Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde Region of Portugal

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Abstract

One thousand six hundred and twenty yeast isolates were obtained from 54 spontaneous fermentations performed from grapes collected in 18 sampling sites of three vineyards (Vinho Verde Wine Region in northwest Portugal) during the 2001-2003 harvest seasons. All isolates were analyzed by mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) and a pattern profile was verified for each isolate, resulting in a total of 297 different profiles, all revealed to belong to the species *Saccharomyces cerevisiae*. The strains corresponding to seventeen profiles showed a wider temporal and geographical distribution, being characterized by a generalized pattern of sporadic presence, absence and reappearance. One strain (ACP10) showed a more regional distribution with a perennial behavior. In different fermentations ACP10 was either dominant or not, showing that the final outcome of fermentation was dependent on the specific composition of the yeast community in the must. Few of the grape samples collected before harvest initiated a spontaneous fermentation, compared to the samples collected after harvest, in a time frame of about 2 weeks. The associated strains were also much more diversified: 267 patterns among 1260 isolates compared to 30 patterns among 360 isolates in the post- and pre-harvest samples respectively. Fermenting yeast populations have never been characterized before in this region and the present work reports the presence of commercial yeast strains used by the wineries. The present study aims at the development of strategies for the preservation of biodiversity and genetic resources as a basis for further strain development.

1. Introduction

Traditionally, wine fermentation is carried out in a spontaneous way by indigenous yeast either present on the grapes when harvested or introduced from the equipment and cellar during the vinification process. All recent research agrees that the predominant species on healthy grapes are apiculate yeasts like *Hanseniaspora uvarum* (and its anamorph form *Kloeckera apiculata*) and oxidative species such as *Candida*, *Pichia*, *Kluyveromyces* and *Rhodotorula* [1]. Contrarily, fermentative species of the genus *Saccharomyces*, predominantly *Saccharomyces cerevisiae*, occur in extremely low number on healthy undamaged berries or in soils [2-4], while damaged grapes are believed to be an important source of *S. cerevisiae* [5]. The prevalence of strains belonging to this species is well documented among the wineries resident flora [6-10]. The grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, geographic localization of the vineyard [4, 9], antifungal applications [11], grape variety and the vineyard's age [12-14], as well as the soil type [15]. Several ecological surveys, using molecular methods of identification, report a large diversity of genetic patterns among the enological fermentative flora. *S. cerevisiae* strains seem to be widely distributed in a given viticultural region [16-19], can be found in consecutive years [20, 21] and there are also strains predominant in the fermenting flora [2, 22], hypothesizing the occurrence of specific native strains that can be associated with a *terroir*.

Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality. In the years following the publication of the *S. cerevisiae* genome sequence [23], enough evidence was provided showing substantial

68 genetic differences among wine yeast strains [24-26]. Therefore, exploring the
69 biodiversity of indigenous fermentative strains can be an important contribution towards
70 the understanding and selection of strains with specific phenotypes.

71 The genetic diversity of *S. cerevisiae* strains has been analyzed by several methods such
72 as karyotyping by pulse field gel electrophoresis [27], mitochondrial DNA restriction
73 analysis (mtDNA RFLP) [28-31], fingerprinting based on repetitive delta sequences
74 [32, 33] and microsatellite genotyping [34-36]. Schuller *et al*. [37] have recently shown
75 that microsatellite typing, using 6 different loci [36], an optimized interdelta sequence
76 analysis [33] and RFLP of mitochondrial DNA generated by the enzyme *Hinf*I had the
77 same discriminatory power. In the present work mtDNA RFLP analysis using *Hinf*I was
78 applied as genetic marker for the distinction of *S. cerevisiae* strains.

79 The aim of the present work was to assess the biodiversity of the fermenting flora found
80 in vineyards belonging to the Vinho Verde Region in order to define strategies for
81 future wine strain selection programs. Another goal was the establishment of a strain
82 collection contributing to the preservation of *S. cerevisiae* genetic resources.

83

84 **2. Materials and methods**

85 *2.1 Sampling*

86 The sampling plan included a total of 18 sites in three vineyards surrounding a winery,
87 located in northwest Portugal (Região Demarcada dos Vinhos Verdes). In each
88 vineyard, six sampling points were defined according to vineyard geography, and the
89 distance between winery and the sampling sites varied between 20 to 400 m, as shown
90 in Figure 1. Two sampling campaigns were performed before (early stage) and after

(late stage) harvest, in a time frame of about 2 weeks, in order to assess the diversity among fermentative yeast communities during the last stage of grape maturation and harvest. This experiment was repeated in three consecutive years (2001-2003). Samples were not always collected from the same rootstock, but from the same area (± 1-2 m). The grapevine varieties sampled were Loureiro (vineyard A), Alvarinho (vineyard P) and Avesso (vineyard C), being all white grapes used in the Vinho Verde Region.

