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ORIGINAL ARTICLE

A molecular genetic study of natural strains of *Saccharomyces* isolated from Asturian cider fermentations

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Abstract

Aims: To analyse the genetic diversity and the dynamics of *Saccharomyces* strains in spontaneous fermentation in ciders. The effect of the cellar, harvest and cider-making technology were evaluated.

Methods and Results: The ecology of spontaneous cider fermentations in the same cellar (Asturias) was studied for two consecutive harvests (2000 and 2001) by using mtDNA restriction analysis. Our results showed that there was a succession of genetically different strains of *Saccharomyces* during cider production. In general, strains of *Saccharomyces bayanus* species predominated at the early fermentation steps (begining and/or tumultuous fermentations), while *Saccharomyces cerevisiae* yeasts were the most abundant at the end of the fermentation. Five *S. bayanus* strains (patterns III, VII, VIII, XV and XVII) were present at significant frequencies in all the experimental tanks during the two consecutive years. The results of the cluster analysis (unweighted pair group method using average linkage) showed higher similarities for the patterns III, XV, VII and VIII. Therefore, these strains should be considered associated with the microbiota of this cellar.

Conclusions: A high polymorphism within populations of *Saccharomyces* was found throughout the different stages of Asturian production of cider. In all the cider fermentations, a variable number of *S. bayanus* and *S. cerevisiae* strains was always present. Our results indicate, over the period of time studied, the existence of the natural microbiota in the cellar.

Significance and Impact of the Study: This study has allowed us to gain a better understanding of the role of wild *Saccharomyces* yeast in Asturian cider fermentations.

Introduction

The fermentation of apple must is a complex microbial reaction involving the sequential development of various species of yeast and bacteria (Beech 1972; Salih *et al.* 1988; Michel *et al.* 1988; Cabranes *et al.* 1990; Dueñas *et al.* 1994; Panon *et al.* 1995). Among these microorganisms, yeasts are primarily responsible for alcoholic fermentation, and therefore of the taste and flavour characteristics of ciders (Beech and Davenport 1970; Mafart 1986; Le Queré and Drilleau 1993, 1998; Cabranes *et al.* 1997; del Campo *et al.* 2003; Suárez *et al.* 2005).

Asturias, a region located at the Atlantic coast of northern Spain, is the country's leading natural cider producer, with an average yearly production of nearly 45 million litres (Suárez and Picinelli 2001). Natural cider is produced by two different processes. In the first (traditional), the apples are crushed by a slow mechanical process and the juice is fermented in wooden barrels. In the second (alternative), a faster, more modern, pneumatic press is used and the juice is fermented in stainless-steel tanks. Nevertheless, alcoholic fermentations in both cider-making procedures are always carried out in a spontaneous way by indigenous yeasts either present on the

apples or introduced from the equipment and cellar during the cider-making process. This type of fermentation is of particular interest to ascertain the ecology of fermentation processes. Preliminary studies were conducted to learn more about the yeast species involved in cider making, and they showed that the genus *Saccharomyces* is usually predominant during alcoholic fermentation while non-*Saccharomyces* grow mainly during the initial stages of the process (Salih *et al*. 1988; Cabranes *et al.* 1990; Cabranes 1994; Dueñas *et al*. 1994; Morrissey *et al*. 2004; Suárez *et al*. 2007).

Identification at the strain level of yeast is a requisite to study the diversity of species and to monitor population dynamics during fermentations. In the last two decades, molecular methods constituted powerful tools for yeast identification and characterization. The genetic diversity of *Saccharomyces* strains has been analysed by several methods, such as pulsed-field gradient electrophoresis (PFGE) of chromosomes, restriction analysis of the mitochondrial DNA (mtDNA-RFLP), methods based on the PCR technique (RAPD, microsatellites, minisatellites, δ sequences, AFLP) among others (Degré *et al.* 1989; Querol *et al.* 1992a; Vezinhet *et al.* 1992; Lieckfeldt *et al*. 1993; Ness *et al*. 1993; Guillamón *et al.* 1994, 1996; Quesada and Cenis 1995; Romano *et al.* 1996; de Barros Lopes *et al*. 1999; Pataro *et al*. 2000; Fernández-Espinar *et al*. 2001; Ayoub *et al.* 2006).

