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Cellar-Associated *Saccharomyces cerevisiae* Population Structure Revealed High-Level Diversity and Perennial Persistence at Sauternes Wine Estates

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ABSTRACT

Three wine estates (designated A, B, and C) were sampled in Sauternes, a typical appellation of the Bordeaux wine area producing sweet white wine. From those wine estates, 551 yeast strains were collected between 2012 and 2014, added to 102 older strains from 1992 to 2011 from wine estate C. All the strains were analyzed through 15 microsatellite markers, resulting in 503 unique *Saccharomyces cerevisiae* genotypes, revealing high genetic diversity and a low presence of commercial yeast starters. Population analysis performed using F_{st} genetic distance or ancestry profiles revealed that the two closest wine estates, B and C, which have juxtaposed vineyard plots and common seasonal staff, share more related isolates with each other than with wine estate A, indicating exchange between estates. The characterization of isolates collected 23 years ago at wine estate C in relation to recent isolates obtained at wine estate B revealed the long-term persistence of isolates. Last, during the 2014 harvest period, a temporal succession of ancestral subpopulations related to the different batches associated with the selective picking of noble rotted grapes was highlighted.

IMPORTANCE

High genetic diversity of *S. cerevisiae* isolates from spontaneous fermentation on wine estates in the Sauternes appellation of Bordeaux was revealed. Only 7% of all Sauternes strains were considered genetically related to specific commercial strains. The long-term persistence (over 20 years) of *S. cerevisiae* profiles on a given wine estate is highlighted.

Saccharomyces cerevisiae is widely distributed and associated with human-related fermentations, as well as with those from the natural environment (e.g., oak trees and fruits). The population genetic structure of *S. cerevisiae* has been shown to correlate with its ecological differentiation (1–4), as well as geographical distance (2, 5). Strains isolated from vineyards and wine-related environments constitute a genetically well-differentiated homogeneous group. In the last 20 years, many studies have described the genetic diversity of *S. cerevisiae* isolates from different grape varieties. Molecular methods, such as pulsed-field gel electrophoresis (PFGE) (6), mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis (7), inter-*delta* analysis (8), and microsatellite analysis (9), were used to describe the genetic diversity of vineyard-associated *S. cerevisiae*. Numerous factors, such as climate conditions, geographical locations of the vineyards, fungicide management, grape varieties, and winemaking practices, impact the natural yeast population's diversity (10). Grapes are thought to be the first source of *S. cerevisiae* strains involved in the winemaking process, and winery surfaces are probably the main microbial reservoir to carry out must spontaneous fermentation (11). Gayevskiy and Goddard were the first to show evidence for region-specific *S. cerevisiae* populations associated with vines and wines in New Zealand using microsatellite markers, and they also pointed out the exchange of strains among these regions (12). The presence of specific fermentative profiles with perennial persistence over successive years in a given wine-producing area has been highlighted by different authors (13). However, a recent study challenged the view of a stable population in a wine environment over time, showing that no *S. cerevisiae* strain was isolated in the same vineyard or cellar during three consecutive years (8). Until now, very few studies have reported long-term observations of the changes in the *S. cerevisiae* population over time (14).

The Sauternes region in France, similar to the Tokaj wine region in Hungary, is one of the most famous and highly esteemed areas for noble rot sweet wines. Musts are obtained from noble rotted grape cluster selective pickings (15). The noble rot development is subject to weather conditions that dictate the number of selective pickings each year, typically up to three or four. The resulting grape musts have specific characteristics, with high sugar, acid, glycerol, and mineral contents; nitrogen deficiency; special polysaccharides; and aroma composition, which provide extremely difficult nutritional and environmental conditions for yeast growth and fermentative metabolism (16). As a consequence, yeasts produce high levels of acetic acid during alcoholic fermentation, ranging from 0.56 to 1.50 g/liter, depending on the

