

THE BIOGEOGRAPHY OF FERMENTATIVE YEAST POPULATIONS FROM THE VINEYARDS OF THE AZORES ARCHIPELAGO

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ABSTRACT

During the harvests of 2009 and 2010, 163 musts were obtained from grape samples collected in eight islands of the Archipelago of the Azores. The sampling plan included traditional and hybrid grape varieties from vineyards in appellations of origin and abandoned vineyards. In 2009, 2640 yeast isolates were obtained from the initial stage of fermentation, belonging to more than 20 species. Differences between most representative yeast species were apparent between islands, grape varieties, and the kind of vineyard. From the final fermentative stages, a total of 2850 isolates were obtained (1470 and 1380 in 2009 and 2010, respectively), whereas 1770 isolates were assigned to the species *Saccharomyces cerevisiae*. These isolates were classified in 275 strains (168 in 2009 and 107 in 2010). The percentage of *S. cerevisiae* and the number of strains per fermentation showed differences between islands, type of vineyard and sampling year.

RESUMO

Nas vindimas de 2009 e 2010 foram obtidos 163 mostos de amostras de uvas colhidas em oito ilhas do Arquipélago dos Açores. A amostragem incluiu castas tradicionais, e híbridas a partir de vinhas em zonas demarcadas e vinhas abandonadas. Em 2009 obtiveram-se 2640 isolados de levedura da fase inicial da fermentação, que pertenceram a mais de 20 espécies. Relativamente às espécies mais representativas, foram observadas diferenças entre ilhas, castas e tipos de vinha. Da fase final das fermentações obteve-se um total de 2850 isolados (1470 e 1380 em 2009 e 2010, respectivamente). Destes, 1770 foram identificados como pertencentes à espécie *Saccharomyces cerevisiae* e foram classificados em 275 estirpes (168 em 2009 e 107 em 2010). A percentagem de *S. cerevisiae* e o número de estirpes revelou diferenças entre ilhas, tipos de vinha e anos de amostragem.

INTRODUCTION

Winemaking is a complex processes in which yeast has a profound impact on the quality of the final product. It depends on the yeast species and strains that conduct the process (indigenous yeast or commercial yeast starter) but also on the wild yeasts that are always present on the grapes. In many wine production areas around the world, numerous studies were performed, aiming the characterization of the indigenous microflora, both from the perspective of biodiversity evaluation and characterization of the oenological potential.

The Azores Archipelago includes three appellations of origin on the islands Terceira (Biscoitos), Graciosa and Pico. Other wine producing regions and also extensive areas of abandoned vineyards can be found on different islands. The Azorean *terroirs* produce wines with well defined profiles, due to the soil, grape varieties, climatic and cultural characteristics. From an ecological point of view, these *terroirs* correspond to particular ecosystems, whose fermentative flora has not yet been characterized. Traditionally, Azorean wines were obtained by spontaneous fermentations by indigenous yeasts species, which may have significantly contributed to the aromatic profiles of wines in a given island or region. However, the current widespread use of commercial yeast may have contributed to the loss of Azorean wines typicality. This study aims to assess and characterize the yeast biodiversity of Azorean vineyards and to constitute a collection of *S. cerevisiae* strains as a resource for future selection and breeding of strains that are most adapted to the characteristics of the wine from Azores.

MATERIAL AND METHODS

Sampling

During the harvests of 2009 and 2010, 88 and 75 grape samples, respectively were collected from 36 locations from eight islands of the archipelago, as shown in Tab. 1 and Fig. 1. The grapes belonged to traditional (Arinto cv, Verdelho cv and Terrantez cv) and hybrid grape varieties. Each sample was composed of approximately two kg of aseptically collected grapes.