Studies carried out in wine have examined the dynamics and variability of wild *Saccharomyces* strains during spontaneous fermentation (Frezier and Dubourdieu 1992; Vezinhet *et al*. 1992; Versavaud *et al.* 1993, 1995; Querol *et al.* 1994; Constantí *et al.* 1998; Sabate *et al.* 1998; Gutiérrez *et al.* 1999; Esteve-Zarzoso *et al.* 2001; Torija *et al.* 2001; Rainieri *et al.* 2003; Cocolin *et al*. 2004; Schuller *et al.* 2005; Legras *et al*. 2005).

However, investigations on natural variability of *Saccharomyces* strains in cider fermentations are scarce. Nevertheless, yeast strains isolated from French ciders were analysed by molecular karyotyping and PCR-RFLP (Masneuf *et al*. 1998; Naumov *et al.* 2001; Coton *et al.* 2006). In addition, physiological test, together with a PCR-based method using intron splice site primers, have been developed to identify Asturian cider strains and to analyse the level of implantation of the starter in inoculated cider fermentations (Pando 2002; Suárez *et al.* 2005).

The objectives of this work were to analyse the genetic diversity and the population dynamics of *Saccharomyces* strains in spontaneous fermentation of apple musts obtained by two making procedures. The study was performed in one cider cellar during two consecutive years. Taxonomic identity was established at the yeast level for *Saccharomyces* by RFLP of mtDNA restriction (López *et al*. 2001).

Materials and methods

Cider fermentations

Two cider-making systems [traditional system (TS) and alternative system (AS)] were studied in one Asturian cellar from Villaviciosa (Asturias, Spain). Apple musts in this cellar had never been inoculated with active dry yeasts. In the TS, the must was obtained by means of a batch mechanical press with a slow pressing (cycle 3 days), the fermentation process being conducted in a 10 000-l wood barrel; in the other system referred to as AS, the must was obtained by a pneumatic press (cycle 8 h), and the fermentation was carried out in a 25 000-l stainless steel tank. The study was carried out in the same conditions, in two consecutive harvests (2001 and 2002). One vat from each system was analysed per year.

Microbiological analysis

A total of four samples were taken from each vat at different stages of the cider-making process: filling of the vat (FV; density: 1050–1047 g l^{-1}), beginning of fermentation (BF; density: 1037–1020 g l^{-1}), tumultuous fermentation (TF; density: 1020–1010 g l^{-1}) and end of fermentation (EF; density $\leq$1000 g l^{-1}). At each stage, the samples were aseptically taken and carried to the laboratory under refrigeration. Aliquots of several dilutions were spread onto malt extract agar (0·3% yeast extract, 0·3% malt extract, 0·5% peptone, 1% glucose, 2% agar) supplemented with 25-mg l^{-1} penicillin G potassium salt (Calbiochem, Nottingham, UK) and 100-mg l^{-1} streptomycin sulfate (Acofarma, Madrid, Spain) to inhibit bacterial growth. The plates were incubated for colony development at 28°C for 2 days; 50 colonies from each sample were randomly taken and preserved onto yeast extract peptone dextrose (YEPD) agar slants and stored at 4°C for further investigations.

Molecular identification of yeast

Yeast identification at species level was performed by PCR-RFLP analysis of the ITS1-5·8-ITS2 region from the nuclear rRNA gene according to Esteve-Zarzoso *et al.* (1999) and Fernández-Espinar *et al.* (2000).