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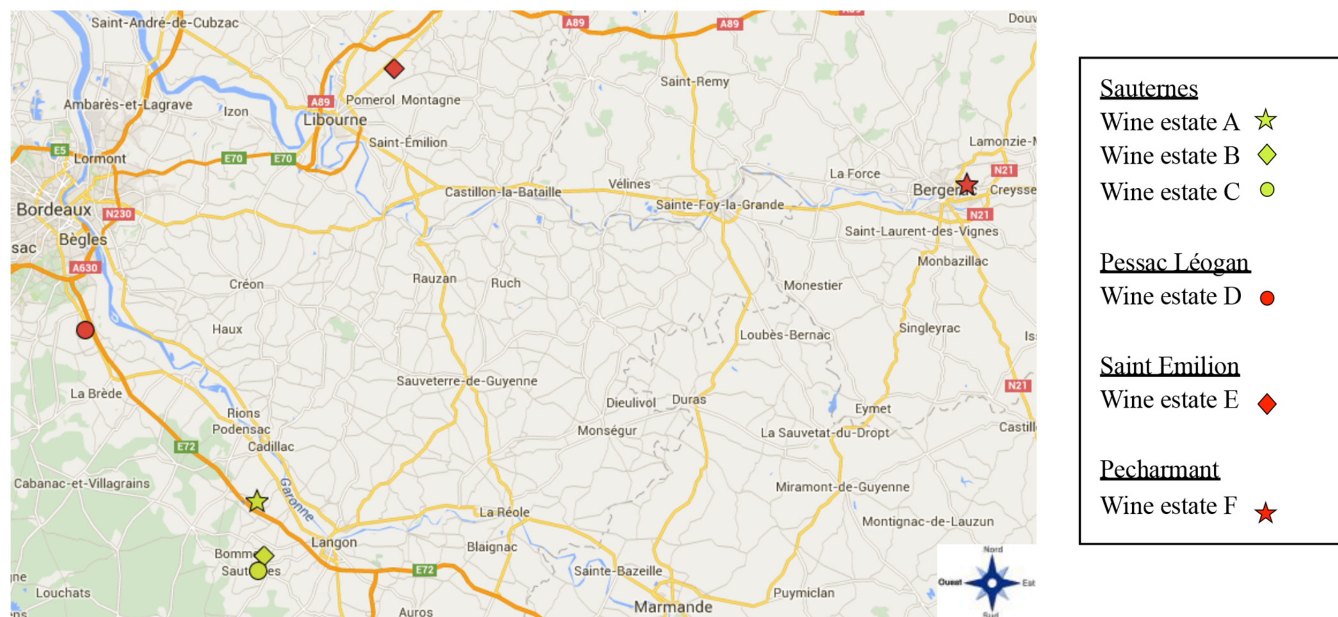

FIG 1 Geographic localization of the wine estates in the appellations of the Bordeaux and Bergerac regions. The yellow labels represent wine estates in the white wine Sauternes appellation, and the red labels represent wine estates in red wine Pessac Léognan, Saint Emilion, and Pecharmant appellations. (Template map source, Google Maps.)

must (17), and fermentation can be a slow process. Thus, the use of selected yeast starters and subcultures, or "pied de cuve," produced from fermenting must is generally recommended (18, 19).

Many previous studies reported the population dynamics on the surfaces of botrytized grapes and revealed a complex microbiota. *Botrytis* infection stimulates a high level of yeast diversity, and the community is likely enriched with fermentative and/or spoilage species (20). The presence of *S. cerevisiae* and *Saccharomyces uvarum* on Tokaj grapes has been described (21). The yeast microbial community of the grape must mirrors the grape microbiota and is highly diverse compared to that of traditional dry wines. *Candida zemplinina*, later renamed *Starmerella bacillaris*, could dominate fermentation during the first stages, and later, *Kluyveromyces*, *Hanseniaspora*, and *Pichia* were frequently isolated from midfermentation (22–25). Non-*Saccharomyces* yeast may contribute significantly to the fermentation of botrytized wines at early stages, but *S. cerevisiae* still dominates the fermentation process, frequently associated with *S. uvarum* (21, 23, 26, 27). The state of the damaged grape berries may impact the *Saccharomyces* yeast diversity and population level, since they may be very rich depositories of *S. cerevisiae* compared with sound berries (28). However, the *S. cerevisiae* population associated with the winemaking process of botrytized must has been poorly investigated until now. In a survey of wine estates in the southern region of Bordeaux, Frezier and Dubourdieu (13) described the existence of dominant *S. cerevisiae* profiles whenever white, red, and botrytized wine spontaneous fermentations were studied during two consecutive years. Later, Masneuf and Dubourdieu (29), using the PFGE method, established the karyotypes of 199 *S. cerevisiae* strains isolated from indigenous fermentation of botrytized must and reported high diversity in the profiles, with no dominant ones.

The occurrence of local and resident *S. cerevisiae* populations in a given viticultural region, and on a smaller scale in a given winery, is a recurring issue for the scientific community and winemakers. The microbial aspect of terroir was recently illustrated by different studies that suggested a link between vineyard environmental conditions and microbial inhabitation patterns and revealed the importance of microbial populations for the regional identity of wine (30, 31).

The objective of this study was to establish the population genetic structure of *S. cerevisiae* on a spatial (region/winery) and temporal (over 20 years) scale in the case of a fermentative system characterized by a highly complex microbiota and difficult nutritional and environmental conditions for yeast growth. For that purpose, we used a robust molecular method based on the analysis of 15 microsatellite markers. *S. cerevisiae* isolates were collected from spontaneous fermented must samples from three wine estates in the Sauternes appellation from 2012 to 2014. We aimed to gain deeper knowledge of cellar-associated *S. cerevisiae* ecology and possible exchanges between populations in the same appellation. We took advantage of having a large collection of *S. cerevisiae* isolates collected from spontaneously fermenting grape must since 1992 on one of the wine estates to survey the long-term diversity and population structure of cellar-associated *S. cerevisiae* and to test the hypothesis of the presence of specific wine cellar populations with perennial persistence in a given region or wine estate.

