Biodiversity of *Saccharomyces* yeast strains from grape berries of wine-producing areas using starter commercial yeasts

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Abstract

The use of commercial wine yeast strains as starters has grown extensively over the past two decades. In this study, a large-scale sampling plan was devised over a period of 3 years in three different vineyards in the south of France, to evaluate autochthonous wine yeast biodiversity in vineyards around wineries where active dry yeasts have been used as fermentation starters for more than 5 years. Seventy-two spontaneous fermentations were completed from a total of 106 grape samples, and 2160 colonies were isolated. Among these, 608 *Saccharomyces* strains were identified and 104 different chromosomal patterns found. The large majority of these (91) were found as unique patterns, indicating great biodiversity. There were differences in biodiversity according to the vineyard and year, showing that the biodiversity of *Saccharomyces* strains is influenced by climatic conditions and specific factors associated with the vineyards, such as age and size. Strains that were *terroir* yeast candidates were not found. The biodiversity of *S. cerevisiae* strains after harvest was similar to that in the early campaign; moreover, a temporal succession of *S. cerevisiae* strains is shown. This fact, together with the differences in biodiversity levels verifies that other factors were more important than commercial yeast utilization in the biodiversity of the vineyard.

Introduction

Traditional wine fermentation is a complex heterogeneous microbiological process involving the sequential development of various yeasts and other microorganisms present in musts, such as moulds and lactic and acetic acid bacteria. However, it is accepted that strains of *Saccharomyces cerevisiae*, known as 'wine yeast', are especially well adapted to this process and play a major role in the fermentation of grape musts (Rankine, 1968; Martini & Vaughan-Martini, 1990; de Barros Lopes *et al.*, 1998). The origin of *S. cerevisiae* in spontaneous fermentation is rather controversial (Pretorius, 2000). Some authors consider that *S. cerevisiae* comes from the microbial community resident in the wineries. In the vineyard, yeasts may be transported from the soil to the grapes by various insects or by the wind. Surprisingly, fermentative species of *Saccharomyces* occur in very low numbers in grapes, the predominant microorganisms being apiculate yeasts and other oxidative species (Fleet & Heard, 1993). On the other hand, Mortimer & Polsinelli (1999) observed that damaged grape berries are rich depositories of *S. cerevisiae*, showing that the vineyard can be a natural store of *S. cerevisiae*. The importance of each yeast source – vineyard or winery – may vary greatly, depending on a large variety of factors, such as climatic conditions, including temperature and rainfall, the geographical location of the vineyard, the amount of SO_2, antifungal applications, the harvest technique, the grape variety, the age of the vineyard, and the soil type (Pretorius, 2000).

Since the beginning of the 1980s, the use of active dried *S. cerevisiae* yeast starters has become increasingly common. Today, the majority of wine production is based on the use of commercial strains, which have been isolated from vineyards or wineries and selected for their superior properties for winemaking. This ensures rapid and reliable fermentations and reduces the risk of sluggish or stuck fermentations and of microbial contamination. The use of selected *S. cerevisiae* strains has greatly improved the reliability of the fermentation process and the quality of wines.

On the other hand, there is increasing interest in both indigenous strains of *S. cerevisiae* and wild yeast species that may contribute to the overall sensorial quality of wine, even in guided fermentations using selected *S. cerevisiae* starter cultures, and in the use of indigenous *S. cerevisiae* strains in mixed starter cultures tailored to reflect the biodiversity of a

given region. Extensive ecological surveys using molecular methods of identification have been carried out with the aim of selecting new yeasts better adapted to local fermentation conditions (Pretorius *et al.*, 1999; Khan *et al.*, 2000; van der Westhuizen *et al.*, 2000a). These and other publications (Versavaud *et al.*, 1995; Lopes *et al.*, 2002) report a great diversity of genetic patterns among the enological fermentative microbial communities. *Saccharomyces cerevisiae* strains seem to be widely distributed in a given viticultural region, and they can be found in consecutive years (Vézinhet *et al.*, 1992; Torija *et al.*, 2001); there are also strains predominant in fermenting microbial communities (Frezier & Dubourdieu, 1992; Sabate *et al.*, 1998), suggesting the occurrence of specific native strains that can be associated with a *terroir*.

Preserving biodiversity is also important in order to ensure the conservation of gene pools of technological importance. With regard to this, several studies have been performed with the aim of assessing the impact of winemaking practices – including the extensive use of active dried yeast – on the natural microbial community. Monoculture practice was described as having a negative effect on the biodiversity of non-*Saccharomyces* wine yeast in a wine-producing region of Chile (Ganga & Martínez, 2004). Our results from a large-scale study in two different wine-producing areas, the Vinho Verde region in the north of Portugal and the Languedoc region in the south of France, show that dissemination of commercial yeast in the vineyard is restricted to short distances and limited periods of time, and that they do not become implanted systematically in the ecosystem (Valero *et al.*, 2005).

Against this background, the present study was performed with two aims: first, to examine *S. cerevisiae* biodiversity and its natural population dynamics over a 3-year period in the vineyards surrounding wineries where active dry yeasts were used as fermentation starters; and second, to establish a strain collection contributing to the preservation of *S. cerevisiae* genetic resources. The results from the Portuguese winemaking area were published recently by Schuller *et al.* (2005), and the present article gives the results from the French winemaking region.

Materials and methods

Sampling plan and fermentation

Grapes were harvested in three vineyards (A, B and C) around a winery, located in the Languedoc region, around the Mediterranean city of Montpellier. The vineyards were situated at distances of 30 and 80 km apart. In each vineyard, six sampling points were defined according to the predominating wind direction at a distance of between 100 and 1000 m from the winery, as shown in Fig. 1.

In order to evaluate the diversity among fermentative yeast communities during the last stage of grape maturation and harvest, two sampling campaigns were performed, before (early campaign) and after (later campaign) harvest. The gap between the two campaigns was about 10 days. This study was carried out over a period of 3 consecutive years (2001–2003); samples were always collected from the same area at a maximum radius of 5 m. With the present experimental design, 36 grape samples were collected each year. The grape variety was always Carignan, with the exception of the sample point situated closest to the north of the winery, where it was Mourvèdre in vineyard A, Cabernet in vineyard B and Merlot in vineyard C.

Approximately 2 kg of grapes, including the stems, were harvested in aseptic conditions from each sampling point and placed directly into sterile plastic bags, which were transported to the laboratory in cool bags. At the laboratory, grapes were crushed by hand in the plastic bags; these were then opened, and 180 mL of juice was poured into 250-mL sterile fermenters. The fermenters were placed in a temperature-controlled room at 20 °C with mechanical agitation. Fermentation progress was monitored daily by weight determinations.