Mitochondrial DNA (mtDNA) restriction analysis

The total DNA extraction and mtDNA restriction analysis were performed by the method of Querol *et al.* (1992b) modified by López *et al.* (2001). Yeast DNA was digested with HinfI restriction enzyme (Roche, Mannheim, Germany) and the fragments were separated in 1%

agarose gels in 1X TAE (tris-acetic acid-EDTA) buffer at 100 V using a Power-Pac 300 (Bio-Rad, Hercules, CA, USA). Gels were stained with ethidium bromide and the DNA fragments were visualized under UV light and scanned using a camera charge-coupled device (CCD) Gene Genius (Syngene, Cambridge, UK).

The restriction fragment sizes were measured as base pairs, calculated in comparison with a λDNA (Roche, Mannheim, Germany) digested with Pst I restriction enzyme (Roche, Mannheim, Germany) using a Gene Tools gel Analysis Software (Syngene, Cambridge, UK).

Data analysis

The similarities between patterns were determined by the fraction of shared bands (Dice coefficient). Clustering was calculated by the unweighted pair group method using average linkage (UPGMA) included in the Numerical Taxonomy and Multivariate Analysis System (NTSYS) package (Exeter Publishing Ltd., Setauket, NY, USA) Version 2.2.

Results

Two cider-making processes (TS and AS) were studied in the same cellar in 2001 and 2002 (Fig. 1). Samples were periodically taken from each experimental vat at four different stages. In a previous work, a total of 800 colonies were identified by RFLP analysis of ribosomal genes, showing that the group of *Saccharomyces sensu stricto* strains represented 59% of the total of the isolates. Among them, the species *S. bayanus* and *S. cerevisiae* were found at frequencies of 58% and 42%, respectively (Suárez *et al*. 2007).

Subsequently, the discrimination between these indigenous *Saccharomyces* species at the strain level was performed using the molecular mtDNA-RFLP analysis. The mtDNA restriction patterns of the 472 colonies isolated revealed 99 different restriction patterns – 52 of them were found among the 199 *S. cerevisiae* colonies, and 47 restriction patterns among the 273 *S. bayanus* colonies. The number of colonies identified, and the variability and diversity of patterns of *Saccharomyces* strains are indicated in Table 1.

These results showed that the degree of variability of *Saccharomyces* strains, measured as the percentage of the different strains found among the colonies analysed, was similar in both cider-making systems and close to 33%.

In 2001, 166 colonies were analysed and 47 different mtDNA restriction patterns were observed; *S. bayanus* strains (29–24%) were dominant in both technology systems. In the second year, 69 different strains were found among the 306 colonies analysed; in this harvest, *S. bayanus* presented a lower variability, irrespective of the cider-making procedures (13%). On the other hand, more than 50% (53 strains) of the patterns were represented by a single colony in one stage of the fermentation (Table 1). These sporadic strains were observed at higher frequency in the traditional tanks comparing with the AS. Among the remaining patterns, 14 were isolated only in one vat at low frequency except in the case of the patterns XXX and XXXI. These two strains were the most abundant at the end of the fermentation in the cider elaborated by AS during 2001.