MATERIALS AND METHODS

Sample collection and processing. A total of 3 wine estates in the Sauternes appellation, which is one of the sweet-wine-producing areas in Gironde, part of the Aquitaine region in southwest France, were selected to conduct this study. The distance between wine estates A and B/C is 10 km, whereas the distance between wine estates B and C is 1.8 km. The three wine estates produce sweet wines from botrytized Sauvignon and Semillon grape varieties (Fig. 1). The initial sugar contents of the grape musts were between 350 and 450 g/liter. Wine estates A and B are managed according to organic practices, whereas wine estate C is managed conventionally. Briefly, sulfur and copper are used in both organic and

TABLE 1 Summary of samples collected in the Bordeaux and Bergerac regions

Wine estate	Yr	No. of samplings	No. of isolates			
			Analyzed by microsatellites	With <4 missing markers	After removing all similar clones	After removing those with >75% similarity to commercial strains
A	2012	2	55	54	52	47
	2014	4	120	114	110	110
B	2012	3	120	118	72	71
	2013	3	48	46	35	19
	2014	2	60	55	55	49
C	1992	5	43	43	43	43
	1993	2	32	25	25	18
	2002	NA[a]	6	3	2	2
	2007	NA	2	2	2	2
	2011	NA	19	15	15	14
	2014	5	148	129	106	105
D	2012	1				12
E	2013	1				9
F	2012	1				28
Total			653	604	517	529[b]

[a] NA, not applicable.
[b] A total of 480 of these isolates were from estates A, B, and C.

conventional farming systems, whereas synthetic fungicides are also used in the conventional system. Alcoholic fermentation was stopped thanks to a massive addition of sulfur dioxide (200 to 300 mg/liter). Samples were taken at 75% of the alcoholic fermentation from barrels containing 225 liters of must. In the wine estates during harvest periods, several pickings were made in different crops, with different numbers of lots from different years for the study (Table 1). At wine estate A, sampling was performed for 2 years, in 2012 and 2014, from 2 and 4 different lots, respectively. At wine estate B, sampling was performed for 3 years, 2012, 2013, and 2014, with 3 lots in 2012 and 2013 and 2 in 2014. Finally, sampling was performed at wine estate C in 2014 in 5 different lots.

Strain isolation. Different dilutions (10^{-4}, 10^{-5}, and 10^{-6}) of the collected samples were plated onto YPD (yeast extract, 1% [wt/vol]; peptone, 1% [wt/vol]; glucose, 2% [wt/vol]; agar, 2%, [wt/vol]) with 100 $\mu g \cdot ml^{-1}$ of chloramphenicol and 150 $\mu g \cdot ml^{-1}$ of biphenyl to delay bacterial and mold growth. A maximum of 40 randomly chosen colonies were collected after incubation (2 days at 26°C). After two subclonings on YPD plates, each colony was stored in glycerol (30% [vol/vol]) at −80°C.

Additional isolates. For wine estate C, *S. cerevisiae* isolates collected since 1992 that were kept in the laboratory collection at −80°C were added to the data set collection, increasing the original data set sampled by 102 new isolates (Table 1).

As a possible external group, 49 new isolates collected from 3 red wine estates belonging to 3 different Bordeaux and Bergerac appellations were added to the data set. Appellation Saint Emilion was represented by wine estate D, Pessac Léognan by wine estate E, and Bergerac by wine estate F.

In addition to cellar samples, 33 yeast strains from diverse origins whose genomes have been sequenced (3, 4) (see Table S1 in the supplemental material) and 35 commercial wine strains (see Table S2 in the supplemental material) widely used as yeast starters were added to the data set.

Molecular methods. Considering that all yeasts collected at the stage of wine must fermentations where 75% of the alcohol is fermented would very likely belong to the *S. cerevisiae* species and that this technique provides complete genotypes only for *S. cerevisiae* strains (32), all colonies were directly analyzed by microsatellites. For each of them, a small amount of fresh colony was suspended in 50 μl of Milli-Q water, and 7 μl of this suspension was dropped on an FTA card for DNA preservation. The samples were then genotyped using 2 multiplex PCRs of 8 and 7 microsatellite loci, respectively, for mixtures 1 and 2 (see Table S3 in the supplemental material) (32–37). Mixtures were prepared in a total volume of 84 μl for 8 samples, with 50 μl of 2× Qiagen Multiplex PCR master mix, 15.5 μl primers, and 18.5 μl water. Mixture 1 had 8 multiplexed primers, and mixture 2 had the other 7; each of them had a specific concentration as specified in Table S3 in the supplemental material. The PCRs were run in a final volume of 12 μl containing 10.5 μl mix and 2 μl of cell suspension. The following PCR program was used: initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 57°C for 2 min, 72°C for 1 min, and a final extension at 60°C for 30 min. The PCR products were sized on an ABI3730 DNA analyzer (Applied Biosystems) using size standard 600LIZ (GeneScan).

