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Quest for wine yeasts—an old story revisited

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Numerous studies have described the yeast biota of grapes, and grape must in order to understand better the succession of yeasts during fermentation of wine. The origin of the wine yeasts has been rather controversial. By using more elaborate isolation methods, classical genetic analysis and electrophoretic karyotyping of monosporic clones, with this study, credible proof now exists that the vineyard is the primary source for the wine yeasts and that strains found on the grapes can be followed through the fermentation process.

Keywords: grape(s); wine yeast(s); *Saccharomyces cerevisiae*; genetic analysis; electrophoretic karyotyping; segregation of chromosomal length polymorphism

Introduction

Scores of studies with various outcomes have been carried out since Pasteur's first report [23] in attempts to describe the yeast biota of grapes, and grape must, and to understand the succession of yeasts during fermentation of wine [1]. Pasteur clearly stated that the conversion of must to wine is a spontaneous process brought about by the resident yeast biota of the grape surface [24]. However, scientists have had unexpected difficulties confirming his finding. The search has been in part interpreted as a 'series of misunderstandings', 'false identification', and resulted in statements such as:

'... though the yeast cells present on the surface of ripe grapes can reach as high a number as 10^6 per gram of fruit, the strict 'wine-associated species' are either consistently absent or in definite minority ...' [2]
'... typical strains of *Sacch. cerevisiae* ... are rarely found on the fruits and berries of wild species of plants ...' [27]
'... wine strains of the taxonomic species *Sacch. paradoxus* actually belong to the biological species *Sacch. cerevisiae* ...' [20]
'... *Sacch. cerevisiae* is practically absent ... In fact, this yeast not being a normal resident of grapes nor being it associated with other natural substrates or sources, its possible origin must be located elsewhere ...' [15]
'... At this moment, on the basis of incontrovertible experimental support from the numerous surveys carried out on the yeast ecology of various natural and man-made environments associated with grape must fermentation, we must exclude a natural origin for *S[acch]. cerevisiae* ...' [39]

On the other hand, there were studies that emphasized that yeasts make an essential contribution to the sensory character of wine by generating a wide range of volatile and non-volatile end-products during fermentation [14]. Fleet *et al* [6] described growth curves for known members of the natural yeast biota of grapes, such as *Kloeckera apiculata*, *Candida stellata*, *C. pulcherrima*, other *Candida* spp and *Saccharomyces cerevisiae* and indicated the significant contribution of these species. Heard and Fleet [9] and Fleet [5] questioned the general assumption that inoculated *S. cerevisiae* suppresses or retards the growth of indigenous species. They showed in parallel experiments with inoculated and spontaneous fermentations using the same grape musts, that despite the ultimate dominance of *S. cerevisiae*, indigenous non-*Saccharomyces* species make a great contribution to the sensory quality of the wine. These species survive longer in the fermentation than previously thought and can grow to maximum populations of 10^6–10^7 cells ml^{-1} [5]. Some exceptionally alcohol-tolerant *Kloeckera* spp were observed in the later stage of fermentations in France (Versavaud and Hallet, personal communication), as well. Herraiz *et al* [10] and Versavaud and Hallet (personal communication) found that the initial growth of *Kloeckera apiculata* has a retarding effect on the subsequent growth of *S. cerevisiae*.

Fleet [5] concluded that indigenous strains of *S. cerevisiae* are, in all probability, far better adapted to grow in the grape must than any inoculated strain. Thus, their potential to contribute to the fermentation of inoculated wines should not be underestimated. Wucherpfennig and Bretthauer [43] and Sponholz and Dittrich [34] showed that inoculation influenced the natural microbiota in musts so that higher alcohols, isoamylacetate and ethylacetate were produced in lower amounts than in spontaneously fermented wines.

Vezinhet *et al* [42], in an ecological survey in France, using molecular methods for wine yeast identification, concluded that '... the wide distribution of strains and their [reoccurrence] over years constitute preliminary evidence for the occurrence of specific native strains, ie strains representative of an enological area, 'terroir' ...'. Other authors have had varying experience with reoccurrence of members of the indigenous yeast population in the vineyard [7]. Insects are the principal vectors for the transportation of yeasts [16,26,28–30,35]. Lachance [12] and Lachance *et al* [13] recently found that yeast community structures and yeast taxa frequencies in *Drosophila* flies were influenced significantly by the habitat and ecological factors. Proximity of fruit trees contributed to maintaining a substantial *Drosophila* community. Yeast colonization on grapes is influenced by the degree of ripening of different sectors of the bunch as well as the berry [32]. Scanning electron micrographs revealed that yeasts are localized in those

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areas of the grape surface where some juice might escape, and are embedded in a gummy secretion of the fruits [3]. Also, it has been shown that some *S. cerevisiae* strains can form pseudohyphal strands that can penetrate the substrate [8; Fink, personal communication]. This ability would mean an additional advantage for yeast localization on the grape surface.

The overwhelming success of several highly rated California wineries performing spontaneous fermentation with their premium wines and the growing interest in it coincided with our curiosity to search for wine yeasts systematically and to study them genetically. This paper is a preliminary report on our observations and already establishes that the vineyards are the paramount source for wine yeasts in Northern California's wineries.

Materials and methods

Ecological studies, sample collection and strain isolation

Based on earlier studies [19,31], efforts were concentrated on four typical but very different Northern-California wineries and their vineyards. Their geographical location, production size, wine making philosophy and style, viticulture techniques and management were as distinct as was their strong commitment to spontaneous fermentation.

Samples summarized in Table 1 were carefully collected under field conditions. In the vineyards, ten vines per vineyard, and three clusters per vine were collected, unbiased — healthy, dry and bird-damaged alike —, in separate plastic bags. In one case only, two vines were sampled by clipping all grape clusters from the vines. On two occasions grapes were collected upon their arrival in the winery. Thus, they were mixed samples; though representative but with no reference to particular plants. All samples were transported on ice and processed within 4 h.

Yeasts from grape samples were washed off by shaking the clusters in 100 ml 0.1% sterile peptone water for 1 h on an orbital shaker operated at 150–200 rpm. Some samples additionally underwent mild sonication. Yeast cells were collected by centrifugation, resuspended in 5 ml 0.1% sterile peptone water and surface plated. Also, direct isolation of yeasts from the grape surface was performed by embedding berries in media. Three media were used for strain isolation: YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar), and two modifications of the ethanol-sulfite-yeast extract medium of Kish *et al* [11]. In one modification sulfite was eliminated and the medium contained 12% ethanol, 0.5% yeast extract, 0.5% peptone, 2% glucose and 2% agar (EYPD). The other modification replaced glucose with maltose (EYPM). Added bromthymolblue (0.008%) increased the selectivity of media: most *Saccharomyces* spp appeared as white colonies mixed within purple-brownish *Metschnikowia* spp and mostly yellow *Kloeckera* spp and other members of the yeast biota.