2.2 Fermentation and strain isolation

From each sampling point, approximately 2 kg of grapes were aseptically collected and the extracted grape juice was fermented at 20ºC in small volumes (500 ml), with mechanical agitation (20 rpm). Fermentation progress was monitored by daily weight determinations. Fermentation progress was monitored by daily determinations of the musts mass loss. When a reduction by 70 g/l was observed, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on YPD plates (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v, agar 2%, w/v), and 30 randomly chosen colonies were collected after incubation (2 days, 28ºC). The isolates obtained from all fermentations throughout this work were stored in glycerol (30%, v/v) at -80ºC.

2.3 DNA isolation

Yeast cells were cultivated in 1 ml YPD medium (36 h, 28ºC, 160 rpm) and DNA isolation was performed as described [28] with a modified cell lysis procedure, using 25

U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 minutes and 1 hour (37°C). DNA was used for mitochondrial RFLP.

2.4 Mitochondrial DNA RFLP

Restriction reactions were preformed as described [37]. The attributed designations for observed distinct patterns were A1-A93, C1-C62 and P1-P135, corresponding to isolates from vineyard A, C and P respectively. Pattern designation ACP10 refers to a strain common to all vineyards and C69P77 and C42P80 were assigned to strains common to vineyard C and P. Pattern profiles that are identical to commercial starter yeasts used by the wineries are designated S1-S6. One representative strain of each of the 297 patterns was withdrawn and tested for growth in a medium containing lysine as sole nitrogen source [38].

2.5 Analytical methods

Sugar concentration was determined by a previously described dinitrosalicylic method [39].

3. Results

In the present work, three vineyards, situated in the Vinho Verde Wine Region, in northwest Portugal, were sampled during the 2001-2003 harvest seasons (Figure 1). In order to obtain a more detailed picture of fermenting yeast temporal distribution, two sampling campaigns were performed, one before and another after the harvest, in a time frame of about two weeks. A total of 108 grape samples have been planned (six

sampling points x two sampling campaigns x three vineyards x three years), from which 54 started a spontaneous fermentation, 36 were not able to start fermentation after 30 days of incubation, whereas 18 samples were not collected due to unfavorable weather conditions and a bad sanitation state of the grapes in 2002. From the 54 fermentations 1620 yeast isolates were obtained. All the isolates were analyzed by their mtDNA RFLP (*Hinf*I) and a pattern profile was attributed to each isolate, resulting in a total of 297 different profiles.

The total yeast count (cfu in YPD medium) ranged between 1.0×10^6 and 8.0×10^7, corresponding to values generally described for grape must fermentations. All isolates belonged to the species *S. cerevisiae* due to their inability to grow in a medium containing lysine as sole nitrogen source and by their capacity to amplify six *S. cerevisiae* specific microsatellite loci. None or only 1 of the loci was amplified in other *Saccharomyces* species occurring in wine such as *S. paradoxus* and *S. bayanus*, respectively. No amplification was observed for species that are generally present at initial stages of fermentation, such as *Candida stellata*, *Pichia membranifaciens* and *Kloeckera apiculata* (not shown).

The results of mtDNA RFLP for the 1620 isolates are summarized in Table 1. Among the total 450 isolates collected in vineyard A, 93 corresponded to unique patterns whereas in C and P a total 450 and 690 strains were isolated, corresponding to 62 and 135 unique patterns, respectively.

For 11 common patterns, found in more than one fermentations (Table 1 and Figure 2), and also for six commercial starter yeast strains (S1-S6), a wider geographical and temporal distribution was verified. Patterns S1 to S6 corresponded to commercial starter

yeasts that had been used in the wineries for the last few years. Perennial strains were associated with more sites of a single vineyard (patterns A06 and S6, P136, P50), but showed also a wider distribution across multiple sampling sites in two or three vineyards (patterns S3, S4, and ACP10). Patterns S1, S2, C63, A11, A13, P03 and P24 were found only in one year but across several sampling sites of a single vineyard, while strain S5 had a wider distribution across several sampling sites of vineyard C and P. Patterns C42P80 and C69P77 appeared only in a single sampling site during 2003 of both vineyards C and P. Pattern ACP10 is the only "regional" isolate with a wider geographical distribution, whereas A06, A11, A13, C63, P03, P27, P50 and P136 can be considered as "vineyard-strains" due to their occurrence in multiple sampling sites and/or years.

The wet weather in the summer 2002 resulted in severe fungal infestations and heavy applications of chemical sprays, being probably the reason for the merely 12 unique patterns among the 150 strains collected in the late sampling stage in 2002 in vineyard P. In 2003, this relation was again more similar to the one found in 2001 (47 and 62 unique patterns among each 180 isolates from the late sampling stages of vineyard P).

As shown in Figure 3, onset of spontaneous fermentation was verified in almost all grape samples collected in the late sampling campaign. This was rarely the case for most of the samples collected some days before the harvest. Must prepared from grapes collected in the early sampling stage in vineyard A, never started to ferment spontaneously. An accidental agrichemical over-dosage occurred in 2001, resulting in delayed spontaneous fermentation onset for three of the four post-harvest samples (II, III and VI). In the following two years, fermentation profiles were similar to samples from C and P, suggesting the recovery of the intervenient flora.