Data analyses. ABI3730 genotyping results were read using Gene Marker (V2.4.0, Demo). The presence of a missing value was allowed up to a maximum of 3 markers per sample. Estimation of population diversity was by rarefaction of 10,000 individuals repeated 10 times. The Shannon index (H'), the equitability index (J'), and the inverse Simpson diversity index ($D - 1$) were calculated with EstimateS (V9) (38) using the individual-based abundance method for intracellar analysis and sample-based abundance data for the whole-region sampling. H' was determined with the following equation: $H' = -\sum_{i=1}^{S} P_i \ln(P_i)$, where S is the total number of genotypes in the population and P_i is the proportion of a specific genotype in the data set. D was determined with the following equation: $D = \Sigma \frac{N_i(N_i - 1)}{N(N - 1)}$. The term P_i was calculated as follows: $P_i = N_i/N$, where N_i is the number of individuals for a specific genotype and N is the total number of unique genotypes. GenClone (V2.0) software was used to remove strains with exactly similar profiles, resulting from potential clonal expansion, from our data set (39). Observed and expected heterozygosity, F_{st} (genetic distance), and analysis of molecular variance (AMOVA) were determined using Arlequin (V3.5.2.2) software (40).

SplitsTree V4.12.6 (41) was used to reconstruct a neighbor net phylogenetic network for *S. cerevisiae* using Bruvo's distance (42), calculated using the R program (43) with the following packages: ape (44) and poppr

TABLE 2 Diversity analysis of the three wineries of the Sauterne appellation

Parameter	Value[a] A	B	C	Total
No. of individuals	168	219	217	604
H'	5.04	4.57	5.11	6.22
J'	0.995	0.90	0.99	1.00
$1 - D$	0.993	0.97	0.992	0.998

[a] The analyses are based on the 604 genotypes obtained after microsatellite typing of the isolates from the 3 different wine estates of the Sauternes appellation. The individual-method estimate was used for each cellar and the sample-based method for the estimates for the whole region.

(45). The population structure was evaluated using a Bayesian clustering method with the software InStruct, which does not account for the Hardy-Weinberg equilibrium (46). Five chains of 150,000 iterations with a burn-in of 5,000 were run for a possible population number (K) of 1 to 25. The most likely number of ancestry populations was selected by choosing the lowest deviance information criterion (DIC). Ancestry profiles were drawn as bar plots from the Instruct output, using a different color for each inferred ancestral population under the R statistical environment. The contribution of each population was then evaluated with ObStruct software (47).

RESULTS

Cellar sample diversity. To investigate *S. cerevisiae* population diversity in the typical appellation of Sauternes in the Bordeaux region, 3 wine estates, A, B, and C, were selected. Samples of spontaneously fermenting must were taken before mutage and at different times of the harvest corresponding to selective pickings. A total of 653 colonies were collected on the wine estates between 1992 and 2014 and analyzed by 15 microsatellite markers. Isolates with genotypes with missing values at more than 3 markers were removed from the data set. A summary of the samplings, years, and numbers of *S. cerevisiae* colonies with completed microsatellite genotypes collected is provided in Table 1. For wine estate C, *S. cerevisiae* strains from the laboratory collection that were isolated between 1992 and 2011 were included in the study (29). After microsatellite analyses, 43 additional *S. cerevisiae* isolates with full microsatellite genotypes were kept, giving a final data set of 604 *S. cerevisiae* isolates for further analysis (Table 1).

In order to compare the diversities of the yeast populations obtained from the three wine estates, we calculated three diversity indices using EstimateS: the Shannon index (H'), which measures the diversity within a population and takes into account both richness and evenness; the inverse Simpson index ($1 - D$), which gives more weight to common or dominant species; and the Pielou evenness index (J'). The different indices were evaluated on the basis of the number of different genotypes (Table 2) and on the standard deviations of H' and $1 - D$; all the results were significantly different. The Shannon index (H') showed strong diversity in all 3 wine estates (over 4.50), with a slight decrease of diversity for wine estate B. The Pielou index (J') was always close to 1, suggesting that genotypes have similar abundances within the population. The inverse Simpson index ($1 - D$) results were in accordance with the H' and J' indices, with high values over 0.97. When considering wine estates as a sample of the Sauternes appellation, the Pielou index value was even higher, reaching 100% diversity and again confirming the results from the inverse Simpson and diversity indices. The diversity index of the Sauternes *S. cerevisiae* population was similar to the diversity index obtained for the Merlot red wine cellar *S. cerevisiae* population (270 individuals), with H' and J' indices of 5.38 and 0.96, respectively (data not shown). The whole-appellation diversity, evaluated by the rarefaction analyses, estimated a number of unique genotypes on the whole-appellation scale greater than 72,533 (with 95% confidence limits of 7,010 to 138,057) and evaluated it with a sampling design including more than 1,000 *S. cerevisiae* samples throughout the region to achieve full diversity. The small number of genotypes shared between cellar sampling populations explains this high diversity.

Strain genetic relationships. Due to clonal expansion of individual genotypes during fermentation, it was necessary to remove all identical genotypes within each sampling site before accessing the genetic relationships. From the initial 604 *S. cerevisiae* isolates, the GenClone software inferred 503 unique genotypes, grouping a total of 517 isolates from all 3 wine estates with 14 genotypes shared between the 3 wine estates (Table 1). Thirty-seven industrial *S. cerevisiae* strains widely used in the Bordeaux region and in Sauternes appellations specifically were then added to this data set in order to detect the potential presence of yeast starters within cellar populations, and 49 *S. cerevisiae* isolates from Bordeaux region Merlot must fermentations were also included in the analysis (Table 1). Finally, 33 *S. cerevisiae* strains of various origins whose genomes have been recently sequenced were also included as an outgroup in our data set.