The washed grape clusters were crushed under aseptic conditions in the original collection plastic bags. To simulate the usually longer skin contact in the case of red wine making, some grape skin was added to the red grape juice. The must underwent fermentation. Appropriate dilutions of grape must and fermentation samples from laboratory tests were plated on the surface of the above media. Large numbers of yeast colonies were examined under the microscope, the presumed wine yeasts collected and used for further characterization of their genetic traits as described earlier [17,19,31]. Isolated wine yeasts were maintained in 210 μl YPD containing 15% glycerol in 96-well microtiterplates at −80°C.

Table 1 Samples used in the study

Winery	Vineyard	Varietal	Sample type and size	Yeast titer
A	n/a	chardonnay	mixed, 1 kg	1.2×10^3 g^{-1}
	n/a	chardonnay	freshly pressed, 50 ml	1.9×10^5 ml^{-1}
	DM	cabernet	10 vines, three clusters each	n/a
	TP	cabernet	10 vines, three clusters each	n/a
B	V	zinfandel	10 vines, three clusters each	n/a
	V	zinfandel	two vines, 10 clusters each	1.8×10^1–2.6×10^2 g^{-1}
C	G	cabernet	mixed, 500 g	10^6 g^{-1}
	G	cabernet	freshly pressed, 50 ml	n/a
D	ST	cabernet	10 vines, three clusters each	1.4×10^1–1.3×10^2 g^{-1}
	CH	cabernet	mixed, 1 kg	7.8×10^1 g^{-1}
	T	cabernet	fermentation, 50 ml	n/a

PCR-based strain screening

PCR-screening was applied to large numbers of isolates by using the primer sequences as published by Pearson and McKee [25] amplifying a 310-bp stretch of the 2-μm plasmid in *S. cerevisiae*. Reactions were run in a GenAmp® PCR System 9600 (Perkin Elmer, Foster City, CA, USA) and performed in a 30-μl volume containing 2 μl intact yeast cells suspended in water (~ 10^8 ml^{-1}), 3 μl 10 × GeneAmp® PCR buffer (Perkin Elmer), 3 μl 2 mM nucleotide mixture (Boehringer, Indianapolis, IN, USA), 3 μl 10 μM primers each, and 5 units AmpliTaq® DNA polymerase (Perkin Elmer). Reaction conditions were quasi-'hot-start', denature at 92°C, 30 s, anneal at 55°C, 30 s, extend at 72°C, 90 s, 35 cycles with a final extension at 72°C for 7 min. PCR products were run on 1.5% SeaKem®LE (FMC, Philadelphia, PA, USA) agarose minigel by loading 5 μl reaction mixture per lane and using 3 μl (300 ng) 1-kb ladder (Gibco BRL, Gaithersburg, MD, USA) as a DNA size standard.

CHEF gel electrophoresis

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used for electrophoretic karyotyping isolates and to examine the segregation of karyotypes in monosporic cultures of dissected tetrads. On one occasion CHEF gel electrophoresis was used for screening strains selected from grapes to prove with certainty that *Saccharomyces* spp were isolated. Conditions for plug preparation and CHEF gel electrophoresis were simplified from earlier descriptions [18,36]. Five milliliters of an overnight culture

Table 2 Phenotype and origin of wine yeast strains isolated from chardonnay grapes and grape juice collected at winery A

Strains	G	M	S	YPG	Sporulation frequency	CUP	H_2S	Origin
7, 13, 16, 19, 21	pos	pos	pos	pos	H	neg	3	clear grape juice, must w/sediment, young wine
4, 9, 27	pos	pos	pos	pos	H	neg	2	clear grape juice, young wine w/sediment
3, 10	pos	pos	pos	pos	H	neg	1	clear grape juice
17	pos	pos	pos	pos	M	neg	3	young wine
1, 23	pos	pos	pos	pos	M	neg	2	grapes embedded w/medium, young wine w/sediment
24, 34	pos	pos	pos	pos	M	neg	1	young wine w/sediment, sonicated grapes
18, 25	pos	pos	pos	pos	L	neg	3	young wine, young wine w/sediment
28, 35	pos	pos	pos	pos	M	pos	3	young wine w/sediment, grapes embedded w/medium
26	pos	pos	pos	pos	M	pos	2	young wine w/sediment
30	pos	pos	pos	pos	M	pos	1	young wine w/sediment
31	neg	pos	pos	pos	H	neg	3	young wine w/sediment
5, 6	neg	pos	pos	pos	H	neg	2	clear grape juice
14	neg	pos	pos	pos	H	neg	1	24 h after fermentation started
22	neg	pos	pos	pos	M	neg	3	young wine
20, 29	neg	pos	pos	pos	L	neg	3	young wine, young wine w/sediment
12	pos	neg	pos	pos	H	neg	3	must w/sediment
32	pos	neg	pos	pos	L	neg	3	grapes washed by shaking
15	pos	neg	neg	pos	H	neg	2	24 h after fermentation started
8	neg	neg	neg	pos	L	pos	2	clear grape juice
11	pos-Y	pos-Y	pos-Y	pos	H	neg	1	clear grape juice
33	pos-Y	pos-Y	pos-Y	pos	M	pos	4	grapes washed by shaking

G, M, S: ability to ferment galactose, maltose, and sucrose, respectively.
YPG: growth on non-fermentable carbon source (3% glycerol and 1% ethanol).
Sporulation frequency: L low sporulation (< 5% of the cells); M medium sporulation (5–80% of the cells); H high sporulation (> 80% of the cells) on McClary agar.
CUP: ability to grow in the presence of 60 mg L^{-1} cupric ions.
H_2S: production of hydrogen sulphide.
Y: 'yellow mutation' [19].

in YPD were harvested by centrifugation. Cells were washed in distilled water and spun again. The pellet was suspended in 500 µl SCE buffer (1 M sorbitol, 100 mM sodium citrate, and 10 mM EDTA, pH 7.8) and mixed with 700 µl 0.7% InCert® (FMC) agarose kept at 45°C. The mixture was instantly dispensed to form inserts using the mold designed by Smith *et al* [33]. Ten solidified inserts per sample were treated with 2 units Zymolase 100T (Seikagaku, Tokyo, Japan) in 1 ml SCE buffer at 37°C for 2–3 h, followed by an overnight incubation in 1 ml ESP (500 mM EDTA, 1% sarcosyl, and 1 mg ml^{-1} Proteinase K [Boehringer], pH 8.0) at 50°C. Then the inserts were rinsed with and stored in TE-50 (10 mM Tris and 50 mM EDTA, pH 7.8) at 4°C.