Yeast isolation

The yeast community present in the fermentation was evaluated when the must weight was reduced by 70 g L^{-1}, corresponding to the consumption of about two-thirds of the sugar content. Must samples were diluted and spread on plates with YEPD medium (yeast extract 1% w/v, peptone 1% w/v, glucose 2% w/v, agar 2% w/v), and incubated for 48 h, after which 30 colonies selected at random were collected from each spontaneous fermentation.

Selection of *Saccharomyces* and molecular identification

To rapidly discriminate between *Saccharomyces* and non-*Saccharomyces*, every isolate was evaluated according to its ability to grow in a medium containing L-lysine as the sole nitrogen source (Barnett *et al.*, 1990). The *Saccharomyces* strains not able to grow on L-lysine medium were further identified by pulsed-field gel electrophoresis (PFGE). To establish chromosomal profiles, yeast chromosomal DNA was prepared in plugs and analysed using the TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman), as previously described (Blondin & Vézinhet, 1988). The gels were run for 6 h at 250 V with a 35 s pulse time, and then for 20 h at 275 V with a 55 s pulse time, at a constant temperature (14 °C). Designations for observed distinct patterns were A1–A5, B1–B25 and C1–C77, corresponding to isolates from vineyards A, B and C, respectively. Identification of commercial yeasts was carried out by comparison of chromosomal patterns of 23 commercial yeasts used in the wineries and the

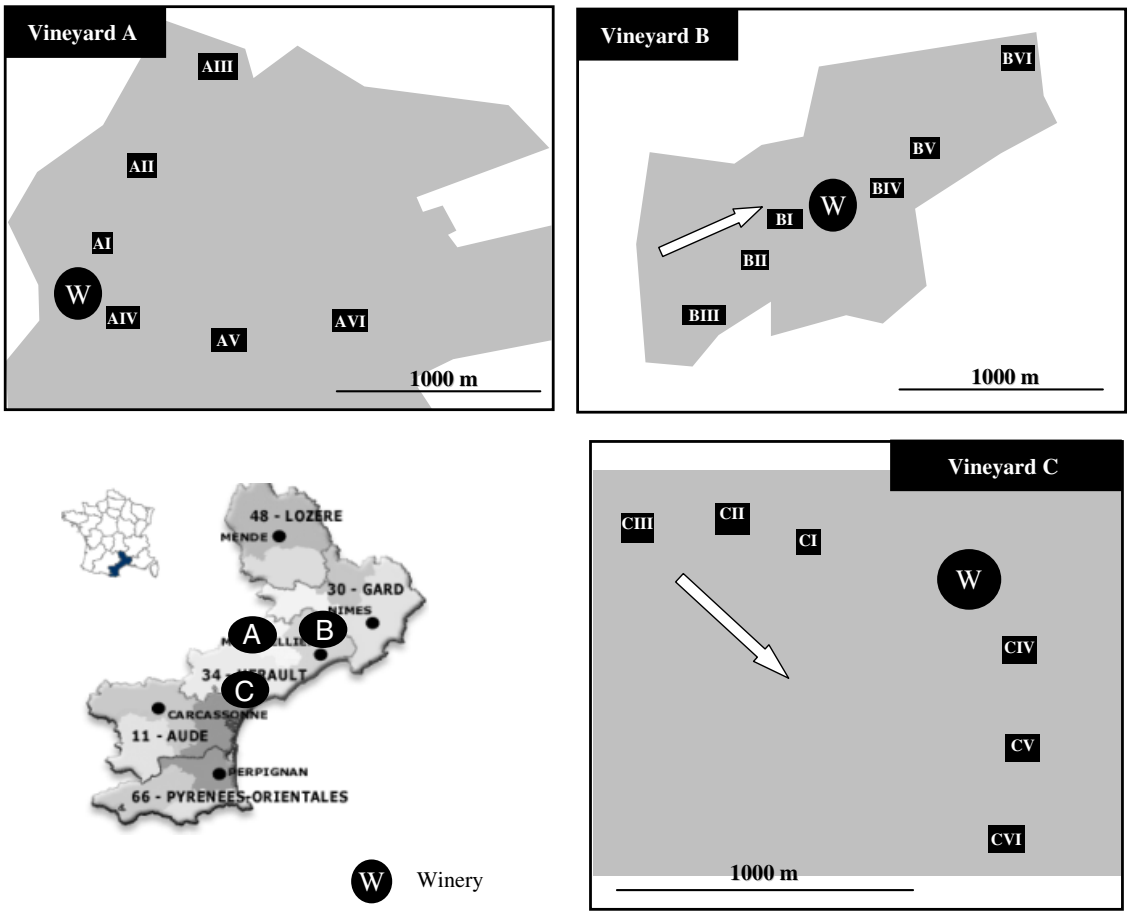

Fig. 1. Geographical localization of the vineyards (A, B and C) in the Languedoc wine region of France, with an indication of the wineries and the sampling sites AI-AVI, BI-BVI and CI-CVI.

different *Saccharomyces* strain isolates (Valero *et al.*, 2005). Some examples of chromosomal patterns of the *Saccharomyces* strains isolated are shown in Fig. 2.

Differentiation between the indigenous *Saccharomyces sensu stricto* strains isolated was performed by PCR restriction fragment length polymorphism (PCR-RFLP) analyses of the internal transcribed spacer (ITS1) region of the 18S rRNA gene. The ITS1 region was amplified with the NS1/ITS2 primer pair, and the PCR products were digested with *Hae*III and *Msp*I restriction endonucleases and separated by electrophoresis as described by Redzepovic *et al.* (2002).

Results

Three vineyards (A, B and C) in the Languedoc region (south of France) were selected to study the evolution of *Saccharomyces* strain populations over a period of three harvest seasons (2001–2003). Two sampling campaigns were performed, one before and the other after the harvest, to evaluate in greater detail the fermenting yeast temporal distribution. In total, 106 grape samples were collected, of which 72 completed spontaneous fermentations. From these fermentations, 2160 colonies were isolated.

A large proportion of non-*Saccharomyces* strains was found in the isolates after fermentation, representing 72% of the total yeasts isolated over the 3 years. Analysis of 79 non-*Saccharomyces* isolates from the four fastest fermentations, by PCR-RFLP of the rRNA gene ITS region (Granchi *et al.*, 1999), showed that these strains mainly belonged to the genus *Kloeckera* (data not shown). It is noteworthy that 2002 was an atypical year, owing to heavy rainfall (50% above normal) before and during the harvest, resulting in a greater application of antifungal sprays, which may explain the reduced number of *Saccharomyces* isolates (12%).