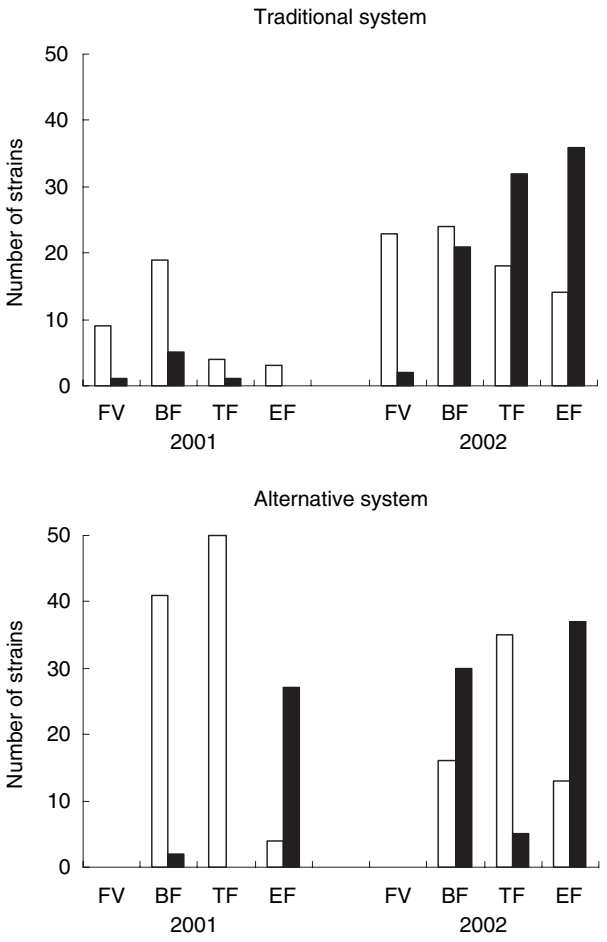

Figure 1 Common patterns of strains in traditional and alternative cider-making: FV, filling of the vat; BF, beginning of fermentation; TF, tumultuous fermentation; EF, end of fermentation; *Saccharomyces bayanus* (□); *Saccharomyces cerevisiae* (■).

Figure 2 shows the common strains present in TS and AS. In the TS, 10 common patterns were observed: eight strains of *S. bayanus* and two strains of *S. cerevisiae* (XLVI, XLVII). These two patterns were dominant at the end of the fermentation in 2002 in the cider elaborated by the TS. In the AS, a total of 12 common patterns were

Table 1 Wild *Saccharomyces* strains isolated from different cider fermentations analysed in the present study

Making system	Harvest	No. of colonies studied	No. of different strains	Variability (%)	No. of strains *of Saccharomyces cerevisae*	No. of strains of *Saccharomyces bayanus*	No. strains isolated one time	Strains isolated in one vat
TS	2001	42	15	35·7	3	12	3	–
TS	2002	170	49	28·8	26	23	15	*S. bayanus*: LXXXV, XCIV *S. cerevisae*: LXXXVIII, XCVI, C
AS	2001	124	42	33·8	12	30	18	*S.bayanus*: XIII, XXIII, XLII *S.cerevisae*: XVIII, XXX, XXI, XXXII
AS	2002	136	45	33·0	27	18	17	*S. bayanus*: L *S. cerevisae*: LVIII

TS, traditional system (slow pressing and wooden barrels); AS, alternative system (pneumatic press and stainless-steel tanks).

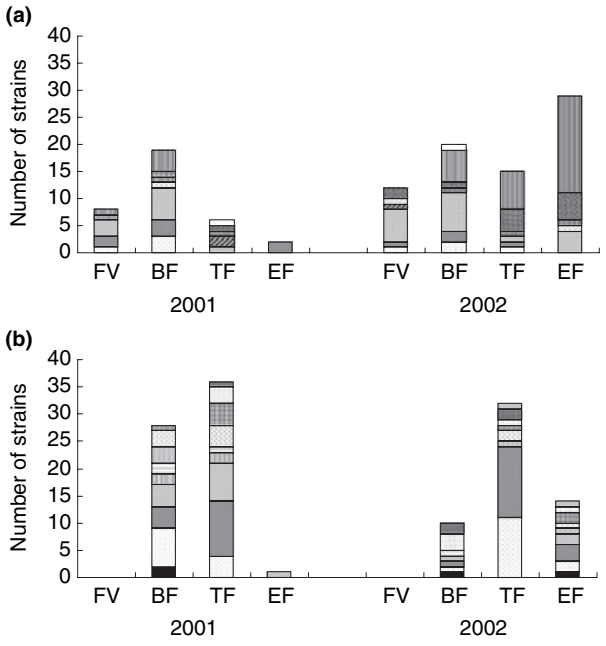

Figure 2 Common patterns of strains in traditional and alternative cider-making: FV, filling of the vat; BF, beginning of fermentation; TF, tumultuous fermentation; EF, end of fermentation; *S. bayanus*: ■ I, □ III, ■ VII, ■ VIII, ▨ X, ■ XI, □ XII, ■ XIV, □ XV, ■ XVII, □ XIX, ■ XXVII, □ LXXXIII; *S. cerevisiae* : ■ XLI, ■ XLVI, ■ XLVII.