Conditions for electrophoresis were adjusted to run 21-cm gels to increase separation of chromosome-size DNA molecules, ie longer running times (average 40–50 h) and wider ramping of the pulse time. Specific running conditions were given in the legend to the figures. The strain YNN 295 (Bio-Rad, Hercules, CA, USA, accession number: YP 80; Yeast Genetic Stock Center, Berkeley, CA, USA) was used in all runs as the yeast chromosome-size DNA standard.

Results

Ecological studies

Winery A is a major Napa Valley, CA, USA winery where spontaneous fermentation had been gradually introduced over several years and then exclusively practiced in the last two seasons. Freshly picked chardonnay grapes were sampled upon their arrival in the winery (a 1-kg mixed sample), as were 50 ml clear grape juice, and another 50 ml grape juice with sediment at pressing. Cabernet sauvignon grapes were picked at vineyard DM, merlot grapes at vineyard TP, several miles from the winery. The yeast titer for the chardonnay grapes turned out to be 1.2×10^3 cells per g. This value was not different even if we applied mild sonication of the samples. The grape juice had 1.9×10^5 ml^{-1} yeasts that caused fermentation to start in less than 24 h. From the grape berries, five wine yeast strains (strains 1, 32, 33, 34 and 35) were isolated by washing or by direct embedding in medium that upon further examination showed four different phenotypes. Eleven strains were isolated from the freshly pressed grape juice on EYED medium. On EYEM medium, 21 wine yeast strains were selected. Another 44 strains were isolated 24 h after fermentation onset and 40 more 2 weeks later. A set of the phenotypes of 35 strains is compiled in Table 2 to show the diversity of the isolates. It is interesting that non-fermenting strains, or strains with the 'yellow mutation' [19] that sporulated were isolated.

The concentrated washes plated from red grape samples revealed a variety of mold, bacterial and yeast colonies. After realizing that the fast growing microorganisms obscured colonies of the rare *Saccharomyces* spp, sterile must was added (two parts wash water and one part must), and a few wine yeast colonies were isolated from most of the washes. Twenty-eight colonies isolated from grapes from vineyard DM and 30 from vineyard TP were analyzed genetically. The red grape samples were then crushed and the grape juice collected, separated by vineyards, in two

sterile 2.8-L Fernbach flasks, and upon adding grape skins to the mixtures spontaneous fermentation took place. At the end of fermentation, 44 yeast strains were isolated from each Fernbach flask and analyzed genetically. Twenty-seven of the 28 colonies off the grapes from vineyard DM were of one type. Similarly, 26 of the 30 colonies isolated from vineyard TP grapes were of the same yeast type that was found on vineyard DM grapes. Upon dissecting large numbers of their asci, however, it was found that the strains fell into the same four types as reported earlier [19] with those that had high (> 80%) spore viability and uniform spore colony size in the majority. Minority wine yeast strains numbered only a few heterozygosities in the scored traits (1–2) and displayed mostly high spore viability.

Winery B is a small, family-owned business with 106-year-old vines, locked in between housing developments and industry in Contra Costa County, CA, USA. The vines stand alone on a hillside conventionally viticultured with no irrigation, open to climatic changes. The ten Zinfandel grape samples were separately washed and crushed using aseptic laboratory techniques. Fermentation proceeded in sterile 50-ml Blue Cap tubes: seven samples started the fermentation and only three completed it. Hence a second collection was necessary. By that time, only the second crop was left on the vines. Fortunately, on two vines 10 small clusters, each, were found. The sample size varied between 11 and 72 g, the yeast titer was 1.8×10^1–2.6×10^2 g^{-1}. Besides yeasts, a great number of bacteria and molds grew on the plates. Again, the grapes were crushed separately and the juice transferred into sterile 50-ml Blue Cap tubes. Of the 20 samples four started the fermentation within 24 h, and 14 fermented vigorously within 72 h. Nevertheless, only four samples completed the fermentation, and two lost pigmentation due to bacterial growth. All but two isolates that completed the laboratory fermentation were of the same phenotype: galactose non-fermenter, no growth on non-fermentable carbon sources, copper sensitive, low H_2S producer. These strains, as expected, did not sporulate at first. After several months at 4°C, however, some colonies produced spores that, upon dissection, were not viable. Of the two other strains that also completed the laboratory fermentation, one was fermenting galactose, the other one was an amino acid auxotroph.

Winery C is a medium-sized facility in the Sonoma Valley, CA, USA. Of special interest was a single-vineyard fermentation. It has behaved differently year after year: grape juice from vineyard G started to ferment within 24 h, while grape juices from other vineyards needed a longer lag-period in the same winery.

For vineyard G, a freshly picked grape sample was available. The yeast titer was 10^6 g^{-1}, and 28 wine yeast strains were isolated. The grapes were crushed and the juice transferred aseptically into a sterile 50-ml Blue Cap tube. Fermentation started in less than 24 h under laboratory conditions. On the 9th day of fermentation, a sample was plated and 36 strains were selected for PCR screening. Although the strains were subdivided into five types (Table 3), they seemed to be basically of two kinds: dominant strains that were present throughout the sampled regions of Northern California — positive in all tested phenotypic features, varying in copper sensitivity, sporulating with high frequency and high viability (class types c and a) — and a wide variety of other members of the wine yeast biota. Tetrad dissection revealed the genetic diversity of the isolates (Tables 4 and 5).

Table 3 Classes of strains isolated in vineyard G of winery C

Class type	Description of class	Grapes	Model fermentation
a	Fermentation of tested carbon sources and growth on tested media positive, high sporulation frequency, high H_2S production, copper resistant	3[a]	6
b	Same as class a, medium sporulation frequency	6	1
c	Fermentation of tested carbon sources and growth on tested media positive, high sporulation frequency, H_2S production varies, copper sensitive	10	9
d	Same as class c, medium sporulation frequency	7	2
e	Fermentation of tested carbon sources and growth on tested media positive, low sporulation frequency, H_2S production varies, copper sensitive	–	7
f	Fermentation of tested carbon sources and growth on tested media positive, no sporulation, high H_2S production, copper sensitive	2[b]	–

[a] Number of strains isolated belonging to a given class.
[b] Both strains carried the 'yellow mutation' [19].