For the relationship between cellars and commercial strains, the 636 isolates were accessed from a phylogenic network built from the Bruvo's pairwise distance matrix (Fig. 2). As expected, one cluster gathered the sequenced strains of different origins (group I), and most of the sequenced strains originating from a wine environment clustered with our wine isolates, except for clinical strains (YJM978, -981, and -975) and baker strains (YS2, YS4, and YS9), which were grouped in the same branches, including Sauternes strains and commercial strains (arrows 2 and 1, respectively). Note that the sequenced strain YIIc17_E5, the genome for which has been sequenced, was isolated from the Sauternes region in 1992 and clustered with strains isolated on wine estate C in 1992 (arrow 3). Concerning the Sauternes cellar *S. cerevisiae* population, some branches clustered isolates from one wine estate with very close genetic relationships (group II), suggesting clonal variants. Those branches were mostly observed for wine estates A and C and to a lesser extent for wine estate B. Other branches were composed of clusters mixing wine estate B and C isolates, with only rare isolates from wine estate A (group III). Finally, there also appeared to be possible links between cellar strains and commercial strains (arrow 4), whichever wine estate was considered. Concerning Merlot isolates, all the strains from wine estate D and most from wine estate F clustered apart from the others (group IV), whereas a few isolates from wine estates E and F were spread over the network.

To further compare the populations by wine estate, F_{st} statistics between all Sauternes and Merlot wine estates were calculated (Table 3). All the population comparisons indicated a significant difference ($P < 0.001$). As suggested by the individual network, there was higher differentiation between wine estates A and B (0.109) than between wine estates B and C (0.038), with the latter being more than twice as low as the comparison of A and C (0.145, the highest Sauternes pairwise F_{st}). Pairwise F_{st} statistics between Sauternes wine estates and Merlot wine estates ranged from 0.103