Yeast isolation

From each sample, 500 ml of must was obtained and fermented, using 500 ml Erlenmeyer flasks with a rubber stopper that was perforated with a syringe needle to allow CO₂ release. Fermentation progress was followed by daily weight loss determinations due to CO₂ production. Right after the preparation of the must, diluted aliquots (10⁻¹ to 10⁻⁵) were spread on plates containing YPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2%, w/v) supplemented with biphenyl (40 mg/l). After incubation (2 days, 30 °C), 30 colonies were collected from plates containing between 30 and 300 colonies. When the weight loss of the must was about 65-70 g/l, corresponding to a stage close to the end of fermentation, must aliquots were again withdrawn, diluted and spread onto plates containing YPD medium (without biphenyl supplementation). Thirty colonies were chosen from each must sample as described above. All isolates obtained were stored in 30% (v/v) glycerol at -80°C.

Molecular identification

Molecular identification of isolates from the beginning of fermentation was performed by restriction fragment length polymorphism analysis (RFLP) of ITS sequences (Esteve-Zaroso *et al.*, 1999; Fernandez-Espinar *et al.*, 2000) and DNA sequencing. Isolates corresponding to the species *S. cerevisiae* were further analyzed by PCR-based interdelta sequence amplification (Legras and Karst 2003; Schuller, *et al.* 2004) for strain delimitation. All isolates from the final fermentative stages were analyzed by this method. When no interdelta amplification patterns were obtained from isolates corresponding to this stage (which indicates the presence of a non-*Saccharomyces* species), molecular identification of species was performed as described above.

Tab.1: Number of collected samples in two sampling years with indication of the island, location, grape variety and type of vineyard (abandoned/non-abandoned)

	Island	Location	Non-abandoned vineyards						Abandoned vineyards					
			Arinto		Terrantez		Verdelho		Hybrid grape varieties					
			2009	2010	2009	2010	2009	2010	2009	2010				
S. Maria	1	2	3	4							1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
São Miguel	5	6	7	8	9	10					1	1		
											1	1		
											1	1		
											1	1		
											1	1		
Terceira	11	12	13	14	3	3					1	1	1	1
											1	1		
											1	1		
											1	1		
Graciosa	15	16	17	18	1	1					2	2	1	1
											2	1	1	1
											1	1	1	1
											1	1	1	1
Pico	19	20	21	22	1	1	1	1	1	1	2	2	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
Faial	26	27	28	29							1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
São Jorge	30	31	32	33	1	1					1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
											1	1	1	1
Flores	36										1			
Total					14	13	3	3	13	11	34	31	24	17