Fermentation started after six to twelve days being generally accomplished by one to twenty strains. Spontaneous fermentations were performed by one or more predominating strains accompanied by no, few or many "secondary" strains, or by a very heterogeneous yeast community with no prevalent strain(s). This is in agreement with other studies reporting the presence of one or two predominating strains constituting more than 50% of total biomass, and a varying number of "secondary" strains [7, 17, 19, 29, 40, 41], or presence of many distinct strains with no prevalence [22, 42]. The occurrence of both situations has also been reported [16, 18, 43].

Apparently no correlation between the number of strains involved in a fermentation and sampling site, year or vineyard was found. The wider distributed strain (ACP10) was dominant in six fermentations (AII-2002, AI-2003, AII-2003, CIII-2003, PIII-2002, PVI-2002) contributing to 77-100% (23 to 30 strains) of the total yeast flora, but was of minor importance in five fermentations (AI-2002, PII-2001, PII-2002, PI-2003, PVI-2003), accounting for only 3-10% (one to three strains), and being accompanied by one to sixteen different strains. The distribution of this strain is not associated with the capability to predominate in fermentation, and competition with accompanying strains seems to play the key role.

Vineyard-specific patterns of samples collected in the early stage did not appear after two weeks at the same site (P, 2001 and 2003, C, 2001) with the exception of the more generalized patterns S1, S2, S3, S4, S5, ACP10 and P136, speaking in favor of a very diversified *S. cerevisiae* flora.

Being the question about origin of wine yeasts still controversial [3, 5, 8, 44], our results clearly indicate that *S. cerevisiae* occurs in vineyard ecosystems belonging to the Vinho

206 Verde Region in sufficient high numbers to conduct a spontaneous fermentation from
207 musts prepared with approximately two kg of grapes. However, some remarks have to
208 be made concerning our experimental approach. Grape must creates selective and very
209 stressful conditions for yeast, totally distinct from the environmental influences in
210 nature. It is therefore clear that our data refer only to *S. cerevisiae* strains capable to
211 survive the conditions imposed by fermentation, under our experimental circumstances,
212 giving therefore a distorted picture (underestimation) of the kind of strains really
213 occurring in vine. As the detection limit of our experimental approach is 3.3% (one
214 strain in 30 isolates), rare strains, although capable to survive fermentation, might also
215 have not been detected. Searching for *S. cerevisiae* in 18 sites, in two campaigns and
216 over three years using a direct-plating method from single grape berries, as described
217 [3] would be highly labor-intensive. Therefore we regard our approach as an acceptable
218 compromise, allowing good estimation of population composition, but preventing a
219 precise description in terms of relative strain abundance in nature.

4. Discussion

Biogeographical large-scale surveys and studies on the genetic diversity of *S. cerevisiae* strains isolated from spontaneous fermentations have documented the dynamic nature of these populations. In the present study, 297 different genetic patterns have been found among 1620 isolates obtained from 54 small scale fermentations performed with grapes from three vineyards located in the Vinho Verde Region, during a three years period. The overwhelming majority of the patterns were unique, demonstrating an enormous biodiversity of *S. cerevisiae* strains in the Vinho Verde Region. Considering the ratio between the number of isolates and the number of patterns as an approximate biodiversity estimative, our results showed similar values to previously published surveys on genetic diversity of autochthonous oenological *S. cerevisiae* strains in other regions with viticulture traditions such as Bordeaux [2], Charentes [17, 45], Campagne and Loire Valley [21], in France; El Penedèz [46], Tarragona [7], Priorato [20, 22] and La Rioja [47] in Spain; Germany and Switzerland [41]; Tuscany, Sicily [48] and Collio [49] in Italy; Amyndeon and Santorini [42] in Greece; Western Cape [16, 18, 43] in South Africa; Patagonia [19] in Argentina.

The present study has been carried out in a viticultural region that has never been characterized before and includes aspects that have not been considered in previous works, such as the appearance of several commercial yeast strains, and the comparison of yeast populations that can be found in grape samples before and after the harvest.

The vast majority of the strains did not display a perennial behavior, being the flora of each year characterized by the appearance of many new patterns. This might be attributed to the sampling of only 12 x 2 kg of grapes per vineyard and year, being not

243 enough to grasp the entire biodiversity wealth of a given area. Another reason for the
244 appearance of new patterns could be attributed to recombination and evolutionary
245 forces, but it seems unlikely that such changes occur from one year to another to justify
246 the presence of many distinct patterns in consecutive years. Mitochondrial DNA RFLP
247 patterns are stable when *S. cerevisiae* cells undergo about five to seven divisions during
248 alcoholic fermentation (our unpublished data).

