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SHORT COMMUNICATION

Molecular analysis of red wine yeast diversity in the Ribera del Duero D.O. (Spain) area

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Abstract Molecular characterization of wine yeast population during spontaneous fermentation in biodynamic wines from Ribera del Duero D.O. located at northern plateau of Spain has been carried out during two consecutive years. A total of 829 yeast strains were isolated from the samples and characterized by electrophoretic karyotype. The results show the presence of three population of yeast differentiated by their electrophoretic karyotypes, (1) non-*Saccharomyces* yeast dominant in the initial phase of the fermentations (NS); (2) *Saccharomyces bayanus* var *uvarum* detected mainly mid-way through the fermentation process at 20–25 °C; and (3) *Saccharomyces cerevisiae* which remained dominant until the end of the fermentation. This is the first study showing the population dynamic of *S. bayanus* var. *uvarum* in red wines produced in Ribera del Duero that could represent an important source of autochthonous wine yeasts with novel oenological properties.

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Introduction

The biodynamic wines featured in this study are made in the Ribera del Duero D.O. located at northern plateau of Spain, using the principles of biodynamic vine cultivation (Joly 1997; Waldin 2004). This type of wine has become popular in recent years for organoleptic reasons, and the health benefits attributed to the wine (Reeve et al. 2005).

The spontaneous fermentation of wine is carried out by a complex population of microorganisms consisting of bacteria and yeasts. Yeasts are mainly responsible for the alcoholic fermentation, and it is characteristic of this process that several populations of different yeast species are active sequentially during the fermentation, each being largely substituted by the next (Raspor et al. 2002; Santamaría et al. 2005; Lopandic et al. 2008). Although *S. cerevisiae* is the most important wine yeast, *Saccharomyces bayanus* var. *uvarum* has also been observed at the end of the fermentation, either in association with *S. cerevisiae* or acting on its own (Torriani et al. 1999; Naumov et al. 2000, 2002; Demuyter et al. 2004). The differentiation of species and yeast strains of *S. cerevisiae* and *S. bayanus* var. *uvarum* has been performed by electrophoretic karyotype (Naumov et al. 2002; Demuyter et al. 2004; Le Jeune et al. 2007); other genetic studies such as analyses of restriction patterns of the *MET*2 gene (Naumov et al. 2000; Le Jeune et al. 2007) have also been described.

The characterization of yeast is necessary because it is important to obtain knowledge of the autochthonous strains implicated in the fermentations process for each geographic wine-producing area. This relevance is based on

the necessity to develop studies that allow to oenologist community the selection of very specifically yeast strains to be used as starter of the wine making process, with the main aim to control the fermentation process and preserving the typical properties and quality of the finished wines (Rodríguez et al. 2010; Di Maio et al. 2012).

In this work, we have characterized by electrophoretic karyotyping the diversity of wild yeasts during fermentation process biodynamic red wines. The aim has been to determine the most representative yeasts, so that in future, wine producers can select autochthonous yeast strains with properties of particular oenological interest and for better control of the process.

Materials and methods

Vinifications conditions, sampling and isolation of wine yeast strains

Seven industrial-scale spontaneous fermentations were analysed during two consecutive years (2008–2009). Five of them were carried out in smaller 2,000 L capacity foudres (designated T1 to T5). In these wooden casks, the temperature was not controlled and oscillated between about 12 and 25 °C. The two other fermentations were carried out in larger 4,000 L capacity stainless steel vessels (designated D10 and D11) in which temperature was controlled between 14 and 25 °C.

We took 25 mL of samples from the vessels daily, until the end of the fermentation process. The samples were transported on ice to the laboratory where the yeast cells were collected by centrifugation and resuspended in 600 µL of the grape must. The samples were mixed with 400 µL of 50 % glycerol stock solution and preserved at −80 °C until further processing. We analysed three samples from each vessel that correspond to the different phases of the fermentation process, initial, middle and final, when the density of the must was about 1100, 1020 and 994, respectively. For these samples, we isolated yeast colonies as described by Rodríguez et al. (2010) for characterization by electrophoretic karyotype.

Molecular characterization of yeast strains by pulsed-field gel electrophoresis

Electrophoretic karyotypes of the yeast isolates were obtained using the procedure described by Rodríguez et al.