detected: 11 strains of *S. bayanus* and one pattern of *S. cerevisiae* (XLI). It is worth nothing that in the apple juices (FV) obtained by pneumatic pressing, *Saccharomyces* yeasts were not found (Fig. 2b). However, in the fresh musts obtained by slow pressing, *S. cerevisiae* and *S. bayanus* species were always present (Fig. 2a).

Comparing by the cider production harvest, nine common patterns, identified as *S. bayanus*, were isolated in 2001, while in the second year, a bigger diversity of patterns was found (Table 2). On the other hand, in 2002, one strain of *S. cerevisiae* (XLVII) was dominant at the end of the fermentation in both cider processes.

The results reveal that five patterns (III, VII, VIII, XV and XVII), identified as *S. bayanus*, were present in the four experimental vats, irrespective of the harvest and cider-making system.

Table 3 shows the frequencies, measured as the percentage of same pattern found among the isolated colonies (50 colonies from each stage of fermentation), of the most representative strains during fermentations.

In the TS (Table 3), the pattern VIII was the more frequent at FV in both harvests. In BF, the more abundant patterns were VIII and XLVII in 2001, and VIII, XIX and XLVII in 2002 harvest. Likewise, in the second year, the percentage of the strain XLVII, identified as *S. cerevisiae*, increased during the last stages of the fermentation (TF, EF), reaching a significant frequency (36%) at the end of cider-making process. The unusual date obtained in 2001 harvest did not allow to observe the dynamics of this yeast strain in this year.

In the AS at FV, *Saccharomyces* strains were not isolated. In BF, the pattern III in 2001 and XLVII in the second year showed similar frequency. At TF, one strain (VII) was predominant in both harvests. And at the EF, these patterns were clearly replaced, being the more abundant patterns XXX and XXXI in 2001, and XLVII in the second year (Table 3).

In order to determine possible relationships between strains, we performed a cluster analysis of similarity between mtDNA restriction patterns of the most frequent strains, that is, those present in more than 6% in at least one sample (Fig. 3).

The results of the dendrogram from UPGMA clustering show the relatedness among mtDNA-RFLP patterns of strains belonging to the same species. Four clusters were observed, and the type strains of *S. bayanus* are included in two different, but closely related clusters (I and II) except the patter XIX, and all of *S. cerevisae* are clustered

Table 2 Number of the mtDNA patterns obtained at each stage of cider fermentations