249 Among all patterns only ACP10 showed a wide regional distribution with a perennial
250 behavior, being a preliminary evidence for a strain representing a "*terroir*" as described
251 [17, 21]. However, the wider distribution of a strain is not necessarily correlated with a
252 better technological fitness. This makes sense from an ecological point of view, since
253 the selective forces that act in a vineyard are completely different from those that yeast
254 may find in a fermenting grape must. Further physiological characterization under wine
255 making conditions is required to evaluate the potentialities of this strain. The
256 appearance of this strain did not obey to a generalized pattern, but rather to sporadic
257 presence, absence and reappearance, due to natural population fluctuations. The
258 perennial appearance of pattern ACP10 is a consequence of its prevalence in the local
259 microflora. In different fermentations, ACP10 was dominant or not, showing that the
260 final outcome of fermentation was dependent on the specific composition of the yeast
261 community in the must, that is influenced by many factors such as the killer effect
262 which depends strongly on the ratio of killer to sensitive cells at the beginning of the
263 fermentation [50].

264 Grape variety of vine A was Loureiro, being Alvarinho and Avesso the cultivars of
265 vineyard P and C, respectively, indicating that the grape variety could contribute to the
266 finding of so many distinctive patterns. Traditional wine-making practices are very

similar in A, C and P, and differences in climatic influences seem to be of minor influence since the three vineyards are geographically close. However, one can not exclude microclimatic influences, not recorded in the present study.

A first sampling campaign was performed some days before the harvest; a second was carried out a few days after the end of harvest. This was accomplished in a time frame of about two weeks, in order to obtain a more detailed picture of the temporal distribution of fermenting yeast populations during the harvest. As grapes mature to full ripeness, yeasts become more abundant. The last stage of the grape maturation can favor fermentative yeast proliferation on grape surfaces, due to the decrease of grape skin integrity and must leakage from the berries. Insects are the probable source of yeast on damaged grapes. Yeast colonization of grapes can reach values of about 10^5-10^6 cfu/berry [51]. Before vintage, only 5% of the grapes harbor yeasts, being this number much higher (60%) during vintage [52]. As expected, only 11 of 42 pre-harvest samples (26%) were able to ferment spontaneously compared to 43 of 48 post-harvest samples (90%). The associated strains were also much more diversified in the late sampling campaign (267 patterns among 1260 isolates) compared to the early stage (30 patterns among 360 isolates). With only one exception (pattern P136), autochthonous strain patterns from the early sampling stage did not appear in the late sampling stage, speaking in favor of a succession of *S. cerevisiae* strains. Alternatively, differences can be attributed to the fact that different grape bunches were harvested, that may have, although in close proximity to each other, a distinct flora. It seems unlikely that the enormous increase in strain variability at harvest time is due to a spreading of winery-resident flora with harvesting equipment.

The present work is the first large-scale approach about the vineyard-associated strains from the Vinho Verde Region in Portugal, being a useful approach to obtain a deeper insight into ecology and biogeography of *S. cerevisiae* strains, even among geographically close regions. We consider these studies indispensable for the developing of strategies aiming at the preservation of biodiversity and genetic resources as a basis for further strain development.

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Figure 1

Geographic location of the three vineyards A, C and P in the Vinho Verde Wine Region with indication of the wineries and the corresponding sampling sites PI-PVI, AI-AVI and CI-CVI.

Table 1

MtDNA RFLP analysis of 1620 yeast isolates from fermented must prepared with grapes collected in vineyards A, C and P of the Vinho Verde Region, indicated in Figure 1, during the harvest of 2001, 2002 and 2003. E - early sampling stage; L - late sampling stage; NF - no spontaneous fermentation; NC - not collected.

Figure 2

Examples of common mitochondrial DNA RFLP (Hin*fI*) patterns, as listed in Table 1, found in yeast strains isolated from spontaneous fermentations of must collected as described in Materials and methods.

Figure 3

Fermentation profile (lines) and sugar content (bars) of must samples collected in the early (open circles and bars) and late (closed circles and bars) sampling campaigns from which yeast strains analyzed in this work were isolated. In each plot, mtDNA RFLP pattern designations of the yeast isolates are inserted. Predominating strains are double

(≥ 50%) or simple (20-50%) underlined. Pattern designations from post-harvest fermentations are bold. Common patterns are in highlighted in grey squares.