Table 1 Distribution (%) of the different yeast populations, obtained by electrophoretic karyotype patterns, in the spontaneous fermentations in the 2008 and 2009 vintages

Vessels		2008				2009			
		T (°C)	NS[a]	*S. bayanus* var. *uvarum*	*S. cerevisiae*	T (°C)	NS[a]	*S. bayanus* var. *uvarum*	*S. cerevisiae*
D10	IF	14	100	0	0	14	95.2	0	4.8
	MF	23	40	10	50	24.5	90	0	10
	EF	22.5	0	0	100	23.5	21.1	5.3	73.6
D11	IF	17	80	20	0	12	40	60	0
	MF	25	30	65	5	21	10	90	0
	EF	23	0	5	95	23	0	5	95
T1	IF	12	90	10	0	16	90	0	10
	MF	20	20	50	30	24	89.4	0	10.6
	EF	19	0	0	100	17	0	0	100
T2	IF	12	100	0	0	15	100	0	0
	MF	19	50	40	10	23	20	55	25
	EF	18.5	0	20	80	17	0	0	100
T3	IF	12	100	0	0	15.5	87.4	0	12.6
	MF	20.5	35	35	30	24	40	45	15
	EF	18.5	0	10.4	89.6	18	0	0	100
T4	IF	15	100	0	0	14	100	0	0
	MF	18	30	25	45	22	65	35	0
	EF	18	0	5	95	19	0	0	100
T5	IF	12	80	15	5	14.5	65	30	5
	MF	18	50	5	45	23	95	5	0
	EF	18.5	5.6	5.6	88.8	20	17.6	17.6	64.8

IF initial fermentation, *MF* middle fermentation, *EF* end fermentation, *NS*[a] non-*Saccharomyces* population

(2010). Chromosomes were separated using a CHEF-DRIII system (Bio-Rad).

Identification of yeasts by *MET*2 and 5.8S-ITS amplification

Genomic DNA from isolates of yeasts, which showed different karyotypes, was purified as described by Querol et al. (1992). Quantification was carried out in a Biophotometer (Eppendorf).

To differentiate between *S. cerevisiae* and *S. bayanus* var. *uvarum,* the *MET*2 gene was amplified using the primers described by Hansen and Kielland-Brant (1994) and following the procedure described by Le Jeune et al. (2007). Gels, which contained 1µl mL^{-1} ethidium bromide, were digitalized in Molecular Imager equipment (Gel-Doc XR) and analysed using Quantity One 1-D software (Bio-Rad). Molecular sizes were estimated using the GeneRuler^{TM} 50 bp DNA Ladder (Fermentas).

The non-*Saccharomyces* (NS) yeasts were identified at genus or species level by amplification of the 5.8S-ITS region of ribosomal genes as describes by Esteve-Zarzoso et al. (1999). Gels were visualized as described above.

Results

We analysed the wild yeast composition in spontaneous fermentations carried out in industrial conditions during two consecutive years (2008 and 2009) in a winery located in Ribera del Duero. For each year, we studied seven fermentations at three stages during the fermentation process: initial (IF), middle (MF) and end (EF). Twenty isolates per sample were characterized by applying the pulsed-field gel electrophoresis (PFGE) technique: 417 strains were identified in 2008 and 412 strains in 2009. We used electrophoretic karyotyping as a suitable technique for discriminating between yeast clones (Schuller et al. 2004).

The results of the dynamic of wild yeast population during spontaneous fermentations are showed in Table 1. We found three different types of yeast, the first type was formed by non-*Saccharomyces* (NS), the second by *S. bayanus* var. *uvarum* and the third population comprised *Saccharomyces cerevisiae* yeast. NS strains showed patterns with the absence of bands running below the region of 500 kb, which are specific to *S. cerevisiae* strains (De Jonge et al. 1986). The strains of *S. bayanus* var. *uvarum* were differentiated from *S. cerevisiae* by the presence of two small chromosomes in the region of 245–370 kb (Fig. 1), instead of three as for *S. cerevisiae,* as reported by Naumov et al. (2000, 2002). Moreover, we confirmed this differentiation by PCR–RFLP of the *MET*2 gene, in 7 and 29 isolates for *S. bayanus* var. *uvarum* and *S. cerevisiae,*