Based on the L-lysine method (Barnett *et al.*, 1990), 608 *Saccharomyces* strains were selected from the 2160 isolates collected during the 3 years. These strains are not distributed in the same way, in terms of either space or time; 323 *Saccharomyces* strains were isolated in vineyard C, 194 in vineyard B, and only 91 in vineyard A. The same

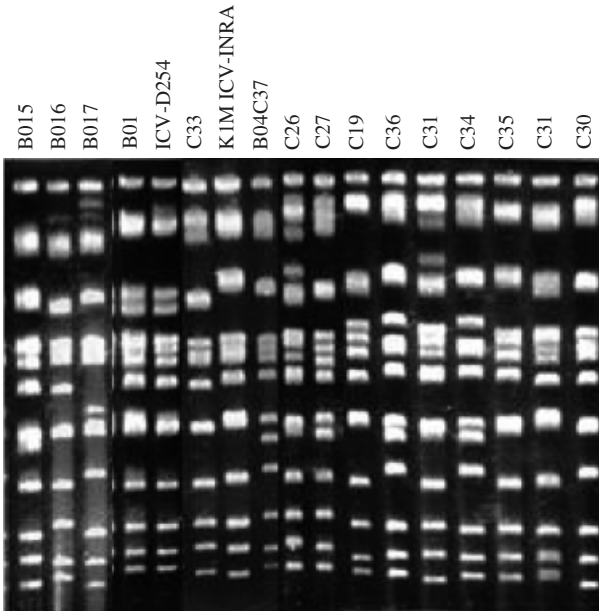

Fig. 2. Examples of chromosomal profiles of commercial yeast and natural isolates of spontaneous fermentations. Profile B01 was identical to ICV-D254.

phenomenon occurred with the different harvests; the largest proportions (50%) of *Saccharomyces* strains were found in 2001 and 2003 (50% and 46%, respectively), and the number was 10 times smaller in 2002 (5.4%).

Molecular identification of the *Saccharomyces* strains by PFGE revealed a total of 104 different chromosomal profiles (Table 1). A large majority of chromosomal profiles (91) were found as unique patterns, and only 13 karyotypes were found in more than one fermentation.

Concerning the geographical distribution of repeated patterns, only two (B04C37 and B23C09) were found in different vineyards (B and C) and 11 in different sites at the same vineyard. Repeated patterns in a single vineyard were always found in vineyard C, with the exception of pattern B01, corresponding to the chromosomal profile of the commercial yeast ICV-D254, found in two different sites in vineyard B in the early campaign in 2001. In vineyard C, patterns C23 and C26 were found in different sites, in the early campaign only, and C33 and C36 in the later campaign, both in 2001. C05 and C19 were found in the early and later campaign in the same years, but in different sampling sites. In 2003, three repeated patterns were found only in the later campaign (C62, C67 and C71) and one other (C18) was found in two fermentations, one in the early campaign in 2001 and the other in the later campaign in 2003. Pattern B23C09 was found in two vineyards, in the early campaigns of 2001 in vineyard C, and in the later campaign of 2003 in vineyard B. The chromosomal profile most widely distributed was B04C37; this was found in five fermentations from two different sites in vineyards B and C in 2001 and in one site in vineyard C in 2003, always in the later campaign.

As mentioned previously, the first sampling campaign was performed some days before the harvest, and the second a few days after the harvest, in a time frame of about 10 days. This study revealed a succession of *Saccharomyces* strains, given that the patterns of autochthonous strains from the early campaign never appeared in the later campaign. Nevertheless, it is possible that some differences can be attributed to the fact that different grape bunches were collected. Although these were situated close together, their microbial communities may have varied. In contrast to the results obtained in the Vinho Verde region of Portugal (Schuller *et al.*, 2005), where spontaneous fermentation was verified rarely from grapes collected some days before the harvest, in our study, 54% of grape samples collected in the early campaign were able to ferment spontaneously compared to 83% of postharvest samples. The numbers of *Saccharomyces* strains collected were 173 and 436, in the early stage and late stage, respectively, a result that, according to Schuller *et al.* (2005), shows that the last stage of grape maturation appears to favour fermentative yeast proliferation on the grape surface.

The fermentation profiles of 72 grape samples that completed spontaneous fermentation are shown in Fig. 3. Whereas the Portuguese results (Schuller *et al.*, 2005) show that only *Saccharomyces* strains were isolated after fermentation, in French wineries many non-*Saccharomyces* strains were involved in the autochthonous fermentations. Fifty-eight percent of fermentations were exclusively carried out by non-*Saccharomyces* strains; the large majority of these fermentations were produced from grape samples collected in 2002.

Fermentations in which *Saccharomyces* strains participated were generally accomplished by a mix of *Saccharomyces* and non-*Saccharomyces* strains in different proportions, varying between 3% and 100% of *Saccharomyces* strains. These strains dominated in 20 fermentations, but only five of these were carried out exclusively by *Saccharomyces* strains. Spontaneous fermentations, mixed or not, were generally carried out by one to 20 *Saccharomyces* strains, with a predominance of one or more strains accompanied by a few or many minority strains, or by a very heterogeneous yeast community with no prevalent strain(s). Studies describing both situations have also been published (Khan *et al.*, 2000; van der Westhuizen *et al.*, 2000a, b).

As can be seen in Fig. 3, the greatest number of strains were involved in fermentations of must from grapes collected in 2001 from vineyard C. Grape samples from vineyard A produced a lower number of spontaneous fermentations, only five in the 3 years studied, all accomplished by only one *S. cerevisiae* strain, which was always

Table 1. Chromosomal patterns of 608 yeast isolates from spontaneous fermentations of collected grape samples from vineyards A, B and C, during the harvest of 2001–2003

Site	Number of isolates	Number of *Saccharomyces* strains	Number of distinct patterns	Number of total patterns	Common patterns
Vineyard A					
2001					
E					
AI	NF	–	–	–	–
AII	30	0	–	–	–
AIII AIV AV	NF	–	–	–	–
AVI					
L					
AI	30	26	1	2	–
AII AIII	NF	–	–		–
AIV	30	30	1		–
AV	30	0	–		–
AVI	NF	–	–		–
2002					
E					
AI	30	0	–	–	–
AII	NF	–	–	–	–
AIII AIV AV	120	0	–	–	–
AVI					
L					
AI AII AIII AIV	120	0	–	1	–
AV	NF	–	–		–
AVI	30	1	1		–
2003					
E					
AI	NF	–	–	1	–
AII	30	30	1		–
AIII AIV AV	NF	–	–		–
AVI	30	0	–		–
L					
AI	30	0	–	1	–
AII AIII	NF	–	–		–
AIV	30	4	1		–
AV	30	0	–		–
AVI	NF	–	–		–
Vineyard B					
2001					
E					
BI BII	NF		–	1	–
BIII	30	10	1		B01
BIV	30	1	–		–
BV	30	5	1		B01
BVI	NF	–	–		–
L					
BI	30	28	2	17	–
BII	30	29	1		B04C37
BIII	30	1	1		B04C37
BIV	30	29	14		–
BV BVI	60	0	–		–
2002					
E					
BI BII BIII BIV	150	0	–	–	–
BV					
BVI	NF	–	–	–	–
L					
BI	30	0	–	2	–
BII	30	14	1		–

Table 1. Continued.