Winery D is situated in the Santa Cruz area (CA, USA), some 70 miles south from the so-called 'wine country', the Napa and Sonoma Valleys. Mountainous terrain and the Pacific Ocean create a different climate. Seven grape samples were from a single vineyard (ST) cultured on a steep terrace hillside, and a mixed one from another vineyard (CH). The yeast titer of the samples was 1.4×10^1–1.3×10^2 g^{-1}. After 72 h, four of the separately kept grape juices started the fermentation. With some samples it took 6–7 days to start the fermentation. Within 1 month, only two samples actually completed the fermentation and another two never started it.

PCR screening

After microscopic examination, a large number of isolated yeast strains were PCR-screened for a 310-bp size piece of the 2-μm plasmid in *S. cerevisiae*. Thirty-six strains tested were isolated in connection with winery C, and 77 more from vineyards belonging to wineries A and B; 25 of the 36, and 31 of the 77 strains were positive.

CHEF gel electrophoresis

CHEF gel electrophoresis is a very sensitive, highly reproducible method of strain characterization. In one case — winery A — this method was used to prove that *Saccharomyces* spp were indeed isolated from grape and freshly pressed grape juice samples. CHEF gel electrophoresis was

Table 4 Summary of yeast strains from the grapes collected in vineyard G of winery C

Class	Strain	Spo. freq.	No. of asci	Viable spores/ascus 4	3	2	1	0	Viab. (%)	HO	G	M	S	CUP	YPG	H_2S	Lethal.	Slow growth	Min.	Sporulation -	L	M	H	#het
a	M5	H	5	0	0	3	2	0	40	+/?	+/+	+/+	+/+	+/–	+/+	4/3	+/–	+/–	+/+	5	2	1	0	7(Y)*
	M14	H	4	1	0	2	1	0	56	+/+	+/+	+/–	+/+	+/+	+/+	3/1	+/+	+/–	+/+	–	–	2	7	4
	E9	H	4	0	1	0	3	0	38	+/+	+/+	+/+	+/+	+/–	+/+	3/2	+/+	+/–	+/+	–	–	–	6	4
b	M3	M	5	1	1	2	1	0	60	+/+	+/+	+/+	+/+	+/+	+/+	3/4	+/+	+/+	+/+	–	2	7	3	2
	E2	M	3	0	0	1	1	1	25	+/+	+/+	+/+	+/+	+/+	+/+	3/4	+/–	+/–	+/+	–	–	–	3	3
	E3	M	4	1	2	2	0	0	75	+/+	+/+	+/+	+/+	+/–	+/+	3/2	+/+	+/–	+/+	–	–	–	12	3
	E7	M	2	2	0	0	0	0	100	+/+	+/+	+/+	+/+	+/+	+/+	3/2	+/+	+/+	+/+	–	–	1	7	2
	E8	M	4	0	1	0	3	0	38	+/+	+/+	+/+	+/+	+/–	+/+	3/2	+/+	+/+	+/+	–	–	–	6	2
	E10	M	4	0	0	1	3	0	31	+/+	+/+	+/+	+/+	+/–	+/+	3/2	+/–	+/+	+/+	–	–	–	5	3
c	M13	H	4	2	2	0	0	0	88	+/+	+/+	+/–	+/+	–/–	+/+	3/4	+/+	+/+	+/+	–	–	–	14	2
	E1	H	3	1	2	0	0	0	83	+/+	+/+	+/+	+/+	–/–	+/+	3/4	+/+	+/–	+/+	–	–	2	8	3
	E4	H	4	0	4	0	0	0	75	+/+	+/+	+/–	+/+	–/–	+/+	3/4	+/+	+/–	+/+	–	–	1	11	5
	E12	H	4	4	0	0	0	0	100	+/?	+/+	+/0	+/+	–/–	+/–	3/1	+/+	+/–	+/+	1	1	3	11	6
	E13	H	4	4	0	0	0	0	100	+/+	+/–	+/+	+/–	–/–	+/+	3/3	+/+	+/–	+/+	1	–	3	12	5
	E14	H	4	2	1	1	0	0	81	+/+	+/–	+/+	+/–	–/–	+/+	3/1	+/+	+/–	+/+	–	1	–	12	5
	M4	H	5	1	2	2	0	0	70	+/?	+/+	+/–	+/+	–/–	+/+	2/4	+/+	+/–	+/–	1	2	2	9	5
	M9	H	5	0	1	0	3	0	30	+/+	+/+	+/+	+/+	–/–	+/+	2/2	+/+	+/+	+/+	–	–	1	5	1
	M11	H	4	2	1	1	0	0	81	+/+	+/+	+/–	+/+	–/–	+/+	2/4	+/+	+/–	+/–	–	1	–	12	5
	M8	H	5	1	2	2	0	0	88	+/+	+/+	+/–	+/+	–/–	+/+	1/4	+/+	+/–	+/+	–	3	–	11	4
d	M2	M	4	4	0	0	0	0	100	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	+/+	+/+	–	–	10	6	1
	M7	M	5	5	0	0	0	0	100	+/+	+/+	+/+	+/+	–/–	+/+	3/4	+/+	+/+	+/+	–	–	–	20	1
	M10	M	4	0	1	1	1	1	38	+/?	+/–	+/–/0	+/–/0	–/–	+/–	3/1	+/+	+/–	+/+	1	–	1	4	7
	E5	M	4	0	0	3	1	0	44	+/?	+/–	+/0	+/–/0	–/–	+/–	3/2	+/–	+/–	+/+	3	1	3	–	10
	E6	M	4	4	0	0	0	0	100	+/+	+/+	+/+	+/+	–/–	+/+	3/3	+/+	+/–	+/+	–	1	–	15	3
	E11	M	4	0	1	3	0	0	56	+/?	+/–/0	+/–/0	+/+	–/–	+/–	3/1	+/+	+/–	+/+	5	3	1	–	8
	M1	M	3	2	1	0	0	0	92	+/+	+/–	+/+	+/+	–/–	+/+	2/4	+/+	+/–	+/+	–	4	2	5	6(Y)*
f	M6	–	–	–	–	–	–	–	–	n/a	+	+	+	–	+	4	n/a	n/a	–	–	–	–	–	n/a(Y)*
	M12	–	–	–	–	–	–	–	–	n/a	+	+	+	–	+	4	n/a	n/a	–	–	–	–	–	n/a(Y)*