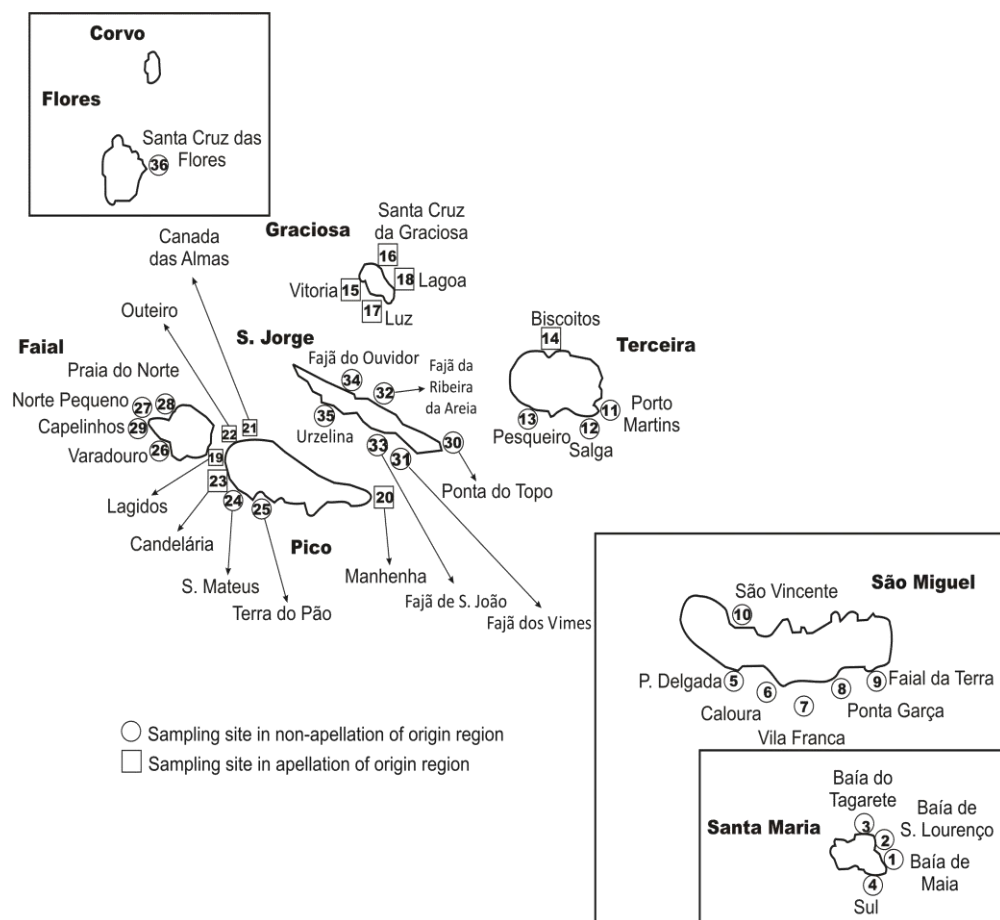


Fig.1: Sampling sites in eight different islands of the archipelago of Azores, with indication of the regions corresponding to appellations of origin.

RESULTS AND DISCUSSION

Yeast species occurring in initial fermentative stages

From the initial stage of fermentations, a total of 4890 isolates were obtained in 2009 and 2010. Herein, we present the data obtained for the 2640 isolates obtained in 2009. As detailed in Tab. 2, 23 different yeast species were identified, belonging to 10 genera.

Over 75% of the isolates belonged to three species: *Hanseniaspora uvarum* (50.8%), *Issatchenkia terricola* (13.5%) and *Candida zemplinina* (10.7%). They were widely distributed and found in all grape varieties, in both types of vineyard and in all islands (with the exception of Flores where *I. terricola* was not found).

Tab 2: Distribution of yeast microflora from initial fermentative stages in 88 musts from traditional and hybrid grape varieties collected in 2009, in abandoned and non-abandoned vineyards, from eight islands of the Azores Archipelago. SA - Santa Maria; SM - São Miguel; TR - Terceira; GR - Graciosa; PC - Pico; FA - Faial; SJ - São Jorge; FL – Flores; AV – abandoned vineyards; NA - non-abandoned vineyards; TV - traditional varieties; HV - hybrid varieties.