Site	Number of isolates	Number of *Saccharomyces* strains	Number of distinct patterns	Number of total patterns	Common patterns
BIII BIV	60	0	–		–
BV	30	22	1		–
BVI	30	0	–		–
2003					
E					
BI BII	NF	–	–	–	–
BIII	30	0	–	–	–
BIV BV BVI	NF	–	–	–	–
L					
BI	NF	–	–	5	
BII	30	0	–		
BIII	30	27	2		
BIV	30	26	3		B23C09
BV	NF	–	–		
BVI					

Site	Number of isolates	Number of *Saccharomyces* strains	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard C					
2001					
E					
CI	30	28	1	27	
CII	NF	–	–		
CIII	30	24	20		C05 B23C09 C18 C19
CIV	30	20	5		C23 C26
CV	30	28	6		
CVI	30	0	–		
L					
CI	30	0	–	24	
CII	30	30	1		C19 C33 C36 B04C37
CIII	30	12	4		
CIV	30	17	10		C33
CV	30	28	2		B04C37
CVI	30	14	12		C05 C19 C36
2002					
E					
CI CII CIII CIV CV CVI	180	0	–	–	–
L					
CI	30	2	1	1	–
CII CIII CIV					
CV CVI	150	0	–		–
2003					
E					
CI CII	NF	–	–	1	–
CIII	30	27	1		–
CIV	NC	–	–		–
CV CVI	NF	–	–		–
L					
CI	30	30	1	20	C62
CII	30	28	8		C67
CIII	30	9	5		C18 C71
CIV	NC	–	–		
CV	30	27	7		B04C37 C62 C67 C71
CVI	30	0	–		

E, early campaign; L, later campaign; NF, not finished; NC, not collected.

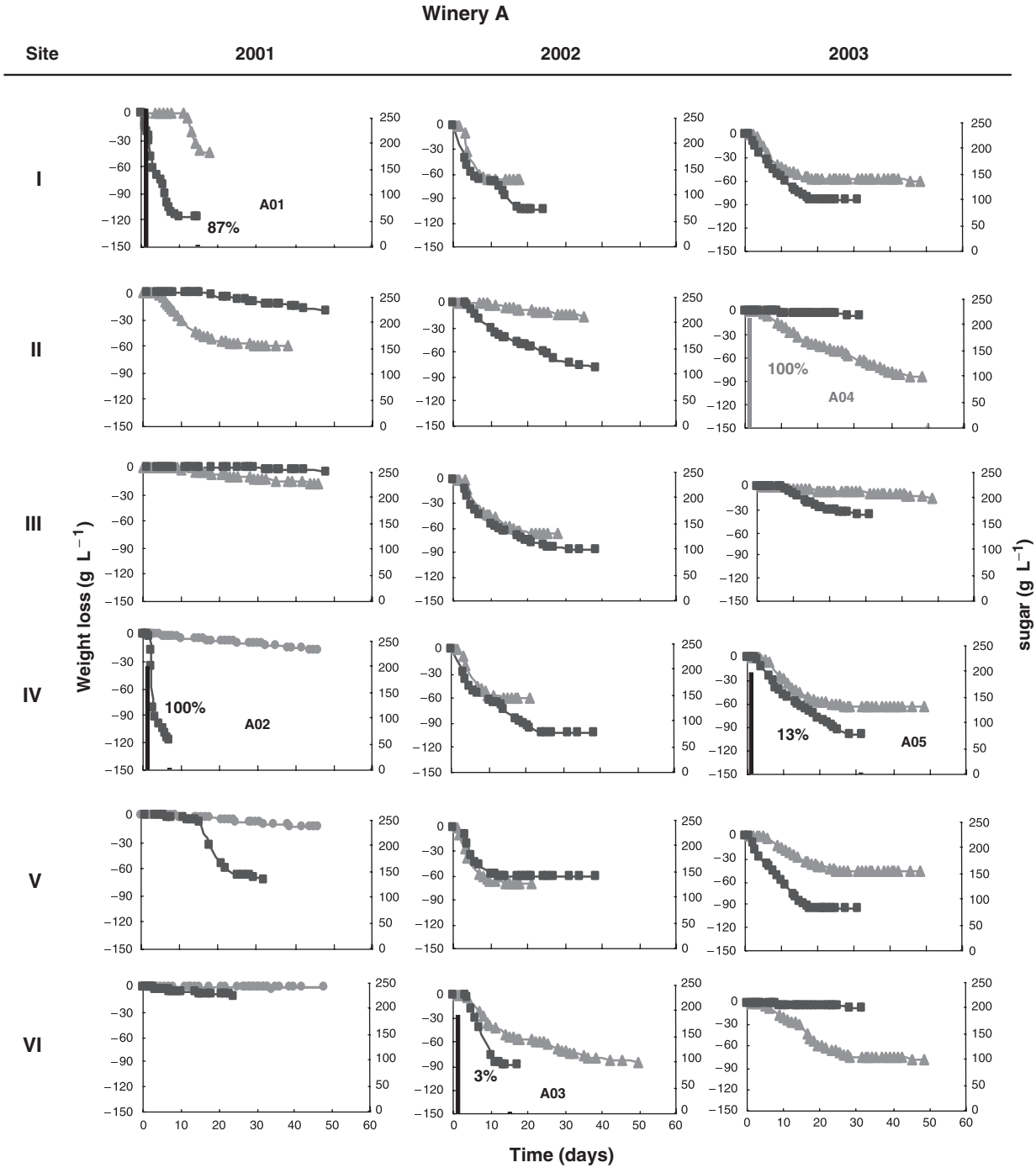

Fig. 3. Fermentation profiles of must samples collected in the early (grey) and late (black) sampling campaigns. Chromosomal patterns of strains isolated from the spontaneous fermentations are indicated. The predominating strains are underlined. Repeated patterns are highlighted in grey. Initial and residual sugar in spontaneous fermentations with *Saccharomyces* strains are indicated by bars.

different. Two of these represented 100% of the yeasts isolated. Grape samples from vineyard B produced 10 spontaneous fermentations, of which six were carried out by only one *Saccharomyces* strain and four by two to 14 strains. Of 15 spontaneous fermentations produced from grapes collected in vineyard C, only four were carried out by a single strain and 11 by more than one strain, varying between two and 20.