Sporulation frequency: – no sporulation; L low sporulation (< 5% of the cells); M medium sporulation (5–80% of the cells); H high sporulation (> 80% of the cells).
No. of asci: number of 4-spore asci dissected.
Viability: number of viable spores divided by the total number of spores dissected in %.
HO: homothallism scored by the ability of individual spore clones to sporulate.
G, M, S: ability to ferment galactose, maltose, and sucrose, respectively (0: no growth).
CUP: ability to grow in the presence of 60 mg L^{-1} cupric ions.
YPG: growth on non-fermentable carbon source (3% glycerol and 1% ethanol).
H_2S: production of hydrogen sulfide.
Lethal(s): presence of lethal mutation(s) was assumed when none of the dissected asci resulted in more than two viable spore clones.
Slow growth: mutation(s) influencing the growth rate of spore clones.
Min: growth on minimal medium (DIFCO Yeast Nitrogene Base, 2% glucose, 2% agar).
Sporulation: sporulation in monosporic clones.
#het: number of traits scored that were heterozygous.
* Strains carried the 'yellow mutation' [19].

Table 5 Summary of yeast strains from the laboratory fermentation (grapes collected in vineyard G of winery C)

Class	Strain	Spo. freq.	No. of asci	Viable spores/ascus 4	3	2	1	0	Viab. (%)	HO	G	M	S	CUP	YPG	H_2S	Lethal.	Slow growth	Min.	Sporulation -	L	M	H	#het
a	E3	H	7	1	1	1	2	2	39	+/+	+/+	+/+	+/+	+/+	+/+	4/4	+/+	+/–	+/+	–	3	3	5	1
	D4	H	6	1	1	2	0	2	46	+/+	+/+	+/+	+/+	+/+	+/+	4/4	+/+	+/–	+/+	–	–	7	4	1
	D8	H	5	0	1	3	1	0	50	+/+	+/+	+/+	+/+	+/+	+/+	4/4	+/+	+/–	+/+	–	–	7	3	1
	E11	H	4	0	0	1	1	2	19	+/+	+/+	+/+	+/+	+/–	+/+	4/4	+/+	–/–	+/+	–	–	–	3	1
	C12	H	4	0	1	3	0	0	56	+/+	+/+	+/+	+/+	+/–	+/+	4/4	+/+	+/–	+/+	–	1	1	7	2
	E12	H	4	2	1	1	0	0	81	+/+	+/+	+/–	+/+	+/+	+/–	4/4	+/+	+/–	+/+	–	1	–	12	3
b	D3	M	6	0	0	3	0	3	25	+/+	+/+	+/+	+/+	+/–	+/+	3/3	+/–	+/–	+/+	1	–	1	4	3
c	D1	H	8	0	2	2	4	0	44	+/+	+/+	+/+	+/+	–/–	+/+	2/3	+/+	+/–	+/+	–	–	–	14	2
	E2	H	6	3	1	1	1	0	75	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	+/–	+/+	–	–	2	16	1
	C6	H	5	5	0	0	0	0	100	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	–/–	+/+	–	–	–	20	0
	D6	H	5	1	1	3	0	0	65	+/–	+/+	+/–	+/+	–/–	+/+	4/4	+/+	+/–	+/+	2	4	5	2	3
	C7	H	6	0	0	4	1	1	38	+/–	+/–	+/–	+/+	–/–	+/+	3/3	+/–	+/–	+/+	4	1	3	1	5
	E7	H	4	4	0	0	0	0	100	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	–/–	+/+	–	–	–	16	0
	D9	H	4	3	0	0	0	1	75	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	–/–	+/+	–	–	–	12	0
	C11	H	5	0	1	1	2	1	35	+/+	+/+	+/+	+/+	–/–	+/+	3/2	+/+	+/–	+/+	–	2	1	4	2
	D11	H	4	3	0	0	0	1	75	+/+	+/+	+/+	+/+	–/–	+/+	4/4	+/+	–/–	+/+	–	1	1	10	0
d	C1	M	7	7	0	0	0	0	100	+/+	+/–	+/+	+/+	–/–	+/+	4/4	+/+	–/–	+/+	–	–	–	28	1
	D10	M	4	0	0	2	2	0	38	+/–	+/–/0	+/–/0	+/–	–/–	+/–	3/2	+/–	+/–	+/+	4	1	1	–	8
e	E1	L	6	0	0	1	3	2	21	+/–	+/–	+/–	+/–	–/–	+/–	4/4	+/–	+/–	+/+	3	1	–	1	7
	C2	L	6	0	0	4	1	1	38	+/?	+/–	+/–	+/–	–/–	+/–	3/1	+/–	+/–	+/+	6	–	1	2	7
	D2	L	6	0	0	1	4	1	25	+/?	+/–	+/–	+/–	–/–	+/–	4/4	+/–	+/–	+/+	1	–	1	4	6
	C3	L	6	0	0	2	2	2	25	+/?	+/–	+/–	+/–	–/–	+/–	3/1	+/–	+/–	+/+	5	–	1	–	7
	C9	L	4	0	0	2	1	1	31	+/?	+/–	+/–	+/–	–/–	+/–	3/1	+/–	+/–	+/+	3	1	1	–	7
	C10	L	4	0	0	0	3	1	19	+/?	+/+	+/–	+/–	–/–	+/–	3/2	+/–	+/–	+/+	2	–	–	1	6
	D12	L	4	0	0	2	2	0	38	+/?	+/–	+/–	+/–	–/–	+/–	4/4	+/–	+/–	+/+	2	–	4	–	6

Sporulation frequency: – no sporulation; L low sporulation (< 5% of the cells); M medium sporulation (5–80% of the cells); H high sporulation (> 80% of the cells).
No. of asci: number of 4-spore asci dissected.
Viability: number of viable spores divided by the total number of spores dissected in %.
HO: homothallism scored by the ability of individual spore clones to sporulate.
G, M, S: ability to ferment galactose, maltose, and sucrose, respectively (0: no growth).
CUP: ability to grow in the presence of 60 mg L^{-1} cupric ions.
YPG: growth on non-fermentable carbon source (3% glycerol and 1% ethanol).
H_2S: production of hydrogen sulfide.
Lethal(s): presence of lethal mutation(s) was assumed when none of the dissected asci resulted in more than two viable spore clones.
Slow growth: mutation(s) influencing the growth rate of spore clones.
Sporulation: sporulation in monosporic clones.
Min: growth on minimal medium (DIFCO Yeast Nitrogene Base, 2% glucose, 2% agar).
#het: number of traits scored that were heterozygous.