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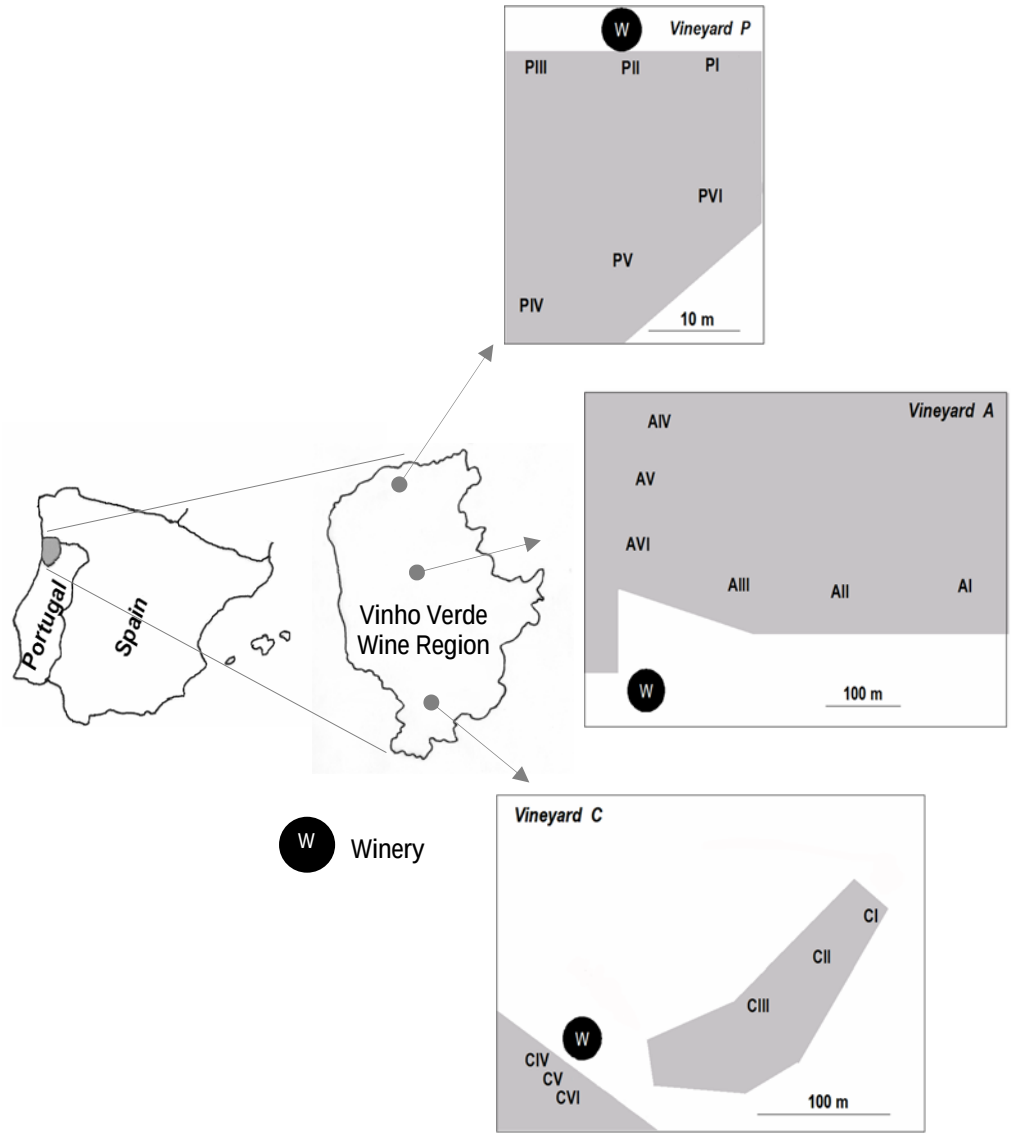
W
Vineyard P
PIII
PII
PI
PVI
PV
PIV
10 m
AIV
Vineyard A
AV
AVI
AIII
AII
AI
W
100 m
Portugal
Spain
Vinho Verde
Wine Region
W
Winery
Vineyard C
CI
CII
CIII
W
CIV
CV
CVI
100 m

Figure 1

Table 1

		Site	Number of isolates	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard A						
2001	**E**	AI AII AIII AIV AV AVI	NF	-	-	-
	L	AI AIV AVI	NF	-	-	-
		AII	90	2	10	A06
		AIII		8		
		AV		1		
2002	**E**	AI AII AIII AIV AV AVI	NC	-	-	-
	L	AI	180	16	34	ACP10 A06 A11
		AII		2		ACP10
		AIII		9		A11 A13
		AIV		6		A06 A13
		AV		9		A13
		AVI		1		-
2003	**E**	AI AII AIII AIV AV AVI	NF	-	-	-
	L	AI	180	3	41	ACP10 S3
		AII		1		ACP10 S3
		AIII		9		A06
		AIV		12		-
		AV		19		-
		AVI		2		-

Table 1 (cont.)

		Site	Number of isolates	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard C	2001 E	CI CII CIII	NF	-	-	-
		CIV	90	5	2	S1 S2 S3 S4 S5
		CV		4		S4 S5
		CVI		1		S1
	2001 L	CI	NF	-	-	-
		CII	150	20	24	S5
		CIII		4		S1 S4 S5
		CIV		2		S3 S5
		CV		4		S1 S3 S4 S5
		CVI		8		S1 S2 S4 S5
	2002 E	CI CII CIII	NC	-	-	-
		CIV	30	1	1	-
		CV CVI	NF	-	-	-
	2002 L	CI CII CIII CIV CV CVI	NC	-	-	-
	2003 E	CI CIII CIV CV CVI	NF	-	-	-
		CII	30	3	3	-
	2003 L	CI	180	8	32	S3 S4 C69P77 C63
		CII		3		C63
		CIII		1		ACP10
		CIV		18		S1, C42P80
		CV		9		-
		CVI		2		S4

Table 1 (cont.)