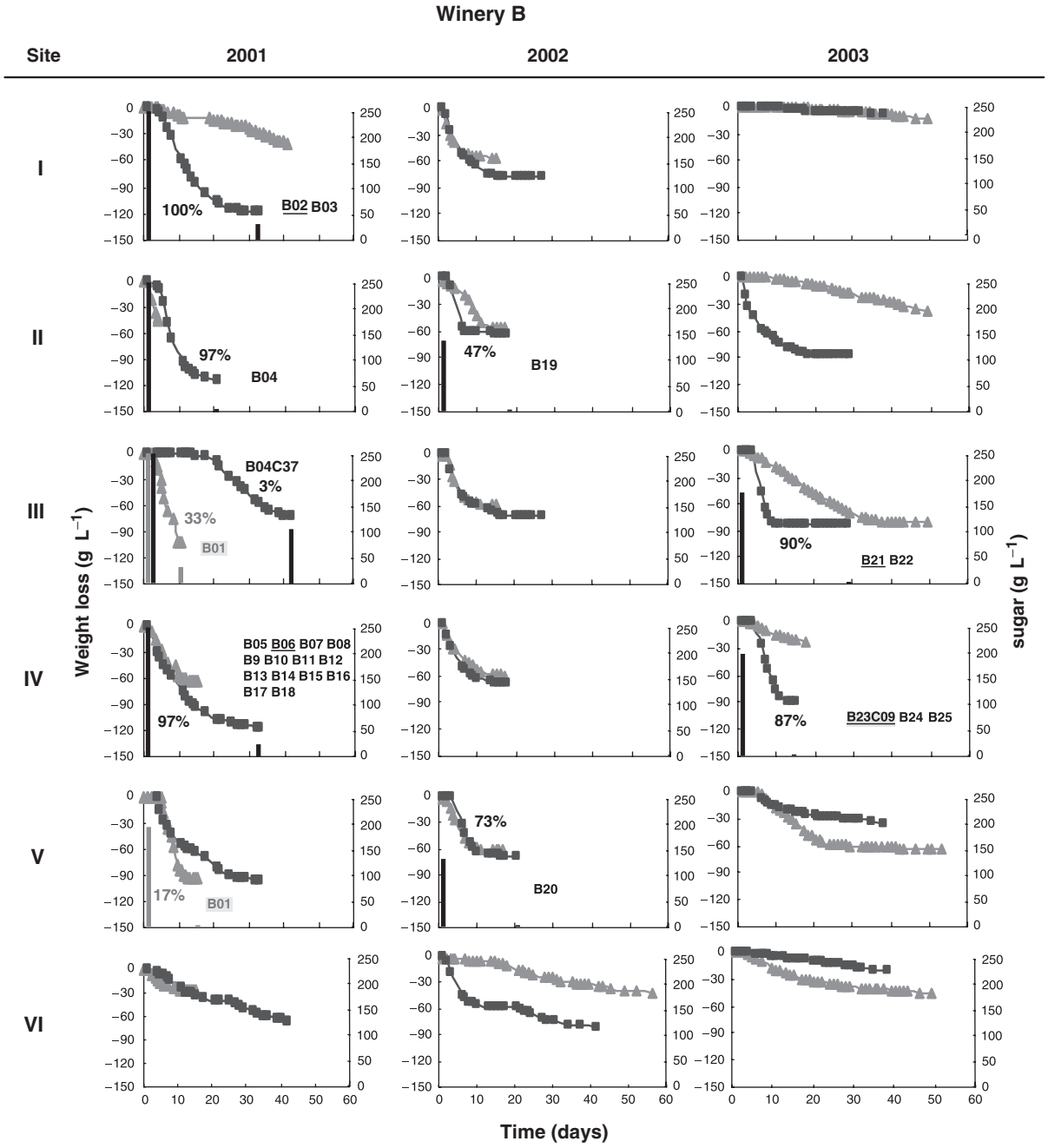

Fig. 3. Continued.

If we consider the number of *Saccharomyces* strains involved in spontaneous fermentation by years, we find that 78 different *Saccharomyces* strains were involved in 18 fermentations of grape samples collected in 2001, four different strains in four fermentations performed in 2002, and 22 strains in seven fermentations from grapes collected in 2003. In addition, the later campaign resulted in a greater number of spontaneous fermentations involving a greater number of *Saccharomyces* strains.

It is important to point out that the distribution of strains is not associated with the capacity to predominate in fermentation. The most widely distributed strain (B04C37) was involved in five fermentations and dominated in only two of these (BII-2001 and CV-2001), being a minority strain in the others (BIII-2001, CII-2001 and CV-2003). In the latter case, this strain accounted for only 3–20% (one to six strains) and was accompanied by one to seven other strains. Commercial yeasts were only found in three fermentations.