Species	Island								Type of vineyard		Grape variety		Azores
	SA	SM	TR	GR	PC	FA	SJ	FL	AV	NA	TV	HV	Total
<i>Candida inconspicua</i>					1.3					0.6	1.2		0.42
<i>Candida azyma</i>	3.3								1.4			0.6	0.38
<i>Candida azymoides</i>					4.3				5.1			2.2	1.41
<i>Candida diversa</i>	0.4	0.4			3.0					1.5		1.7	1.08
<i>Candida fructus</i>				0.3						0.1	0.1		0.05
<i>Candida fukuyamaensis</i>				0.9	0.1				0.3	0.1	0.3	0.1	0.19
<i>Candida quercitrusa</i>	0.8								0.3			0.1	0.09
<i>Candida railenensis</i>	8.4								1.5	0.7		1.5	0.94
<i>Candida zemplinina</i>	25.5	43.1	8.9	8.5	1.3	0.7	1.9	10.5	5.0	12.9	63	13.3	10.73
<i>Hanseniaspora opuntiae</i>				17.3						3.8	7.6		2.78
<i>Hanseniaspora uvarum</i>	58.2	47.4	22.7	56.4	51.5	49.3	65.7	89.5	65.6	45.2	47.2	53.4	50.82
<i>Hanseniaspora vineae</i>		0.4			0.3	9.0			2.1	0.2	0.3	1.0	0.71
<i>Issatchenkia hanoiensis</i>		1.7	0.4		1.4	9.7			1.7	1.2		2.1	1.32
<i>Issatchenkia terricola</i>	0.8	0.4	48.6	7.6	5.8	31.3	26.6		12.8	13.8	14.6	12.7	13.51
<i>Metschnikowia pulcherrima</i>				8.5	21.6				0.5	11.6	19.2	2.3	8.52
<i>Meyerozyma guilliermondii</i>					1.0					0.5	0.1	0.4	0.33
<i>Pichia cecembensis</i>				0.3	1.1					0.6	0.4	0.4	0.42
<i>Pichia fermentans</i>					1.1					0.5		0.6	0.38
<i>Pichia membranifaciens</i>		2.6			0.4		5.3		0.2	1.2	0.8	1.0	0.94
<i>Saccharomyopsis vini</i>			4.0				0.5			0.7	0.1	0.7	0.52
<i>Wickerhamomyces anomalus</i>				0.3						0.1	0.1		0.05
<i>Williopsis californica</i>		0.4								0.1		0.1	0.05
<i>Zygoascus meyeriae</i>		3.4	15.0		5.8				3.4	4.3	1.8	5.3	4.05
Unidentified species A	2.1									0.3		0.4	0.24
Unidentified species B	0.4									0.1		0.1	0.05
Unidentified species C			0.4							0.1		0.1	0.05
Number of species	9	8	7	9	15	5	5	2	13	23	15	22	26

Concerning the geographic distribution of the yeast species, some differences became evident. *H. uvarum* was the most representative species in every island, except for Terceira, where the most representative species was *I. terricola* (48.6%), followed by *H. uvarum* (22.7%). In addition, *I. terricola* appears in the vineyards of Faial (31.3%) and S. Jorge (26.6%) in percentages considerably higher than in the other islands. *I. terricola* was one of the predominant species in the three central islands of the archipelago. In the

islands of the eastern group (São Miguel and Santa Maria) this species was quite rare and occurred in low percentages (0.4% and 0.8%, respectively). In these two islands, the second most represented species was *C. zemplinina*, occurring in percentages of 43.1% and 25.5%, respectively. This value was considerably lower in the other islands (between 0.7 and 10.5%). *Metschnikowia pulcherrima* was the fourth most representative species on the total of analyzed isolates (8.5%). This species was just found in the central islands Graciosa and Pico.

Concerning the grape variety, differences were observed in the yeast microflora composition between traditional and hybrid varieties. *H. uvarum* and *I. terricola* were still the predominant species in both groups of varieties. While *M. pulcherrima* corresponded to 19.2% of the isolates obtained in traditional varieties, that value was just 2.3% in samples of hybrid varieties. As mentioned before, this species occurred only on the islands Pico and Graciosa, where the proportion of grape samples from traditional varieties compared to grape samples from hybrid varieties was highest.

When the microflora composition from the grapes collected in abandoned and non-abandoned vineyards was compared, the main difference was observed in the percentage of the predominant species *H. uvarum*. In abandoned vineyards this species corresponds to more than 2/3 of the total isolates, whereas a considerable lower percentage of 45.2% was found in non-abandoned vineyards. The opposite was observed for the species *M. pulcherrima* that was occurred predominantly in non-abandoned vineyards (11.6%, compared to 0.5 % in abandoned vineyards). This might be related to the fact that traditional varieties did not occur in abandoned vineyards.

As it was expected, the number of species per island increased with the number of collected samples, which augmented the probably to find rare species. To our knowledge, the following species were not previously described as grape- or vineyard-associated: *Candida azymoides*, *Candida fructus*, *Candida fukuyamaensis*, *Pichia cecembensis* and *Williopsis californica*. In addition, three putative new species were found.