		Site	Number of isolates	Number of distinct patterns	Number of unique patterns	Common patterns
Vineyard P	2001	E	PI	60	2	2	P136
			PII		2		P136
			PIII PIV PV PVI	NF	-	-	-
		L	PI	180	6	62	S3 S5 S6 P136
			PII		17		S3 S6 ACP10 P03 P136
			PIII		8		-
			PIV		21		P03 P24 P50 P136
			PV		15		S3 P24
			PVI		13		S3 S6 P136
	2002	E	PI PII PIII	NF	-	-	-
			PIV PV PVI	NC	-	-	-
		L	PIV	NF	-	-	-
			PI	150	5	12	
			PII		4		ACP10 S6 P136
			PIII		1		ACP10
			PV		10		S3 S6 P50 P136
			PVI		1		ACP10
	2003	E	PIV PV	NF	-	-	-
			PI	120	10	12	-
			PII		1		-
			PIII		1		P136
			PVI		2		ACP10
		L	PI	180	15	47	ACP10, S3 C69P77
			PII		1		-
			PIII		9		P136
			PIV		18		S3
			PV		5		C42P80
			PVI		5		-

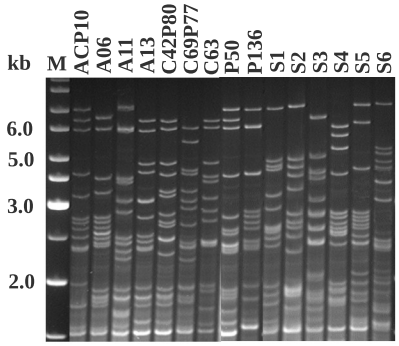
kb
M
ACP10
A06
A11
A13
C42P80
C69P77
C63
P50
P136
S1
S2
S3
S4
S5
S6
6.0
5.0
3.0
2.0

Figure 2

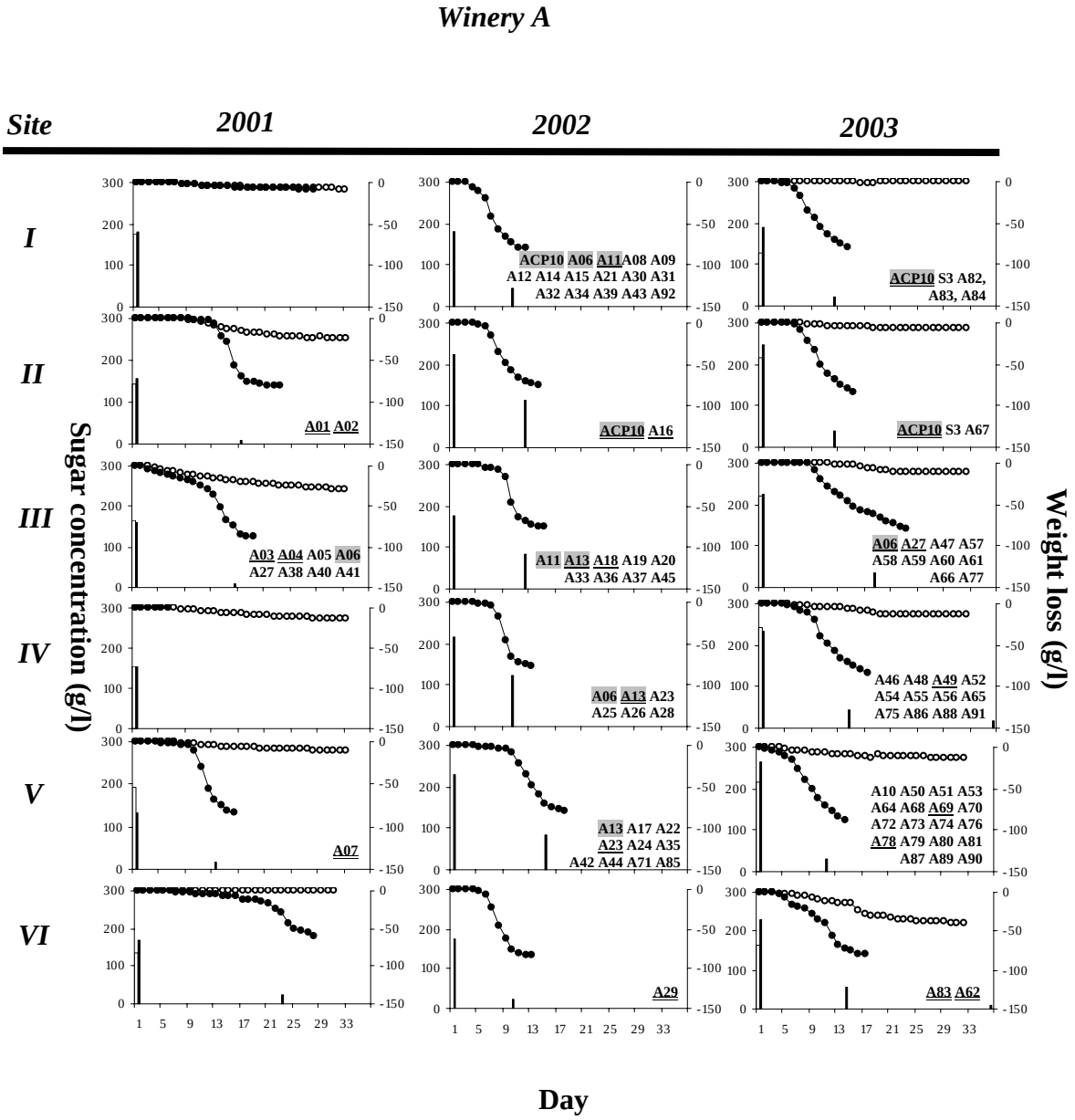
Winery A
Site
2001
2002
2003
I
II
III
IV
V
VI
A01 A02
ACP10 A06 A11A08 A09
A12 A14 A15 A21 A30 A31
A32 A34 A39 A43 A92
ACP10 S3 A82,
A83, A84
ACP10 A16
ACP10 S3 A67
A03 A04 A05 A06
A27 A38 A40 A41
A11 A13 A18 A19 A20
A33 A36 A37 A45
A06 A27 A47 A57
A58 A59 A60 A61
A66 A77
A06 A13 A23
A25 A26 A28
A46 A48 A49 A52
A54 A55 A56 A65
A75 A86 A88 A91
A07
A13 A17 A22
A23 A24 A35
A42 A44 A71 A85
A10 A50 A51 A53
A64 A68 A69 A70
A72 A73 A74 A76
A78 A79 A80 A81
A87 A89 A90
A29
A83 A62
Sugar concentration (g/l)
Weight loss (g/l)
Day