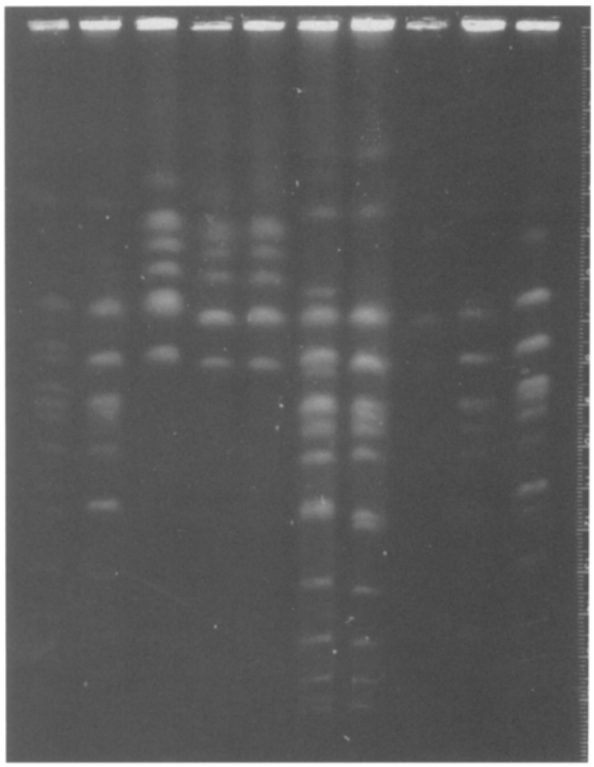

Figure 1 CHEF gel electrophoresis used for screening yeast isolates. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lanes 2–10: new yeast strains isolated from chardonnay grapes and grape juice at winery A. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 25 s for 33 h and from 80 s to 15 s for 7 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

performed after microscopic screening of fully grown colonies on EYED and EYEM media. Figure 1 shows a typical example with nine unknown yeast strains. Lanes 2, 6, 7, 9, and 10 clearly reveal the typical *Saccharomyces* sensu stricto spp electrophoretic karyotype. However, the number of bands and their position compared with that of the standard strain *S. cerevisiae* YP 80 (= YNN 295), lane 1, vary because the strains were most certainly at least diploid-loaded with chromosome-length polymorphism (CLP) and/or they might belong to different species. Lanes 3, 4, 5, and 8 show a similar but different electrophoretic karyotype of non-*Saccharomyces*-sensu stricto-type yeasts.

Also, the segregation of chromosome length polymorphism was characterized in monosporic cultures of dissected tetrads. Four kinds of results were observed:

- The four monosporic cultures of a tetrad showed an identical karyotype. Figure 2a shows a typical example of two dissected tetrads of the same wine yeast strain. Compared with the standard YP80 strain, chromosomes I and VI, V and VIII, XI, and XIV of the wine yeast are somewhat larger. The bands in the size of the standard strain's chromosomes II, and XV and VII seem to be doublets. The band around 1300 kb accounts for a 'missing' band — probably chromosome XVI.
- The four monosporic cultures of a tetrad segregated chromosome-length polymorphism in a mostly 2 : 2 pattern, and if one was to 'push' the four lanes together,

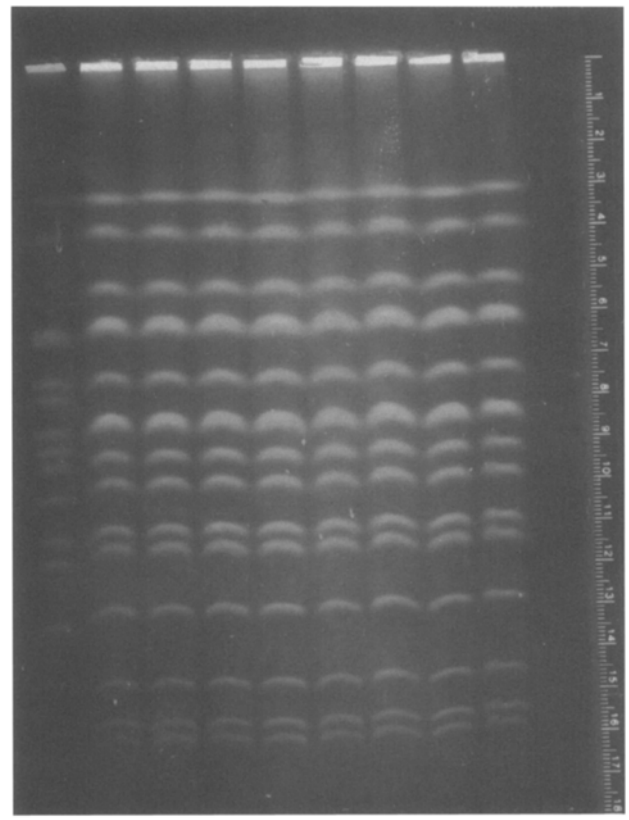

Figure 2a Electrophoretic karyotypes of two dissected tetrads of the wine yeast strain T7.264. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lanes 2–5: monosporic clones of tetrad 1; lanes 6–9: monosporic clones of tetrad 2. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 20 s for 40 h and from 75 s to 15 s for 7 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

they would add up to the karyotype of the diploid. Figure 2b was a shorter run so chromosomes IV and XII are compressed. All the other bands of the monosporic clones (lanes 3–6) show a 2 : 2 segregation of chromosome-length polymorphism that is remarkably well separated in the parental diploid (lane 2) resulting in 20 bands. Figure 2c shows another example: although the lower part of the gel is somewhat compressed, it shows independent segregation of chromosome-length polymorphism of chromosomes IV and XII in the monosporic clones (lanes 2–5).
- The 'sum' of the karyotypes of the monosporic segregants was different from that of the diploid. Figure 2d gives an example: the segregating chromosome polymorphism in the lower part of the gel — first seven bands — of the monosporic clones (lanes 3–6) clearly do not 'add up' compared with the karyotype of the parental strain (lane 2).
- A fourth scheme is possible, too, though with much lower frequency: most lanes or all four electrophoretic karyotypes of the monosporic clones of a dissected tetrad can be unique. Figure 2e shows such a case: two tetrads of the same wine yeast strain were dissected. Lanes 2, 5 and 7 look unique within the respective tetrads. On the other hand, a unique karyotype may have similarities