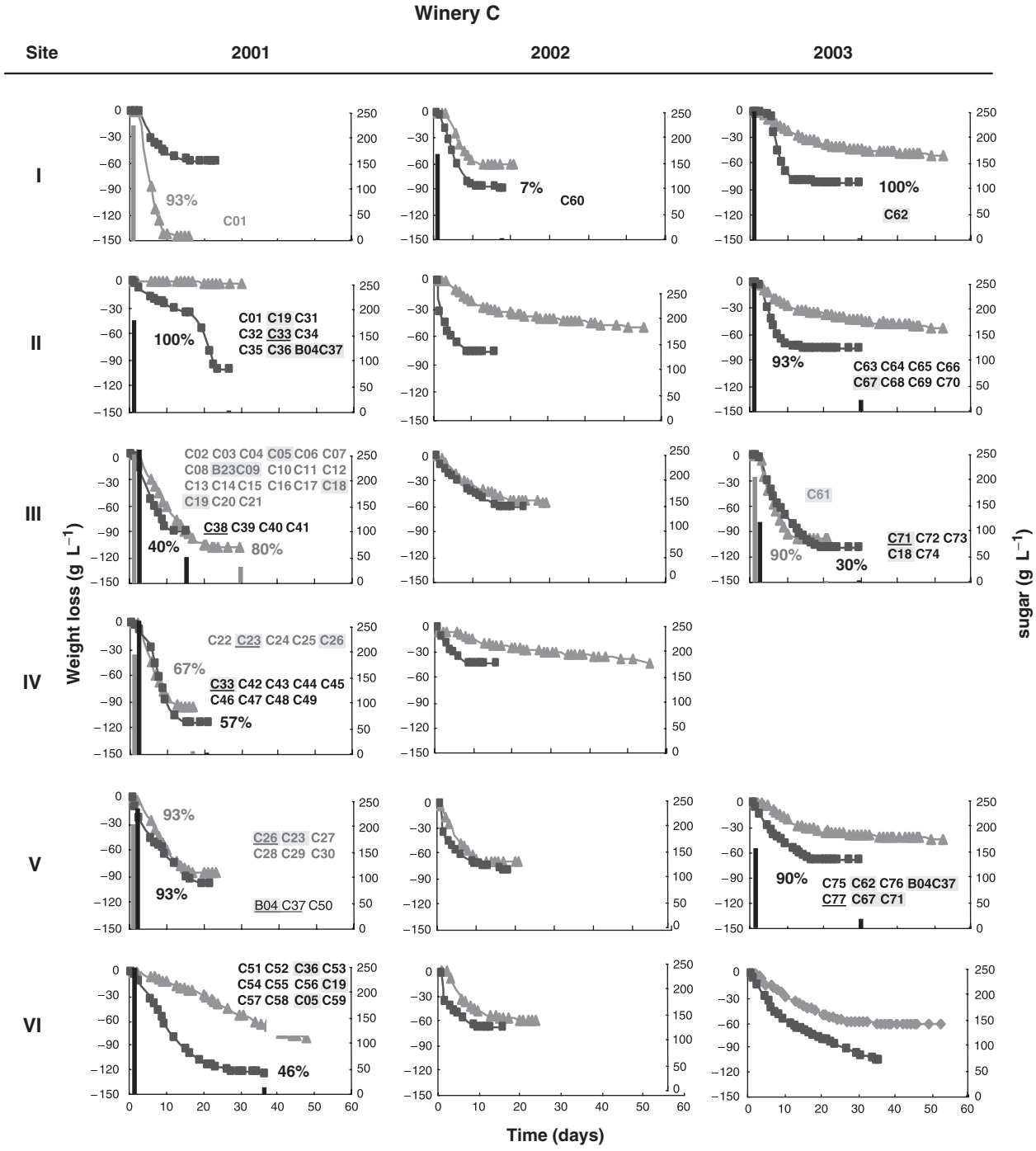

Fig. 3. Continued.

Chromosomal pattern B01 was identical to that of commercial yeast ICV D254, initially isolated in this region, and was found in two fermentations (BIII-2001 and BV-2001) in the early campaign. Pattern C72 was identical to that of K1M-ICV INRA, found in fermentation CIII-2003 in the later campaign. These three fermentations were mixed *Saccharomyces* and non-*Saccharomyces* and did not dominate the fermentations in any case. Whereas ICV D254 was the only *Saccharomyces* strain found in these fermentations, K1M-ICV INRA was accompanied by another four *Saccharomyces* strains, the majority strain being pattern C71 (Fig. 3).

After the surprising observation that *S. paradoxus*, normally associated with oak species (*Quercus robur* or *Quercus mongolica*) in Europe, the Far East and North America

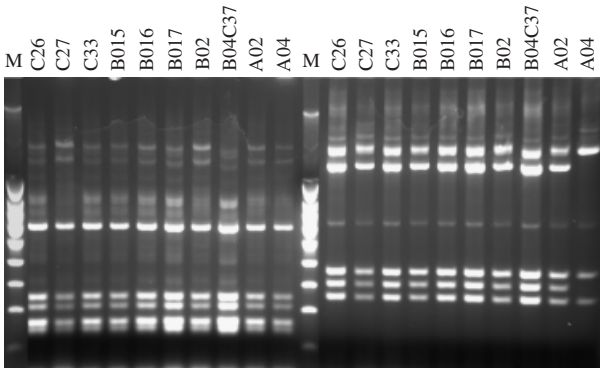

Fig. 4. Examples of PCR-RFLP patterns of the ITS1 region of some *Saccharomyces* strains isolated from spontaneous fermentations. Pattern A04 was identified as *Saccharomyces paradoxus* and the others as *Saccharomyces cerevisiae*.

(Naumov *et al.*, 1992, 1998), appeared to occur in far greater numbers than *S. cerevisiae* in the indigenous population of *Saccharomyces sensu stricto* in Croatian vineyards, we wanted to investigate whether this was a particular case or could occur in the Languedoc region of the south of France. The analysis of the 104 indigenous strains with different karyotypes isolated in this region by PCR-RFLP analyses of the ITS1 region of the 18S rRNA gene (Redzepovic *et al.*, 2002) indicated that only one (pattern A04) of the 104 strains with different chromosomal profiles was *S. paradoxus* (Fig. 4). This strain was found in the later campaign of 2003, in vineyard A, and completely dominated the fermentation, as can be seen in Fig. 3 (AII-2003). This strain exhibited a bad fermentation performance compared with the majority of *S. cerevisiae* strains, taking more than 45 days to complete the fermentation.

Discussion

It is well known that grape yeast communities vary from area to area and from vintage to vintage (Frezier & Dubourdieu, 1992; Vézinhet *et al.*, 1992; Schütz & Gafner, 1994). Several of these studies have been carried out in wineries of different regions of France, from spontaneous fermentations. Although these studies gave interesting conclusions, a larger-scale study of grape-associated yeast in the vineyards was necessary, in order both to evaluate the biodiversity and natural dynamics of autochthonous populations of *Saccharomyces* and to evaluate the impact of the use of commercial selected yeasts on biodiversity.

In the present study, 104 different chromosomal patterns were found among 608 *Saccharomyces* strains selected from 2160 isolates obtained from three different vineyards in the Languedoc region, over a 3-year period. This same study was carried out in the Vinho Verde region in the north of Portugal, published recently by Schuller *et al.* (2005). Important differences were observed between the two studies with regard to the proportion of *Saccharomyces* strains found in the isolates after fermentation. In Portugal, mtDNA RFLP (*Hin*fI) patterns of all the isolates after fermentation showed a *Saccharomyces*-type profile, whereas in France a large proportion of non-*Saccharomyces* strains were found (Fig. 2). The non-*Saccharomyces* strains represented 66% of the total yeasts isolated over the 3 years. These data confirm previous reports indicating that *S. cerevisiae* is not present in large numbers in vineyards (Pretorius, 2000). The majority of non-*Saccharomyces* strains were isolated in 2002, probably due to heavier than usual rainfall. As previously described (Longo *et al.*, 1991; Angulo *et al.* 1993; Ganga & Martínez, 2004), these conditions both produced musts with lower sugar content and slower fermentations and made it necessary to increase the antifungal treatment of the vines, which may at least in part explain the decrease in *S. cerevisiae* strains during the fermentation. Nevertheless, some fermentations carried out exclusively by non-*Saccharomyces* strains were also able to complete the fermentation (e.g. AI-2002 or AIV-2002 in postharvest campaigns), producing 10–11% (v/v) of ethanol. This fact was previously observed by Torija *et al.* (2001), who showed the presence of non-*Saccharomyces* strains in fermentation stages with a high ethanol content. However, in practice the impact of non-*Saccharomyces* strains would be less, as a result of the addition of SO_2 to industrial fermentations in wineries.