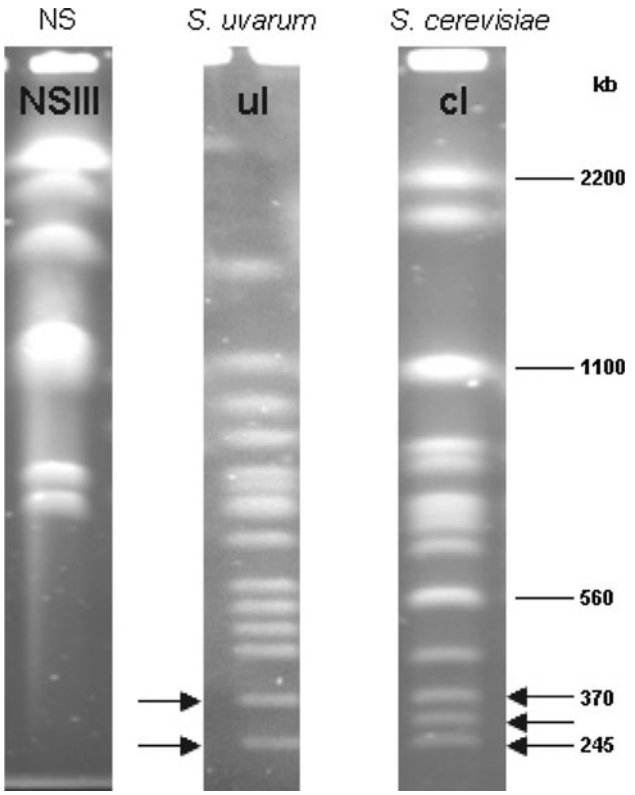

Fig. 1 Electrophoretic karyotype of *Hanseniaspora uvarum/guilliermondii* (pattern NSIII), *S. bayanus var. uvarum* (pattern uI) and *S. cerevisiae* (pattern cI). The *arrows* indicate the chromosomes characteristic of these species

respectively, which present different karyotypes. We have not found isolates with profiles combining the typical patterns of *S. cerevisiae* and *S. bayanus* var. *uvarum* previously detected for hybrid strains in Alsatian wine (Le Jeune et al. 2007).

NS yeasts composed by *Metschnikowia pulcherrima*, *Zygosaccharomyces fermentati/cidri*, *Hanseniaspora uvarum/guilliermondii*, *Torulaspora delbrueckii* and *Hanseniaspora osmophila* were dominant in the initial phase of fermentation but were displaced in the subsequent and final phases of the process by *Saccharomyces* species. NS were identified by the 5.8S-ITS region of ribosomal genes. *S. bayanus* var. *uvarum* yeasts were present mainly mid-way through the fermentation but were in competition with the NS and *S. cerevisiae*. Interestingly, this yeast was detected as the majority species when the temperature of fermentation was in the range between 20 and 25 °C (see Table 1). With respect to *S. cerevisiae*, in the majority of the vessels analysed, the population of this yeast displaced the NS and *S. bayanus* var. *uvarum* yeasts and remained dominant until the end of the fermentation.

Within each yeast population, we detected the presence of several different stains, differentiated by their

Table 2 Global distribution of karyotype patterns (%) of the yeast strains isolated from spontaneous fermentation of wines during the two consecutive years studied

Karyotype	2008							2009						
	D10	D11	T1	T2	T3	T4	T5	D10	D11	T1	T2	T3	T4	T5
NS[a]	46.6	36.7	36.6	49.9	45.7	43.3	46.5	70.0	16.6	59.3	40.0	39.4	55.0	61.4
uI	1.7	6.6	10.0	14.9	5.1	1.7	3.5	1.7	25.0		8.3	12.5	3.3	10.7
uII	1.7	8.3	3.3		3.4	1.7	1.7		3.3		1.7	1.8		1.7
uIII		6.6	1.7	1.7	1.7				5.0				1.7	3.5
uIV		5.0		1.7	1.7	1.7								
uV		3.3	3.3	1.7	3.4	3.3	3.5		13.4		3.3		6.6	
uVI			1.7											
uVII						1.7								
uVIII									1.7					
uIX									3.3		3.3			
uX											1.7			
uXI														1.7
uXII												1.8		
cI	1.7						1.7			1.7			1.7	
cII	1.7					1.7	1.7						1.7	
cIII	21.6	3.3	20.0	6.6		8.3		1.7		1.7	8.3	3.6	9.9	10.7
cIV	3.3			1.7							1.7			
cV	3.3	1.7		1.7									1.7	
cVI	6.6	1.7	8.2	3.3	1.7			1.7				8.8		
cVII	1.7									1.7				
cVIII	1.7		1.7	1.7										
cIX	3.3		1.7	1.7	5.1	6.6	7.0							
cX		1.7	1.7	10.0			1.7						1.7	3.5
cXI	1.7	13.3			23.7	6.6	10.3	16.5	18.3	18.6	8.3	10.7	3.3	
cXII		5.0				11.6	12.1			3.4	5.0	8.8	1.7	
cXIII		1.7	1.7	1.7										
cXIV		1.7												
cXV	1.7	1.7				1.7			1.7					
cXVI		1.7												
cXVII			3.3							1.7				
cXVIII	1.7		1.7			1.7								
cXIX			1.7									3.6		
cXX			1.7		1.7									
cXXI				1.7		1.7								
cXXII					1.7	5.0	3.5			5.1				
cXXIII					1.7									
cXXIV					1.7		1.7		1.7			1.8		1.7
cXXV						1.7								
cXXVI							1.7							
cXXVII							1.7							
cXXVIII							1.7							
cXXIX					1.7									
cXXX								1.7			1.7			
cXXXI								6.7	6.6	3.4				
cXXXII									1.7					
cXXXIII									1.7		1.7			