Figure 3

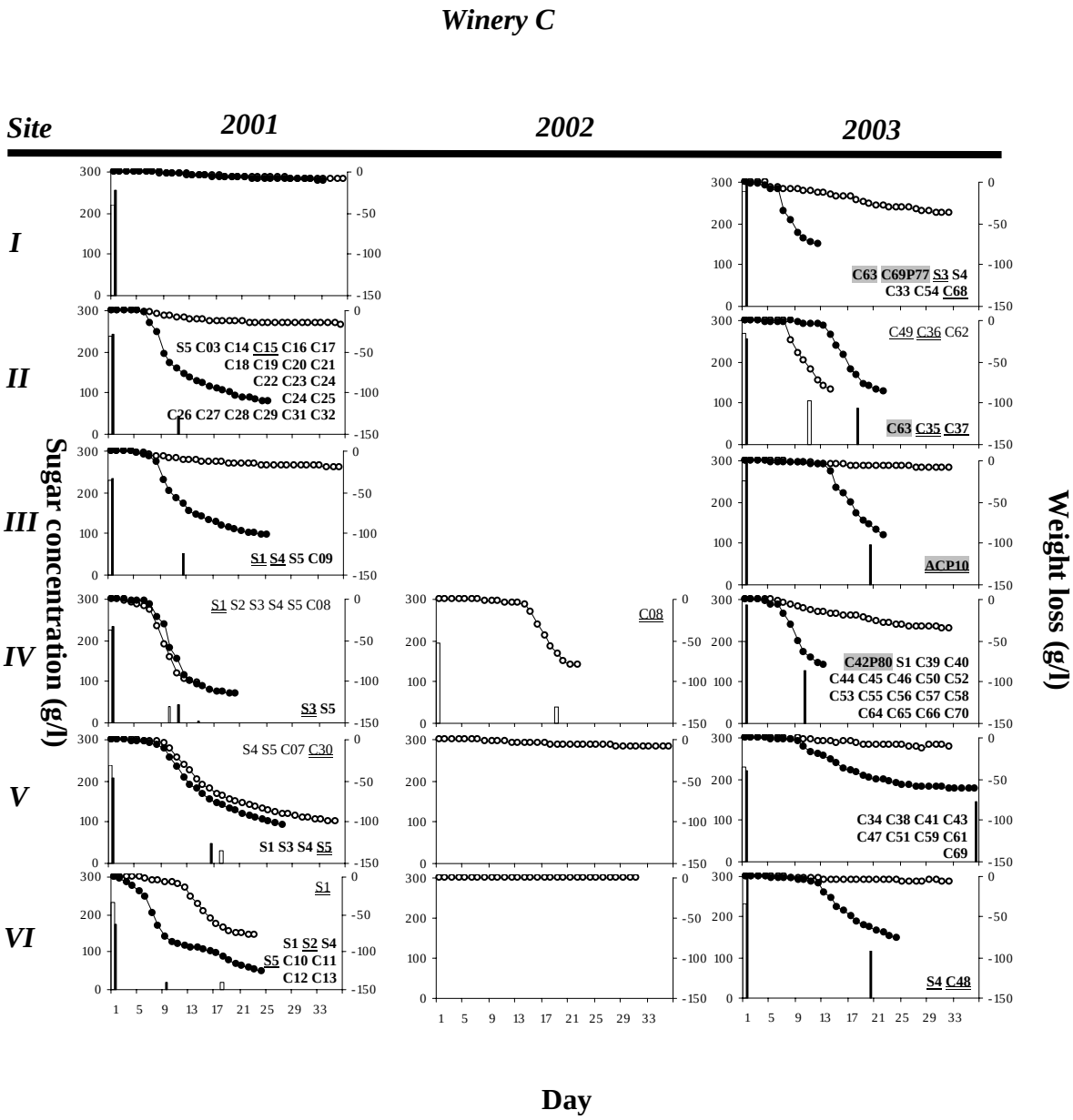

Figure 3 (cont.)

