel A, et al. (2010) Diversity and oenological characterization of indigenous *Saccharomyces cerevisiae* associated with Zilavka grapes. World Journal of Microbiology & Biotechnology 26: 1483–1489.

39. Pramateftaki PV, Lanaridis P, Typas MA (2000) Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. Journal of Applied Microbiology 89: 236–248.

40. van der Westhuizen TJ, Augustyn OHP, Khan W, Pretorius IS (2000) Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. South African Journal of Enology and Viticulture 21: 10–16.

41. van der Westhuizen TJ, Augustyn OHP, Pretorius IS (2000) Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the western Cape in South Africa. South African Journal of Enology and Viticulture 21: 3–9.

42. Khan W, Augustyn OHP, van der Westhuizen TJ, Lambrechts MG, Pretorius IS (2000) Geographic distribution and evaluation of *Saccharomyces cerevisiae* strains isolated from vineyards in the warmer, inland regions of the Western Cape in South Africa. South African Journal of Enology and Viticulture 21: 17–31.

43. Lopes CA, van Broock M, Querol A, Caballero AC (2002) *Saccharomyces cerevisiae* wine yeast populations in a cold region in Argentinean Patagonia. A study at different fermentation scales. Journal of Applied Microbiology 93: 608–615.

44. Chavan P, Mane S, Kulkarni G, Shaikh S, Ghormade V, et al. (2009) Natural yeast flora of different varieties of grapes used for wine making in India. Food Microbiology 26: 801–808.

45. Sun HH, Ma HQ, Hao ML, Pretorius IS, Chen SW (2009) Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. Annals of Microbiology 59: 69–76.

46. Cordero-Busso G, Arroyo T, Serrano A, Tello J, Aporta I, et al. (2011) Influence of the farming system and vine variety on yeast communities associated with grape berries. Int J Food Microbiol 145: 132–139.

47. Cordero-Busso G, Arroyo T, Serrano A, Valero E (2011) Influence of different floor management strategies of the vineyard on the natural yeast population associated with grape berries. Int J Food Microbiol 148: 23–29.

48. Longo E, Camacho J, Angelo D, Villa TG (1991) Effect of climatic conditions on yeast diversity in grape musts from northwest Spain. American Journal of Enology and Viticulture 42: 141–144.

49. Rosini G (1982) Influenza della microflora saccaromicetico della cantina sulla fermentazione del mosto d’uva. Vigne Vini 9: 45–46.

50. Pretorius IS, van der Westhuizen TJ, Augustyn OHP (1999) Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. South African Journal of Enology and Viticulture 20: 61–74.

51. Mortimer RK, Romano F, Suzzi G, Polinelli M (1994) Genome renewal - a new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. Yeast 10: 1543–1552.

52. Franco-Duarte R, Mendes I, Gomes AC, Santos MA, de Sousa B, et al. (2011) Genotyping of *Saccharomyces cerevisiae* strains by interdelta sequence typing using automated microfluidics. Electrophoresis 32: 1447–1453.

53. Granchi L, Bosco M, Messini A, Vincenzini M (1999) Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. Journal of Applied Microbiology 87: 949–956.

54. Schneider S, Roesti D, Escoffier I, Arlequin ver 2.000: A software for population genetic data analysis: Genetics and Biometry Laboratory, Department of Anthropology and Ecology, University of Geneva, Switzerland.

55. Liu K, Muse SV (2005) PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21: 2128–2129.

56. Gao H, Williamson S, Bustamante CD (2007) A Markov chain Monte Carlo approach for joint inference of population structure and inbreeding rates from multilocus genotype data. Genetics 176: 1635–1651.

57. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801–1806.