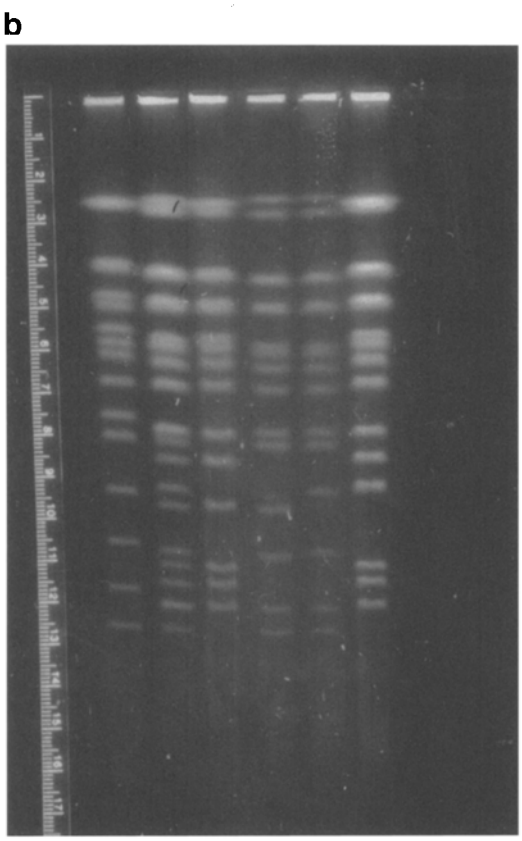

Figure 2b Electrophoretic karyotypes of a dissected tetrad of the wine yeast strain Bb 33(4)1. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lane 2: parental diploid; lanes 3–6: monosporic clones. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 20 s for 33 h and from 75 s to 15 s for 7 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

with clones in another tetrad, eg lanes 5 and 8, of the same strain. In this given case, the monosporic clones of the two tetrads were homozygous for all tested traits.

Discussion

Saccharomyces spp are in the vineyard

The results shed light on the question whether wine yeasts are present in nature or are 'found almost exclusively in man-created fermentation environments' [40]. This question has always been debatable because it never resolved how wine yeasts first got into 'man-created fermentation environments'. The suggestion to have *S. paradoxus* to be the 'sole natural species' of *Saccharomyces* sensu stricto [20,28,38,40] has not aided in resolving this debated question. By using intensive washing procedures and more selective media, we were able to isolate wine yeast strains from grape clusters, directly from grape berries, from grape juices crushed under aseptic laboratory conditions, as well as from laboratory fermentations. PCR- or CHEF gel electrophoresis-based screening were reliable tools in validating the isolation results. Identification is not complete yet because '... growth without vitamin supplement, maximum growth temperature, growth on D-mannitol as a sole carbon source, low to nil fermentation of maltose and ecology ...' [40] and the similar karyotyping patterns for

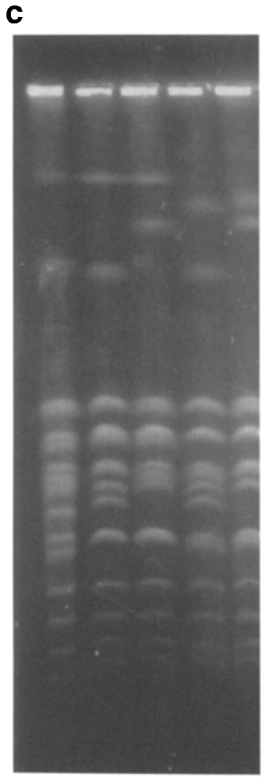

Figure 2c Electrophoretic karyotypes of a dissected tetrad of the wine yeast strain GgE.12-3. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lanes 2–5: monosporic clones. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 100 s for 38 h and from 75 s to 15 s for 8.5 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

S. cerevisiae and *S. paradoxus* vs a presumably distinct one for *S. bayanus* [21,22] are features that can vary within the four monosporic clones of a dissected tetrad of a *Saccharomyces* sensu stricto strain. They are therefore not practical for species delimitation. If we assume that the most characteristic feature of *S. paradoxus* is to sporulate readily on rich media like malt agar [28], about 20–25% of our wine yeast isolates from the grapes and different phases of fermentation showed this feature on YPD. On the other hand, there are literature reports on strains 'belonging' to *S. paradoxus* with only 40, 54, 60, 75 or 79% DNA relatedness to the type strain [4,37,40]. Our preliminary spore-to-spore crosses between wine yeasts and *S. cerevisiae* tester strains show 50–100% viability, while they gave 0% viability when crossing them with *S. paradoxus* tester strains that have either low or high DNA relatedness to the type strain (unpublished results). More spore-to-spore crosses, DNA/DNA reassociation studies and hybridization with known probes are being planned to finalize the identification.

A problem with isolating wine yeasts lies in their extremely low frequency, usually less than 0.1% of the naturally occurring yeast biota in the vineyard while the actual yeast titer may vary between 10^0–10^6 g^{-1}. Furthermore, our experience shows that each plant and every grape cluster is different, inferring that the crop of every vine or the berries of every cluster may not necessarily harbor wine yeasts. The use of more selective media and direct embedding of berries increased the chance of isolating wine yeasts

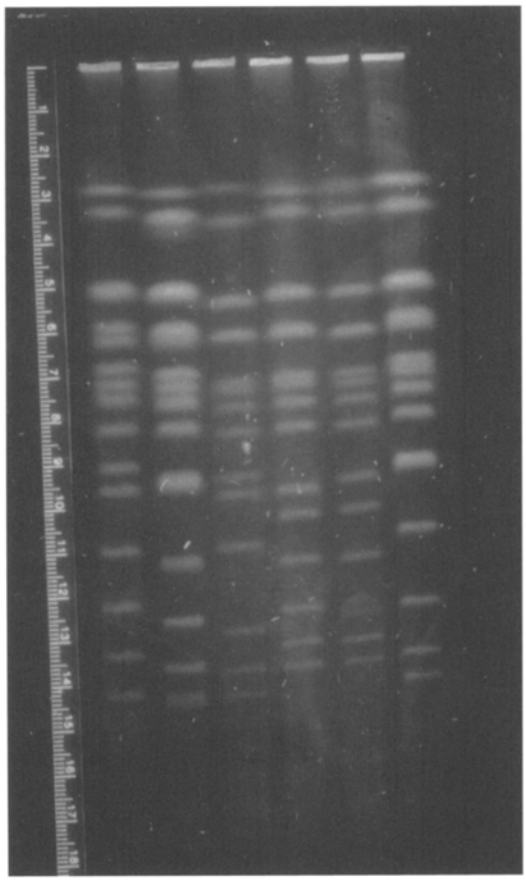

Figure 2d Electrophoretic karyotypes of a dissected tetrad of the wine yeast strain Bb 30(5)1. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lane 2: parental diploid; lanes 3–6: monosporic clones. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 20 s for 33 h and from 75 s to 15 s for 7 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