The methodology used, based on analysis of the yeast community after spontaneous fermentation, permitted the selective isolation of *Saccharomyces* wine yeasts, which do not appear on the grapes in great abundance. As a contribution to the still vigorous debate about the origin of wine yeast (Vaughan-Martini & Martini, 1995; Martini *et al.*, 1996; Mortimer & Polsinelli, 1999; Martini, 2003), our results indicate the presence of a sufficient number of *S. cerevisiae* strains in the vineyard to carry out a spontaneous fermentation if the sample size permits, as proposed by van der Westhuizen *et al.* (2000b). It should be noted that among the 30 colonies analysed per fermentation, an average of about four different *Saccharomyces* biotypes per sample was observed, varying between one and 21 different biotypes. This indicates that the number of colonies analysed per sample was high enough to reflect the initial biodiversity. Nevertheless, our data refer only to yeast strains capable of surviving the conditions imposed by fermentation, and therefore give a distorted picture (underestimation) of the kinds of strain that really occur in vine. However, we regard our approach as an acceptable compromise that allows good estimation of population composition, but no precise description in terms of relative strain abundance in nature is possible.

Differentiation between the four species of the *Saccharomyces sensu stricto* group (*S. cerevisiae*, *S. bayanus*, *S. pasteurianus* and *S. paradoxus*) of the *Saccharomyces* strains isolated in the Languedoc region in France indicated that all belonged to the species *S. cerevisiae*, except for one strain of *S. paradoxus*. The previously reported large distribution of *S. paradoxus* in Croatian vineyards (Redzepovic *et al.*, 2002) is a particular case, not generalizable to other winemaking areas such as that analysed in this study, supporting the idea suggested by the authors of the presence of specific indigenous yeasts that are better adapted to a specific grape-growing area.

The large majority of the 104 chromosomal patterns of *S. cerevisiae* strains identified were unique, demonstrating an enormous biodiversity of indigenous *S. cerevisiae* strains in this region of France. Considering the ratio between the number of *Saccharomyces* isolates and the number of patterns as an approximate biodiversity estimation, our overall results (about six strains per pattern) showed similar values to those found in Portugal by Schuller *et al.* (2005) and in previously published studies on the genetic diversity of indigenous *S. cerevisiae* strains in other viticultural regions of France (Vézinhet *et al.*, 1992; Versavaud *et al.*, 1995). In our study, this general estimation includes different situations, in contrast to the Portuguese results, where no apparent correlation between the number of strains involved in a fermentation and sampling site, year or vineyard was found (Schuller *et al.*, 2005). If we make an estimation per vineyard, we find that the biodiversity was significantly greater in vineyard C, where only four strains per chromosomal pattern were found, and estimated biodiversity was much less in vineyard A, where 18 isolates of *Saccharomyces* per karyotype were found. The value for vineyard B was eight. We could not consider the influence of the grape variety in this study, given that Carignan was principally used in the three vineyards, with the exception of one sample per vineyard, corresponding to fermentations AI, BI and CI from the grape varieties Mourvedre, Cabernet and Merlot, respectively, for vineyards A, B and C. As the three vineyards are geographically close, they are included in the same climatic zone, meaning that intra-annual differences in terms of greater or lesser biodiversity of autochthonous *S. cerevisiae* strains per vineyard must be attributed to specific factors associated with the vineyard, such as the age and size (Pretorius *et al.*, 1999), which would have a positive effect on the biodiversity of *S. cerevisiae* strains. As a reference, winery C, where the greatest biodiversity was found, was established in 1937 and is the largest winemaking area in the region, as well as one of the largest in Europe, with 2250 ha of vineyard, whereas winery A, where biodiversity was less, was established in 1951 and has *c.* 700 ha.

We also observed important differences when estimating biodiversity per year; whereas in 2001 the number of strains per karyotype was five, in 2002 it had doubled, and in 2003 it was in between (seven). The strong decrease in the biodiversity of *S. cerevisiae* strains in 2002, in accordance with the observations of other authors (Longo *et al.*, 1991; Angulo *et al.*, 1993), was probably due, as we mentioned previously, to the particular climatic conditions of this year. An increase in the biodiversity of *S. cerevisiae* strains was observed when weather conditions returned to normal in 2003. Further studies could be designed in order to explore each of these factors in greater depth.

The yeast community of each year was characterized by the appearance of many new patterns, indicating the fact that the behaviour of the large majority of the strains was not perennial. This may be attributable to the fact that only 12×2 kg of grapes per vineyard and year were sampled, and this may have been insufficient to detect the entire biodiversity of the given area. The last stage of grape maturation appears to favour fermentative yeast proliferation on the grape. This is due to damage to the grape skin, and leakage of must from the berries, attracting insects, which are the probable source of yeast on these grapes. A first sampling campaign was performed some days before the harvest, and a second a few days after the end of the harvest, in a time frame of 10 days, in order to assess the temporal distribution of fermenting yeast populations during the harvest. According to Rosini *et al.* (1982), only 5% of the grapes collected before vintage contain yeast, this number being much higher (60%) during vintage. Our results show that before vintage, 40% of samples were able to ferment spontaneously, although only 11% contained *S. cerevisiae* strains, compared to 60%, of which 30% contained *S. cerevisiae* strains, in postharvest samples. The estimated biodiversity of associated strains in the early and later campaign was five and six strains per chromosomal pattern, respectively; therefore, the biodiversity of grape-associated yeast in the later campaign did not seem to increase significantly in our studied area, in contrast to the results from the Vinho Verde region of Portugal (Schuller *et al.*, 2005). Furthermore, as occurred in the Portuguese study, autochthonous strain patterns from the early campaign did not appear in the later sampling campaign, showing a temporal succession of *S. cerevisiae* strains.

With respect to the impact of the utilization of commercial yeast as a fermentation starter in the wineries, our study appears to show that the biodiversity of autochthonous species of *S. cerevisiae* remains very close to that reported in other studies, including fermentations in wineries where no commercial wine yeast strains have been used (Frezier & Dubourdieu, 1992; Vézinhet *et al.*, 1992; Versavaud *et al.*, 1995; Sabate *et al.*, 1998; Torija *et al.*, 2001). Furthermore, the fact that we found very different levels of biodiversity in the three vineyards studied (A, B and C) around the wineries that had utilized commercial yeast in large quantities for a

long time verifies that other factors were more important than commercial yeast utilization for the biodiversity of the vineyard. This is because dissemination of commercial yeast in the vineyard surrounding the winery was almost completely absent (Valero *et al.*, 2005). Only two chromosomal patterns identical to that of commercial yeasts were found: B01, which corresponds to the profile of *S. cerevisiae* strain ICV D254, found in vineyard B; and C72, which corresponds to the profile of *S. cerevisiae* strain K1M ICV-INRA, in vineyard C. This fact could be an indication of previous dissemination, but this cannot be confirmed, as strain ICV D254 was initially isolated from the same region of the south of France where the study was carried out. No commercial yeasts were found from winery A, and one colony, isolated in 2003 in winery C, had the same profile as K1M ICV-INRA, used in all three French wineries for the last 5–15 years. Furthermore, no implantation in the fermentation was produced, as the presence of indigenous strains was not affected, and only one isolate corresponding to this profile was found, accompanied by non-*Saccharomyces* and another four *S. cerevisiae* strains.