Yeast microflora occurring in final stages of spontaneous fermentations

As detailed in Tab 3, almost all fermentations started one or two days after must preparation. Concerning fermentation onset, differences were not observed between islands, grape variety or sampling year. However, fermentations of samples collected in abandoned vineyards started, on average, about 0.4 to 1 days earlier compared to the other samples. This fact may reflect the previously described microflora differences between grapes from abandoned and non-abandoned vineyards.

Ninety five spontaneous fermentations (49 and 46 in 2009 and 2010, respectively) were achieved. In 2009, about 83.3% of the musts obtained from traditional varieties finished fermentation, while this value was 47.1% and 33.3% for musts from hybrid varieties in non-abandoned and abandoned vineyards, respectively. In 2010 those values were 63.0%, 67.7% and 47.1%, respectively. Samples collected from the island Graciosa showed the highest percentage of completed fermentation in both sampling years (78.6% in 2009 and 87.5% in 2010).

In 2009 the percentage of *S. cerevisiae* in fermentations performed with grapes from abandoned vineyards was significantly higher (75%) compared to the values determined for non-abandoned vineyards (56%, for both traditional and hybrid varieties). Contrarily, the number of *S. cerevisiae* strains per fermentation was lower (3-11) in samples from abandoned vineyards compared to non-abandoned vineyards (1-23). The highest number of *S. cerevisiae* strains per fermentation was observed in samples collected from the Graciosa Island.

In 2010, however, the percentage of fermentations finished by *S. cerevisiae* was higher in the samples of traditional grape varieties (76.5%). This value was 61.9% and 50.0% for

grape samples of hybrid varieties in non-abandoned and abandoned vineyards, respectively.

Besides, the number of *S. cerevisiae* strains per sample found in abandoned vineyards ranged between 2 and 16, being higher than in non-abandoned vineyards, for both hybrid and traditional varieties (1-10 and 1-8, respectively). The highest number of *S. cerevisiae* strains per island was obtained from Terceira, contrarily to the previous year, where the highest number of strains derived from the island Graciosa (in particular from non-abandoned vineyards).

Tab 3: Fermentation data and composition of the *S. cerevisiae* microflora that was found in 95 must samples from traditional and hybrid grape varieties collected in abandoned and non-abandoned vineyards from seven islands of the Azores Archipelago in 2009 and 2010.