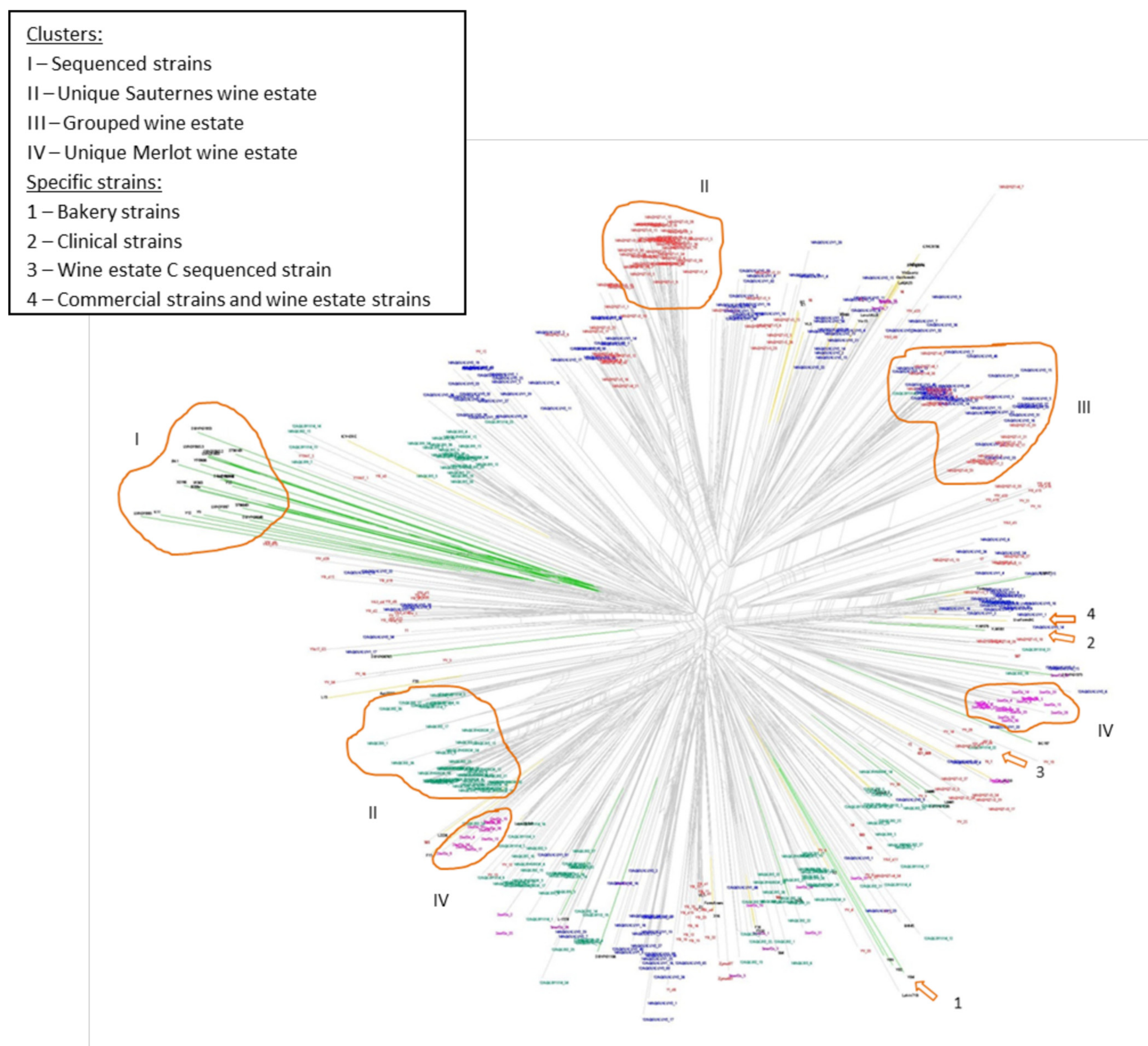

FIG 2 Neighbor net network of 636 strains from the cellars of the 3 wine estates, 3 commercial strains, 33 strains from the *S. cerevisiae* sequenced database, and 49 strains from Merlot must fermentations. The network was constructed from Bruvo's distance between strains based on the polymorphism at 15 loci. Green, wine (red dots) and nonwine sequenced strains; yellow, commercial strains; green labels, domain A; blue labels, domain B; red labels, domain C; pink labels, wine estates D, E, and F.

TABLE 3 Pairwise F_{st} statistic values between the 6 different Sauternes and Merlot wine estates after combining the different years of sampling

	F_{st} or *P* value for estate[a]:					
Estate	A (162)	B (162)	C (193)	D (12)	E (9)	F (28)
A		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
B	*0.109*		**0.001**	**0.001**	**0.001**	**0.001**
C	*0.145*	*0.038*		**0.001**	**0.001**	**0.001**
D	0.222	0.299	0.325		**0.001**	**0.001**
E	0.103	0.105	0.139	0.327 (underlined)		**0.001**
F	0.147	0.120	0.165	0.399 (underlined)	0.138 (underlined)	

[a] F_{st} values are in lightface, and *P* values are in boldface. All values are significant ($P < 0.001$). Italics indicate a comparison between Sauternes wine estates; underlining indicates a comparison between Merlot wine estates. The numbers of strains are in parentheses.

to 0.165, indicating a moderate but still notable differentiation. However, pairwise F_{st} statistics between Sauternes wine estates (A and C) could be higher than pairwise F_{st} statistics between Sauternes and Merlot wine estates (e.g., A and E or B and F), whereas the geographical distance was greater in these cases. Surprisingly, the pairwise F_{st} distances between Merlot wine estate D and any other Sauternes or Merlot wine estates were high (0.222 to 0.399), indicating strong differentiation, shown by the external positions of individuals in the network, which might also be explained by the lower number of strains from the sample.

As highlighted in the network, some cellars' isolates appeared to be very close to commercial strains. Cellar isolates were further

TABLE 4 AMOVA analyses, F_{st} values, and distribution of variance components based on microsatellite data for *S. cerevisiae* isolates obtained from the indicated groups of wine estates and vintages

Fixed parameter[a]	Variable parameter[a]	% variation			F_{st}	$P (r < 0)$
		AG	APWG	AIWP		
A	2012, 2014	6.23	5.14	88.62	0.113	<0.000001
B	2012, 2013, 2014	1.66	5.06	93.26	0.063	<0.000001
	2012, 2013	3.75	5.16	91.08	0.089	<0.000001
	2012, 2014	1.56	3.92	94.51	0.055	<0.000001
	2013, 2014	0	7.17	93.21	0.067	<0.000001
C	2014, 1993, 1992	13.14	15.02	71.83	0.281	<0.000001
	1992, 1993	0	11.91	91.14	0.088	<0.000001
	1992, 2014	13.91	15.51	70.57	0.294	<0.000001
	1993, 2014	19.31	16.28	64.41	0.355	<0.000001
2012	A, B	11.69	4.19	84.11	0.158	<0.000001
2014	A, B, C	22.42	8.68	68.89	0.311	<0.000001
	A, B	12.27	4.06	83.66	0.163	<0.000001
	A, C	31.04	8.77	60.18	0.398	<0.000001
	B, C	12.53	14.12	73.34	0.266	<0.000001

[a] Vintages (years) or wine estates (A, B, and C).

considered genetically related to the industrial strains when they shared at least 75% of the alleles. For example, one isolate from wine estate B was related to commercial strain VL3 and 4 isolates to strain X5. For commercial strain UvaFerm BC, 20 cellar isolates were related to the starter strain, 1 obtained from wine estate A, 18 from wine estate B, and 1 from wine estate C. Finally, 4 cellar isolates from wine estate A were related to commercial strain Levuline BRG. Commercial strains VL3, X5, UvaFerm BC, and Levuline BRG have been frequently used in the Sauternes region during the last 30 years, even by wine estates following organic agricultural practices. Nevertheless, only 7% of all Sauternes strains were considered genetically related to specific commercial strains, indicating a minor but substantial relationship between cellar and commercial strains (Table 1). In order to limit the potential impact of yeasts related to commercial starters on the detection of yeast population structures, they were removed from the data set, and differentiations between Sauternes and Merlot wine estates were estimated again, but the results did not change in a substantial manner (data not shown).