		Traditional system (TS)				Alternative system (AS)			
Patterns	YI	FV	BF	TF	EF	FV	BF	TF	EF
Harvest 2001									
III	*S. b*	1	3	0	0	0	7	4	0
VII	*S. b*	2	3	0	2	0	4	10	0
VIII	*S. b*	3	6	1	0	0	4	7	0
X	*S. b*	0	0	2	0	0	1	0	0
XII	*S. b*	0	2	0	0	0	2	0	0
XV	*S. b*	0	1	0	0	0	3	4	0
XVI	*S. b*	0	1	0	0	0	3	2	0
XVII	*S. b*	1	1	1	0	0	0	3	0
XXVIII	*S. b*	0	2	0	0	0	0	1	0
Harvest 2002									
I	*S. b*	0	2	0	2	0	1	0	1
III	*S. b*	1	2	1	0	0	1	11	2
VII	*S. b*	1	2	1	0	0	1	13	3
VIII	*S. b*	6	7	1	4	0	1	0	2
XI	*S. b*	3	0	0	1	0	0	1	0
XIV	*S. b*	0	0	1	0	0	0	0	1
XIX	*S. b*	2	8	5	4	0	3	0	1
XV	*S. b*	1	0	0	1	0	0	2	1
XVII	*S. b*	0	1	1	0	0	0	1	2
XXVII	*S. b*	0	0	1	0	0	2	2	0
LV	*S. b*	0	0	2	0	0	1	0	0
LXVII	*S. b*	2	0	0	0	0	0	3	0
XLI	*S. c*	0	0	1	3	0	0	0	1
XLV	*S. c*	0	1	0	2	0	5	0	5
XLVI	*S. c*	2	1	4	5	0	4	0	5
XLVII	*S. c*	0	6	7	18	0	7	0	12
LII	*S. c*	0	0	1	0	0	1	0	1
LIII	*S. c*	0	0	1	0	0	1	0	0
LIV	*S. c*	0	0	3	0	0	1	0	2
LVI	*S. c*	0	0	0	1	0	1	0	0
LIX	*S. c*	0	0	1	0	0	1	0	0
LX	*S. c*	0	2	2	2	0	1	0	0
LXII	*S. c*	1	4	3	1	0	1	0	5
LXV	*S. c*	0	0	1	0	0	0	2	0
LXXX	*S. c*	0	0	1	1	0	0	0	1

FV, filling of the vat; BF, beginning of fermentation; TF, tumultuous fermentation; EF, end of fermentation

YI, Yeast identification; *S. b, Saccharomyces bayanus*; *S. c*, *Saccharomyces cerevisiae*.

in another two groups (III and IV). The similarities between the patterns of the *S. bayanus* strains were very high (cluster I: 0·88–0·65, cluster II: 0·70–0·58); however, the similarities between the *S. cerevisiae* strains were much lower (cluster III: 0·44–0·28, cluster IV: 0·36–0·22%). According to the cluster analysis, the patterns that showed major similarities were coincident with those that were present in the four experimental vats irrespective of the harvest and cider making (cellar effect). Thus, the higher similarity between mtDNA restriction patterns was observed for the strains III and XV (similarity: 0·88), followed by the strains VII and VIII (similarity: 0·70). This analysis did not show correlations neither with harvest nor cider-making technology.

Discussion

The application of a technique based on mtDNA restriction analysis has allowed us to extend our knowledge about the intraspecific diversity and the population dynamics of wild *Saccharomyces* strains during spontaneous cider fermentations. Numerous authors have applied mtDNA-RFLP analysis to differentiate *S. cerevisiae* and *S. bayanus* species at the strain level in foods and beverages (Fernández-Espinar *et al.* 2006). Nevertheless, this technique has some limitations. That is, the interspecies hybrids (*S. cerevisiae* × *S. bayanus*), which contain mtDNA of one parent only, cannot be differentiated from the parent strain by this technique (Masneuf *et al.* 1998; Groth *et al.* 1999; Naumova *et al.* 2005).

The big number of different patterns found in the spontaneous fermentation of the apple musts (Table 1) was higher than that reported for *Saccharomyces* population from some wine-growing regions over the world (Frezier and Dubourdieu 1992; Querol *et al.* 1994; Gutiérrez *et al.* 1999; Torija *et al.* 2001; Schuller *et al.* 2005). Two reasons could explain the high biodiversity within *Saccharomyces* strains in Asturias. On the one hand, the use of dried active yeast to direct fermentations is not a common practice in our cider cellars; in fact, in this particular cellar, commercial yeasts have never been inoculated. In this sense, it is well known that the use of commercial starter in a cellar colonizes the environment, thus reducing the diversity of strains (Querol *et al.* 1992b; Constantí *et al.* 1998; Beltran *et al.* 2002). On the other hand, the existence of yeasts with the killer phenotype markedly reduces the variability of the natural population. Previous ecological studies carried out in Asturian cellars showed that the killer phenotype is not frequent (Cabranes 1994).