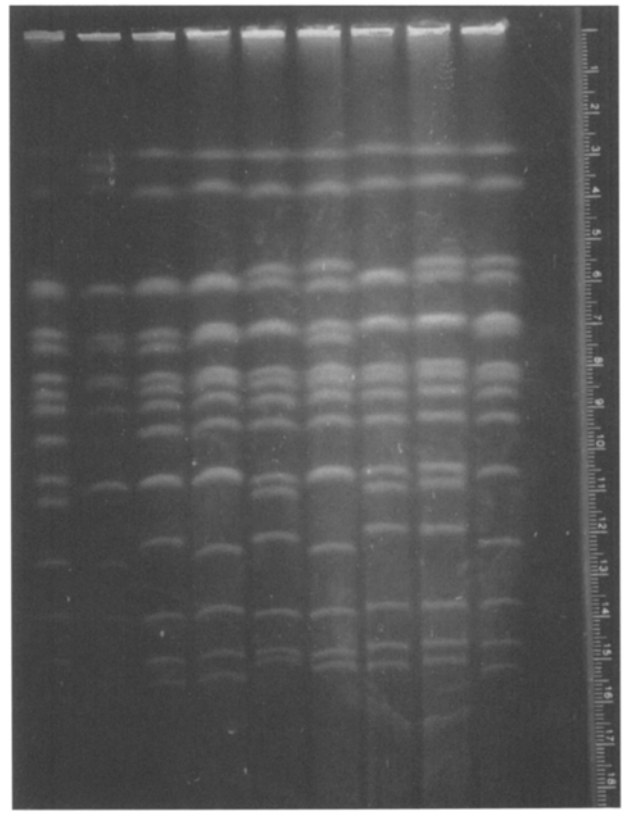

Figure 2e Electrophoretic karyotypes of two dissected tetrads of the wine yeast strain E2. Lane 1: yeast chromosome-size DNA standard YP 80 [YNN 295]; lanes 2–5: monosporic clones of tetrad 1; lanes 6–9: monosporic clones of tetrad 5. Running conditions: 6 V cm^{-1}, pulsing time ramping from 140 s to 20 s for 40 h and from 75 s to 15 s for 7 h, 0.9% SeaKem® LE agarose in 0.5 × TBE at 12°C.

'without enrichment' [40]. Nevertheless, enrichment is legitimate as long as the presence vs the absence of wine yeasts and not their quantification is the question being asked. PCR screening was only 50% effective either due to the procedure employed or because not all wine yeast strains carried the 2-μm plasmid.

Due to usually limited sample size, yeasts isolated from grapes and freshly extracted juice may represent only a fraction of the total wine yeast population present. Strains isolated at the end of fermentation and not found throughout the whole process may be falsely interpreted as coming from 'winery sources'. In this study, finding the same strains on the grapes, in the juice, during fermentation and at the end of fermentation was reassuring as was the reoccurrence of the same dominant strains in successive seasons. Nonetheless, there were cases where the yeast strains in the same single-vineyard fermentation in the '94 season were different from the ones isolated in '93. This also negates the winery-origin of the wine yeasts. Just recently, Versavaud presented similar results analyzing the occurrence and diversity of the spontaneous yeast population of the Charentes vine-growing region in France. He confirmed the polyclonality of the spontaneous *S. cerevisiae* population and showed that only a small number of dominant strains, representing together more than 60% of the monospecific population, is responsible for the fermentation process.

Electrophoretic karyotyping

Electrophoretic karyotyping is a useful tool in yeast taxonomy and is commonplace in quality control throughout the fermentation industry. Fortunately, electrophoretic karyotyping is reliable, and reproducible, relatively simple and affordable. Unfortunately, the information content of these karyograms is limited and interpretation of data should always be done with care. In the case of a vividly contested group such as the *Saccharomyces* sensu stricto complex, karyograms of monosporic clones should be compared only [22]. Comparison should be based on electrophoretic karyotyping of several dissected four-spore asci. If homozygous or only slightly heterozygous spore clones show an identical chromosomal banding pattern, it is easy to declare strain identity. In contrast, however, it is not sound to draw any conclusion with regard to conspecificity, species delimitation or differentiation, genome size, chromosomal length polymorphism or ploidy based on the number of chromosomal bands, their size, or ethidium bromide staining intensity alone. Especially, because different parts of the gel are more or less compressed depending on electro-

phoretic running conditions, separation of bands is compromised, and chromosome length polymorphism can be missed. In our hands, karyotypes of wine yeasts showed sometimes greater differences among the four monosporic clones of a dissected tetrad than among compared tetrads of the same isolate or random clones. Number of bands could go as high as 18–19 for a monosporic culture while we were still confident that we dealt with the same yeast species. Chromosomal-length polymorphism was widely spread in our samples and segregated most typically in a 2 : 2 pattern. In spite of running larger gels (21 cm long) with an extended pulsing protocol, we did not resolve many doublets, typically chromosomes VIII and V, XIII and XVI, and XV and VII. We also observed that sets of monosporic cultures of dissected tetrads of a given strain had very different electrophoretic karyotypes. One explanation could be increased heterozygosity of the strain. However, this is hard to prove because it results in reduced spore viability and allows fewer four-spore tetrads to be karyotyped. On the other hand, homozygous tetrads with daunting differences in their karyotypes were found.

Conclusions

To assess the large genetic diversity of naturally-occurring wine yeasts is a great challenge. It is important not only to isolate yeasts and show strain succession during fermentation, but to also understand biological processes such as 'genome renewal' [19], the cycle of events in the vineyard and their influence on the yeast biota, fermentation and wine complexity. With this study, credible proof now exists that the vineyard is the primary source for the wine yeasts and that strains found on the grapes can be followed through the fermentation process.

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