		Nº of days until the beginning of fermentation		Nº of days corresponding to fermentation		Percentage of finished fermentations		Percentage of fermentations that were finished by <i>S. cerevisiae</i> strains		Total number of <i>S. cerevisiae</i> strains		Lowest number of <i>S. cerevisiae</i> strains in a spontaneous fermentation		Highest number of <i>S. cerevisiae</i> strains in a spontaneous fermentation	
		2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
Tradicional varieties	SM	1.8	1.5	17.5	16.3	80.0	100.0	100.0	75.0	15	12	1	1	7	6
	TR	2.0	4.7	18.5	31.0	80.0	60.0	50.0	66.7	3	12	1	4	2	8
	GR	2.5	1.3	25.0	20.3	100.0	75.0	66.7	100.0	46	8	2	1	23	5
	PC	2.8	2.0	16.3	15.4	75.0	41.7	44.4	80.0	16	7	1	1	7	2
	SJ	2.0	2.0	25.0	12.0	100.0	100.0	0.0	50.0		2		2		2
	AZ	2.4	2.2	19.6	18.8	83.3	63.0	56.0	76.5	80	41	1	1	23	8
HV non - abandoned	SA	2.0	1.0	28.7	39.0	75.0	33.3	66.7	100.0	16	2	1	2	14	2
	SM	3.0	2.3	39.0	22.3	25.0	75.0	100.0	66.7	3	12	3	2	3	10
	TR	3.0	2.5	17.0	11.5	25.0	66.7	100.0	100.0	9	4	9	2	9	2
	GR	2.5	1.8	39.0	14.0	50.0	100.0	50.0	100.0	22	11	1	2	21	3
	PC	2.8	2.5	31.8	16.0	75.0	50.0	50.0	50.0	1	4	1	1	1	3
	FA	3.0	2.7	39.0	39.0	25.0	75.0	0.0	0.0						
	SJ	2.0	2.3	33.5	39.0	40.0	80.0	50.0	50.0	1	4	1	2	1	2
AZ	2.6	2.2	32.3	23.4	47.1	67.7	56.3	61.9	52	37	1	1	21	10	
HV abandoned	SA	1.0	2.0	39.0	11.0	25.0	25.0	100.0	100.0	6	7	6	7	6	7
	TR	1.0	1.5	24.0	11.0	33.3	100.0	100.0	50.0	4	16	4	16	4	16
	GR	1.0		30.7		75.0		100.0		23		3		11	
	PC	4.0	1.8	39.0	19.5	14.3	66.7	100.0	25.0	3	4	3	4	3	4
	FA	2.0		33.0		50.0	0.0	0.0							
	SJ	1.0	2.0	39.0	39.0	25.0	25.0	0.0	100.0		2		2		2
	AZ	1.5	1.8	33.3	18.4	33.3	47.1	75.0	50.0	36	29	3	2	11	16
Total	SA	1.8	1.5	31.3	25.0	50.0	28.6	75.0	100.0	22	9	1	2	14	7
	SM	2.0	1.9	21.8	18.9	55.6	87.5	80.0	71.4	18	24	1	1	7	10
	TR	2.0	3.2	19.2	19.3	50.0	70.0	66.7	71.4	16	32	1	2	9	16
	GR	2.1	1.6	29.1	16.7	78.6	87.5	72.7	100.0	91	19	1	1	23	5
	PC	2.9	2.1	23.6	16.9	59.3	50.0	50.0	53.8	20	15	1	1	7	4
	FA	2.5	2.7	36.0	39.0	33.3	60.0	0.0	0.0						
	SJ	1.8	2.0	31.2	27.0	45.5	63.6	20.0	57.1	1	8	1	2	1	2
	AZ	2.29	2.15	26	20.9	55.7	61.3	59.2	65.2	168	107	1	1	23	16

HV- hybrid grape varieties; SA - Santa Maria; SM – São Miguel; TR - Terceira; GR - Graciosa; PC – Pico; FA – Faial; SJ – São Jorge; AZ- Azores.

CONCLUSIONS

Within this study we showed that the vineyards of the Azores islands harbor a high yeast biodiversity and a high genetic diversity of *S. cerevisiae* strains.

The biodiversity wealth of these islands is certainly much higher than determined in this study, considering that our cultivation methods in YPD medium (with or without biphenyl) might have prevented the growth of numerous other yeast species. The comparison of the microflora from the different ecosystems of the islands showed correlations between the predominating species, geographic location of the islands, type of vineyard and grape variety.

The species *H. uvarum* was predominant in seven islands of the Azorean Archipelago, independent of the grape varieties and the type of vineyard. However, this species was present in abandoned vineyards in considerably higher (20% more) proportions than in non-abandoned vineyards. The high percentage of *C. zemplinina* in Santa Maria and São Miguel differentiates the yeast microflora of this group of islands, pointing towards a correlation between geographic origin of the grapes and the yeast microflora composition. The species *M. pulcherrima*, appears clearly associated with the traditional grape varieties, being only found in Graciosa and Pico, the islands with higher proportion of such grape varieties. However, these data will be completed with results obtained from the samples collected in 2010.

The genetic diversity of *S. cerevisiae* was more dependent on the sampling year than on the geographic origin, grape variety or type of vineyard, showing that ecologically meaningful affirmations require repeated sampling campaigns. This points to additional biotic and abiotic factors that affect the distribution and abundance of yeast communities in vineyards of the Azorean islands.

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