This study has been carried out in one cellar from Asturias. This region is located at the Atlantic coast of northern Spain. The population of *S. bayanus* is generally found in the cold areas of Europe (which is the case of Asturias), and it is associated to cryotolerant species (Castellari *et al.* 1992; Naumov 1996; Feuillat *et al.* 1997). In our survey, *S. bayanus* yeasts were prevalent in all cider fermentations. The presence of cryotolerant yeast in Asturias is not surprising, given the low temperature kept during cider making (<15°C). This fact was in agreement with a previous report for neighbouring regions (Basque Country). Thus, Rementeria *et al.* (2003) show that *S. bayanus* strains were the most frequent species during spontaneous fermentation of the white wine 'Txakoli de

Table 3 Most representative frequencies (%) of the mtDNA patterns at each stage of sampling

		Traditional system								Alternative system							
		Harvest 2001				Harvest 2002				Harvest 2001				Harvest 2002			
Patterns	YI	FV	BF	TF	EF	FV	BF	TF	EF	FV	BF	TF	EF	FV	BF	TF	EF
III	*S. b*	2	6	0	0	2	4	2	0	0	14	8	0	0	2	22	4
VII	*S. b*	4	6	0	4	2	4	2	0	0	8	20	0	0	2	26	6
VIII	*S. b*	6	12	2	0	12	14	2	8	0	8	14	0	0	2	0	4
XI	*S. b*	0	0	0	0	6	0	0	2	0	4	2	0	0	0	2	0
XIII	*S. b*	0	0	0	0	0	0	0	0	0	2	6	0	0	0	0	0
XIV	*S. b*	0	0	0	0	0	0	2	0	0	6	8	0	0	0	0	2
XV	*S. b*	0	2	0	0	2	0	0	2	0	6	8	0	0	0	4	2
XVI	*S. b*	0	2	0	0	0	0	0	2	0	6	4	0	0	0	0	0
XVII	*S. b*	2	2	2	0	0	2	2	0	0	0	6	0	0	0	2	4
XIX	*S. b*	0	0	0	0	4	16	10	8	0	2	0	0	0	6	0	2
XXV	*S. b*	0	0	0	0	2	2	0	0	0	0	10	0	0	0	0	0
LXVII	*S. b*	0	0	0	0	4	0	0	0	0	0	0	0	0	0	6	0
XXX	*S. c*	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0
XXXI	*S. c*	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0
XXXII	*S. c*	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0
XLI	*S. c*	0	0	0	0	0	0	2	6	0	0	0	2	0	0	0	2
XLV	*S. c*	0	0	0	0	0	2	0	4	0	0	0	0	0	10	0	10
XLVI	*S. c*	0	0	2	0	4	2	8	10	0	0	0	0	0	8	0	10
XLVII	*S. c*	2	8	0	0	0	12	14	36	0	0	0	0	0	14	0	24
LIV	*S. c*	0	0	0	0	0	0	6	0	0	0	0	0	0	2	0	4
LXII	*S. c*	0	0	0	0	2	8	6	2	0	0	0	0	0	2	0	10
LXXXVIII	*S. c*	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0