Spontaneous fermentations, mixed or not, were generally carried out by one to 20 *Saccharomyces* strains. This is in agreement with other studies reporting the presence of one or two predominating strains, and a varying number of 'secondary' strains (Querol *et al.*, 1992a, b; Schütz & Gafner, 1993; Versavaud *et al.*, 1995; Constanti *et al.*, 1997; Lopes *et al.*, 2002), or the presence of many different strains with no prevalence (Sabate *et al.*, 1998; Pramateftaki *et al.*, 2000). The occurrence of both situations has also been described (Khan *et al.*, 2000; van der Westhuizen *et al.*, 2000a, b). The most widely distributed strain in this study (B04C37) did not show a perennial appearance or wider geographical distribution, as it was involved in only five fermentations, four of which were in 2001. For this reason, we cannot conclude that any one strain can be considered as a *terroir* yeast.

The present work, together with that carried out in Portugal (Schuller *et al.*, 2005), is a large-scale survey of vineyard-associated strains performed in order to obtain a better understanding of the ecology of *S. cerevisiae* strains. We consider that these studies give interesting conclusions, allowing improved determination of factors influencing the biodiversity of indigenous populations of wine yeast. Studies of this nature are indispensable for the preservation of biodiversity and genetic resources, and as a basis for further biotechnological applications.

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References

Angulo L, López E & Lema C (1993) Microflora present in kefir grains of the Galician region (north-west of Spain). *J Dairy Res* **60**: 263–267.

Barnett JA, Payne RW & Yarrow D (1990) *Yeast Characteristics and Identification*. Cambridge University Press, Cambridge, MA.

Blondin B & Vézinhet F (1988) Identification de souches de levures oenologiques par leurs caryotypes obtenus en électrophorèse en champs pulsée. *Rev Fr Oenol* **28**: 7–11.

Constanti M, Poblet M, Arola L, Mas A & Guillamon JM (1997) Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am J Enol Vitic* **48**: 339–344.

de Barros LM, Soden A, Martens AL, Henschke PA & Langridge P (1998) Differentiation and species identification of yeasts using PCR. *Int J Syst Bacteriol* **48**: 279–286.

Fleet GH & Heard GM (1993) Yeasts: growth during fermentation. *Wine Microbiol Biotechnol* (Fleet GH, ed.), pp. 27–75. Harwood Academic Publishers, Chur, Switzerland.

Frezier V & Dubourdieu D (1992) Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. *Am J Enol Vitic* **43**: 375–380.

Ganga MA & Martínez C (2003) Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *J Appl Microbiol* **96**: 76–83.

Granchi L, Bosco M, Messini A & Vicenzini M (1999) Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J Appl Microbiol* **87**: 949–956.

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. *S Afr J Enol Vitic* **21**: 17–31.

Longo E, Cansado J, Agrelo D & Villa TG (1991) Effect of climatic conditions on yeast diversity in grape musts from northwest Spain. *Am J Enol Vitic* **42**: 141–144.

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. *J Appl Microbiol* **93**: 608–615.

Martini A (2003) Biotechnology of natural and winery-associated strains of *Saccharomyces cerevisiae*. *Int Microbiol* **6**: 207–209.

Martini A & Vaughan-Martini A (1990) Grape must fermentation: past and present. *Yeast Technology* (Spencer JFT & Spencer DM, eds), pp. 105–123. Springer-Verlag, Berlin, Germany.

Martini A, Ciani M & Scorzetti G (1996) Direct enumeration and isolation of wine yeasts from grape surfaces. *Am J Enol Vitic* **47**: 435–440.

Mortimer R & Polsinelli M (1999) On the origins of wine yeast. *Res Microbiol* **150**: 199–204.

Naumov G, Naumova E & Korhola M (1992) Genetic identification of natural *Saccharomyces* sensu stricto yeasts from Finland, Holland and Slovakia. *Antonie van Leeuwenhoek* **61**: 237–243.

Naumov GI, Naumova ES & Sniegowski PD (1998) *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* are associated with exudates of North American oaks. *Can J Microbiol* **44**: 1045–1050.

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. *J Appl Microbiol* **89**: 236–248.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16**: 675–729.

Pretorius IS, van der Westhuizen TJ & Augustyn OHP (1999) Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *S Afr J Enol Vitic* **20**: 61–74.

Querol A, Barrio E, Huerta T & Ramon D (1992a) Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl Environ Microbiol* **58**: 2948–2953.

Querol A, Huerta T, Barrio E & Ramon D (1992b) Dry yeast strain for use in fermentation of Alicante wines – selection and DNA patterns. *J Food Sci* **57**: 183–1987.

Rankine BC (1968) Formation of α-ketoglutaric acid by wine yeasts and its oenological significance. *J Sci Food Agr* **19**: 624–629.

Redzepovic S, Orlic S, Sikora S, Majdak A & Pretorius IS (2002) Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. *Lett Appl Microbiol* **35**: 305–310.

Rosini G, Frederichi F & Martini A (1982) Yeast flora of grape berries during ripening. *Microbiol Ecol* **8**: 83–89.

Sabate J, Cano J, Querol A & Guillamon JM (1998) Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. *Lett Appl Microbiol* **26**: 452–455.

Schütz M & Gafner J (1993) Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J Appl Bacteriol* **75**: 551–558.

Schütz M & Gafner J (1994) Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett Appl Microbiol* **19**: 253–257.

Schuller D, Alves H, Dequin S & Casal M (2005) Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde region of Portugal. *FEMS Microbiol Ecol* **51**: 167–177.

Torija MJ, Rozes N, Poblet M, Guillamon JM & Mas A (2001) Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie van Leeuwenhoek Int J Gen Mol Microbiol* **79**: 345–352.

Vaughan-Martini A & Martini A (1995) Facts, myths and legends on the prime industrial microorganism. *J Ind Microbiol* **14**: 514–522.

Valero E, Schuller D, Cambon B, Casal M & Dequin S (2005) Dissemination and survival of commercial wine yeast in the vineyard: a large-scale, three-years study. *FEMS Yeast Res* **5**: 959–969.

van der Westhuizen TJ, Augustyn OHP & Pretorius IS (2000a) Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa. *S Afr J Enol Vitic* **21**: 3–9.

van der Westhuizen TJ, Augustyn OHP, Khan W & Pretorius IS (2000b) Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. *S Afr J Enol Vitic* **21**: 10–16.

Versavaud A, Courcoux P, Roulland C, Dulau L & Hallet J-N (1995) Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. *Appl Environ Microbiol* **61**: 3521–3529.

Vézinhet F, Hallet J-N, Valade M & Poulard A (1992) Ecological survey of wine yeast strains by molecular methods of identification. *Am J Enol Vitic* **43**: 83–86.