Table 2 continued

Karyotype	2008							2009						
	D10	D11	T1	T2	T3	T4	T5	D10	D11	T1	T2	T3	T4	T5
cXXXIV											3.3		1.7	
cXXXV										1.7	6.6		5.0	1.7
cXXXVI										1.7	1.7			
cXXXVII												3.6	3.3	
cXXXVIII												1.8		
cXXXIX											3.3			1.7
cXL												1.8		
cXLI													1.7	1.7
Total isolates analysed	60	60	60	60	59	60	58	60	60	59	60	56	60	57

NS[a] non-*Saccharomyces*

karyotypes. The diversity of the *S. cerevisiae* yeast was higher than for *S. bayanus* var. *uvarum* and this variability are shown in Table 2. The most representative strains in 2 years of study were those with patterns uI, uII, uIII and uV for *S. bayanus* var. *uvarum*; and those with patterns cIII, cVI, cXI and cXII for *S. cerevisiae*. These strains will also be studied for properties of oenological interest for their selection.

The wine of this study had a high content of glycerol, approximately 3 g/L more respect to the other wines of the Ribera del Duero, which could be related to the presence of *S. bayanus* var. *uvarum* during spontaneous fermentation process.

Discussion

This study reports for the first time an analysis of the population dynamics of the *S. bayanus* var. *uvarum* yeast in wine fermentations carried out under industrial-scale vinification in the Ribera del Duero region, which has a relatively warm climate. This species has been found previously in fermentations carried out at low temperature, associated with a continental-type climate (Usseglio-Tomasset et al. 1980; Torriani et al. 1999; Naumov et al. 2000, 2002). Thus, *S. bayanus* var. *uvarum* is considered a cryophilic yeast; however, we have found this yeast dominating during the middle stage of the fermentation process, when the temperature range was between 20 and 25 °C. The low frequency of detection of *S. bayanus* var. *uvarum* at the end of fermentation could be indicative of its lower ethanol tolerance compared to *S. cerevisiae*. The yeast population dynamics observed in this study were different from those observed in other studies in a continental climate for white Alsatian wines, where *S. bayanus* var. *uvarum* was dominant during the entire spontaneous process, in which the fermentation temperature was regulated between 18 and 22 °C (Demuyter et al. 2004).

In spite of *S. bayanus var. uvarum* has been previously detected in several wine regions (Somavilla et al. 1997; Naumov et al. 1994), we describe the successive participation of *S. bayanus var. uvarum* and *S. cerevisiae* yeasts in fermentation of red wines produced in the Ribera del Duero D.O. (Spain) area, for the first time.

This yeast species has a typical fermentation profile in grape must that is significantly different from *S. cerevisiae*; it produces lower and higher amounts of acetic acid and glycerol, respectively, compared with *S. cerevisiae*, and produces relatively large amounts of alcohols such as 2-phenylethanol in wine (Tosi et al. 2009). In other studies, significant differences have been reported in the aroma profile of Malvasia delle Lipari wines between batches fermented by *S. cerevisiae* and others by *S. bayanus* var *uvarum*, and a higher panel score was attributed to the latter in a tasting (Muratore et al. 2007). In our study, the high content of glycerol detected in the wines produced could be associated with the presence of this yeast species during the fermentation process.

Additionally, the increased knowledge of the diversity of yeast strains implicated in industrial-scale fermentations obtained in this work should be valuable to the industry when selecting autochthonous wine yeasts for future production, mainly due to: (1) their use as starter cultures in pure form or as mixtures; (2) improving the fermentation process; and (3) innovation, diversification and quality improvement. The Spanish wine industry is now aiming at diversifying through the creation of new styles of wines, in response to more sophisticated consumer demands. The knowledge of specific yeast strains and the utilization of autochthonous yeast strains to inoculate fermentations is of increasing interest to wineries. This should allow producers to obtain new wines with particular organoleptic profiles

while maintaining the typical characteristics of regional wines and exercising more precise control of the total winemaking process, in line with current world trends in oenology.

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