Population structure. An AMOVA was then further performed in order to understand how genetic variations at these 15 microsatellite loci are structured (Table 4). For wine estate C, only samples from 2014, 1993, and 1992 were taken into account for the analysis, since isolates from several samples were available, and different groups were tested according to the year of sampling or the wine estate. The contributions of variation among individuals within groups (AIWP) always explained most of the global variation, ranging from 60 to 94% of the total variance. The percentage comparisons of variation among groups (AG) and among populations within a group (APWG) indicate different patterns. The comparison of genetic diversity from the different wine estates indicates that the wine estate has the highest impact on genetic diversity, as this factor explains from 11.7 to 31% of the genetic variability, according to the comparison. Wine estate A appeared to be more differentiated from wine estate C (31%) than from B (12.3%), whereas B and C were similarly differentiated (12.5%). Notably, these comparisons led to a moderate within-group variability (from 4.2 to 8.8%, except for the B-C comparison, with 14.1%) and a low variation among individuals within a population (AIWP). On the other hand, the vintage contributed less to the global variation for close vintages on a wine estate, with 0 to 6% global variation and a low intersample variation (5 to 7% variation in APWG) and the highest values for variation AIWP (88.6 to 94.5%). However, winery C presents a unique picture, as the differences between the most distant vintages explain the highest genetic variation (13.1 to 19.3%), whereas the differences between the 1992 and 1993 vintages were the lowest, similar to what can be observed for 2012 and 2013 and for 2013 and 2014 for wine estate B. Interestingly, this wine estate also shows the highest sample-to-sample variation (APWG explains 12 to 16% of the global variation).

We used InStruct to evaluate the population structure from shared ancestry. The deviance information criterion indicated the most likely population number (K) to be a value of 19 (Fig. 3). In an overall view, whatever year or wine estate was considered, numerous strains were composed of a mosaic of ancestral subpopulations. Unique ancestral populations associated with a given wine estate were highlighted (A, D, and F in 2014). Strains from wine estate E presented mainly a mosaic ancestry, which may explain their dispersion over the individual network. Wine estate A shared few ancestral populations with wine estates B and C, whereas one of the main ancestral populations of wine estate C was shared with wine estate B, which also accounts for the mixed group seen in the network. The relationship between wine estates B and C was illustrated by shared ancestral populations in 2014 and, to a lesser extent, in 1992 and 1993. Except for wine estate B, where ancestral populations persisted through vintages (2012 to 2014), for wine estates A and C, only a few ancestral populations were shared from one year to another. Moreover, in 2014, 2 new ancestral subpopulations appeared to be predominant and absent from the former vintages for both wine estates A and C. When focusing on the population structures of wine estates A and C in 2014, a temporal succession of two ancestral subpopulations was clearly related to the different batches associated with the selective picking of noble rotted grapes (Fig. 4).

The ObStruct program permits an evaluation of the significance of different factors in the ancestry profile obtained from Instruct. Here, we can test the effect of the wineries on the population of Sauternes or Merlot wine estates (see Fig. S1 and Table S4a in the supplemental material). The two Merlot wine estates D and F had a strong influence on the global population structure. Sauternes wine estate B also contributed to this population structure shaping, but at a lower level. To focus on the Sauternes appellation, Merlot wine estates were removed from the data set. The ObStruct results for Sauternes wine estates only (see Table S4b in the supplemental material) showed that wine estate A still had the strongest influence on the shape of the Sauternes population structure, in agreement with the fact that it is clearly distinguished from the other 2 wine estates. Wine estates B and C had a lesser influence on the population structure, with B contributing slightly more than C.

DISCUSSION

Sauternes is a particular appellation of the Bordeaux region producing high-quality sweet wines. The development of noble rot on