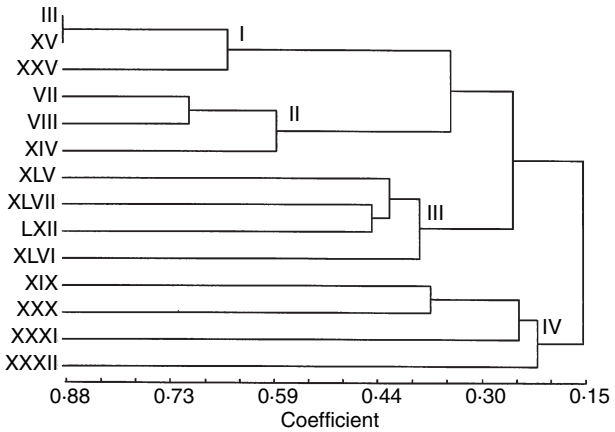

Figure 3 Dendrogram derived from UPGMA (Dice coefficient) comparison of patterns obtained for the most frequent *Saccharomyces* (>7%) strains by mtDNA restriction analysis: *S. bayanus*: III, VII, VIII, XIV, XV, XIX, XXV; *S. cerevisiae*; XXX, XXXI, XXXII, XLV, XLVI, XLVII, LXII.

Bizkaia'. The specific ecological niche of *S. bayanus* is also found in yeast strains isolated from French ciders (Naumov *et al.* 2001; Coton *et al.* 2006).

In our study, some predominant strains were found at a certain stage of fermentation but none of them were capable of outgrowing the others. The sequential presence of different species throughout the fermentations was consistent in both processes. In the TS, *S. bayanus* strains were predominant in the must and at the beginning of the fermentations among the *S.sensustricto* yeasts; whereas, in the AS, this species was clearly dominant in the TF. Likewise, *S. cerevisiae* patterns took over the process in the final stages of the fermentation in the AS (two harvests) and in the cider obtained by the other technology in 2002.

Furthermore, it is well known the infrequent presence of *Saccharomyces* strains in grape must (Martini *et al.* 1996; Guillamón *et al.* 1998; Torija *et al.* 2001) and the surprising presence of *Saccharomyces* strains at the FV in the TS could be explained by the characteristics of the slow pressing system. Thus, the great cycle times of pressing (3 days) enabled the development and growth of the fermentative yeasts coming from the pressing equipment (Beech and Carr 1977).

The number of different strains detected for each cider-making technology and its frequency of appearance varied from one year to another. However, the same strains found over the two consecutive years (28% of the patterns of the 2002 harvest were isolated in the first year of study) suggested the presence of natural microbiota resident in the cellar. Indeed, five *S. bayanus* strains (patterns

III, VII, VIII, XV and XVII) were present at significant frequencies in all experimental tanks during the two consecutive years. These results agreed with many others obtained in wine studies and may be an example of the 'cellar effect', that is, the retention of strains in the cellar from one harvest to another (Vezinhet *et al.* 1992; Versavaud *et al.* 1995; Sabate *et al.* 1998, 2002; Epifanio *et al.* 1999; Gutiérrez *et al.* 1999; Esteve-Zarzoso *et al.* 2001).

On the other hand, during the 2001 harvest, with independence of the cider-making technology, an anomalous abundance of *Hanseniaspora valbyensis* was always isolated at the end of fermentations (Suárez *et al.* 2007) and the little number of patterns of *Saccharomyces* did not allow obtaining more conclusions from this year.

In 2002, the important presence of patterns (*S. cerevisae*) XLVI and XLVII during all the stages of fermentation, irrespective of the cider-making technologies, seem to indicate the influence of the year of making, and therefore of the climatic conditions (Schütz and Gafner 1994; Gutiérrez *et al.* 1999).

In the present study, we have shown for the first time, the diversity and the dynamics of *Saccharomyces* yeast from alcoholic fermentation in the production of cider, being a useful approach to obtain a deeper insight into the ecology of this yeast species. Our results showed that there was a succession of genetically different strains of *Saccharomyces* during the process of cider production. In general, the strains of *S. bayanus* species predominated at early fermentation steps, while *S. cerevisiae* yeasts were the most abundant at the end of the fermentation. Five *S. bayanus* strains (patterns III, VII, VIII, XV and XVII) were present at significant frequencies during the production of cider, irrespective to the cider-making technology and harvest. Therefore, these strains should be considered associated as microbiota of this cellar.

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