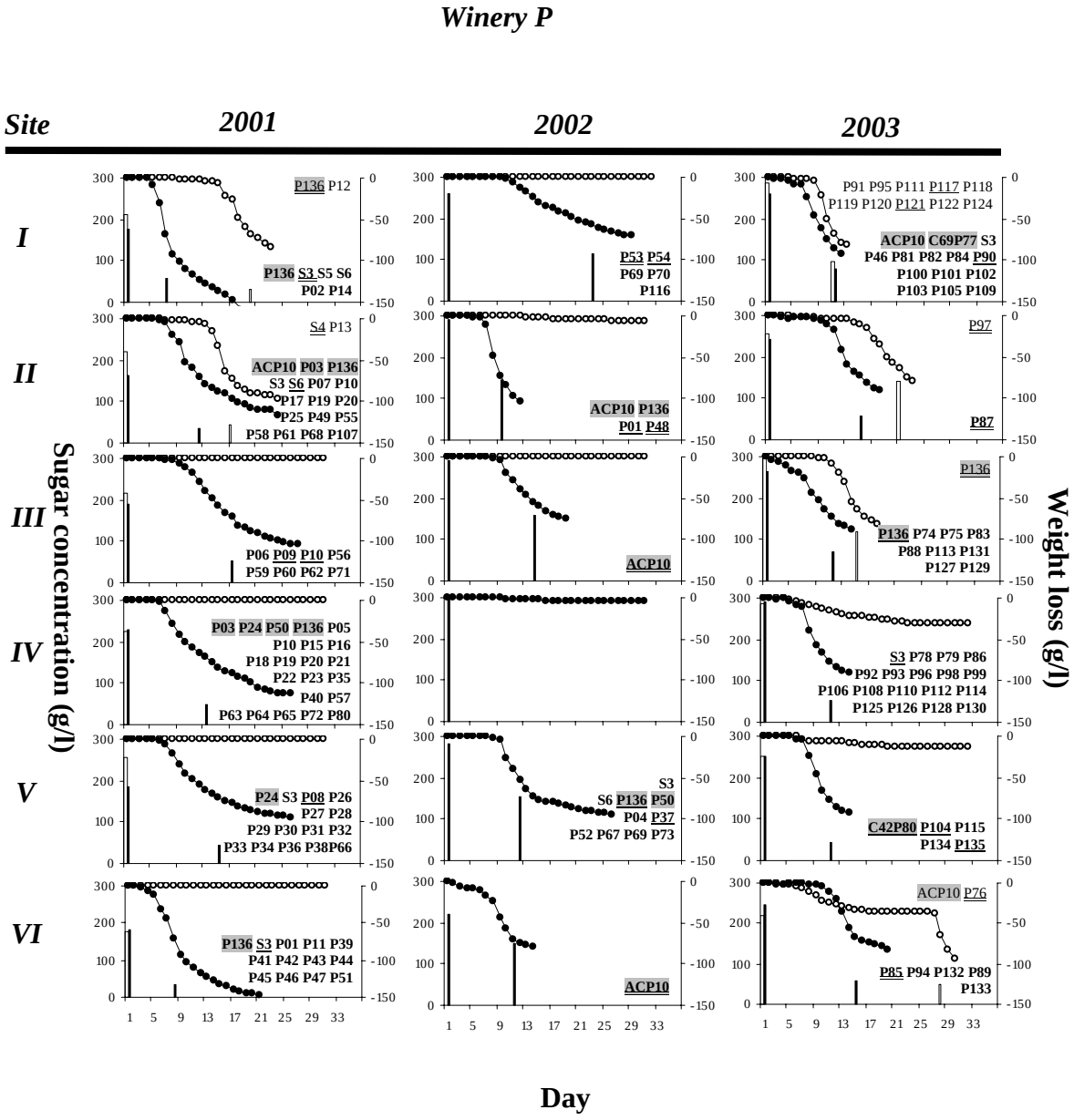

Figure 3 (cont.)