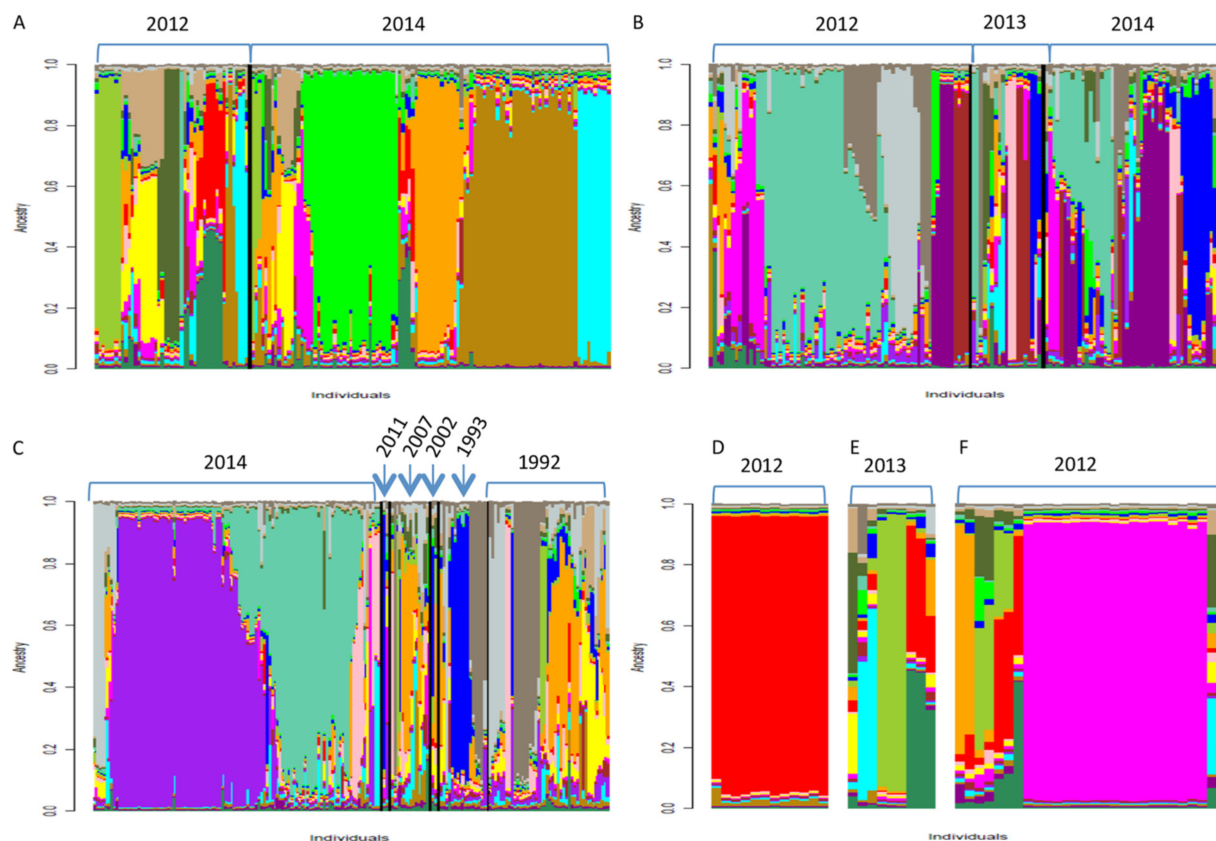

FIG 3 Inference of populations using the InStruct program on the 604 *S. cerevisiae* cellar strains with the optimal *K* value of 19 classified according to years for each wine estate. (A) Wine estate A. (B) Wine estate B. (C) Wine estate C. (D) Wine estate D. (E) Wine estate E. (F) Wine estate F.

grapes results in the production of highly concentrated grape musts and typical wines (19). Fermentation conditions are highly stressful for wine yeast, mainly due to a high sugar content and a low level of assimilable nitrogen. A total of 653 isolates were collected over 3 consecutive years (2012, 2013, and 2014) on 3 different wine estates. Moreover, 102 additional strains collected from 4 to 23 years ago on wine estate C were added to our population sample. A highly discriminating method based on 15 microsatellite markers specific to *S. cerevisiae* was used for molecular typing at the strain level. In comparison to previous studies based on other methods, such as PFGE, mtDNA RFLP, and inter-*delta* analysis (6, 8, 48), this *S. cerevisiae* ecological study used deep sampling and relied on the robustness of the microsatellite marker method (36, 49–51), performed with 15 loci as microsatellite markers and providing more sensitivity than previous studies. Multilocus microsatellite analysis allowed us to evaluate the genetic diversity of our population. A total of 503 genotypes were revealed from an initial population of 653 *S. cerevisiae* isolates (77% of the different genotypes), indicating high genetic diversity, and 97% of the genotypes were wine estate specific. By sampling 21 different white and red ferments across three different regions in New Zealand, Gayevskiy and Goddard (12) obtained 353 *S. cerevisiae* isolates and 274 genotypes (78%) using 10 microsatellite markers, which is similar to our data but with lower diversity. However, our estimate of yeast diversity suggests that the Sauternes region is expected to contain an extremely high diversity of *S. cerevisiae* strains, with an underlying population of more than 72,533 unique genotype strains (with a wide confidence interval), a figure far higher than the estimate of 1,700 inferred for the New Zealand vineyard (52). The diversity index obtained for the Sauternes *S. cerevisiae* population was also similar to the diversity index obtained for the Merlot red wine cellar *S. cerevisiae* population. Several causes might explain these differences between our data and those obtained from New Zealand vineyards: the first obvious explanation might lie in the use of additional markers, enabling a deeper exploration of the diversity; however, as the most polymorphic markers are shared by the two studies, we doubt that this factor alone explains the differences. Because damaged grape berries may be very rich depositories of *S. cerevisiae*, in comparison to sound berries (28), we might expect to obtain higher diversity index values for botrytized ferment populations. However, on the contrary, the specific botrytized grape must composition, with a high sugar content, and the interaction with the action of *Botrytis cinerea* may constitute a highly selective medium, potentially limiting *S. cerevisiae* strain diversity. Finally, the recent origin of the New Zealand vineyard and its associated yeast population (M. R. Goddard, personal communication) may also provide another explanation. Indeed, the yeast diversity may carry the signature of the initial bottleneck associated with the founder effect of its introduction into New Zealand, resulting today in lower diversity despite its partial recovery.

The main objectives of the study were to define the population genetic structure and diversity of *S. cerevisiae* on both the Sauternes appellation and wine estate scales. The impact of commer-

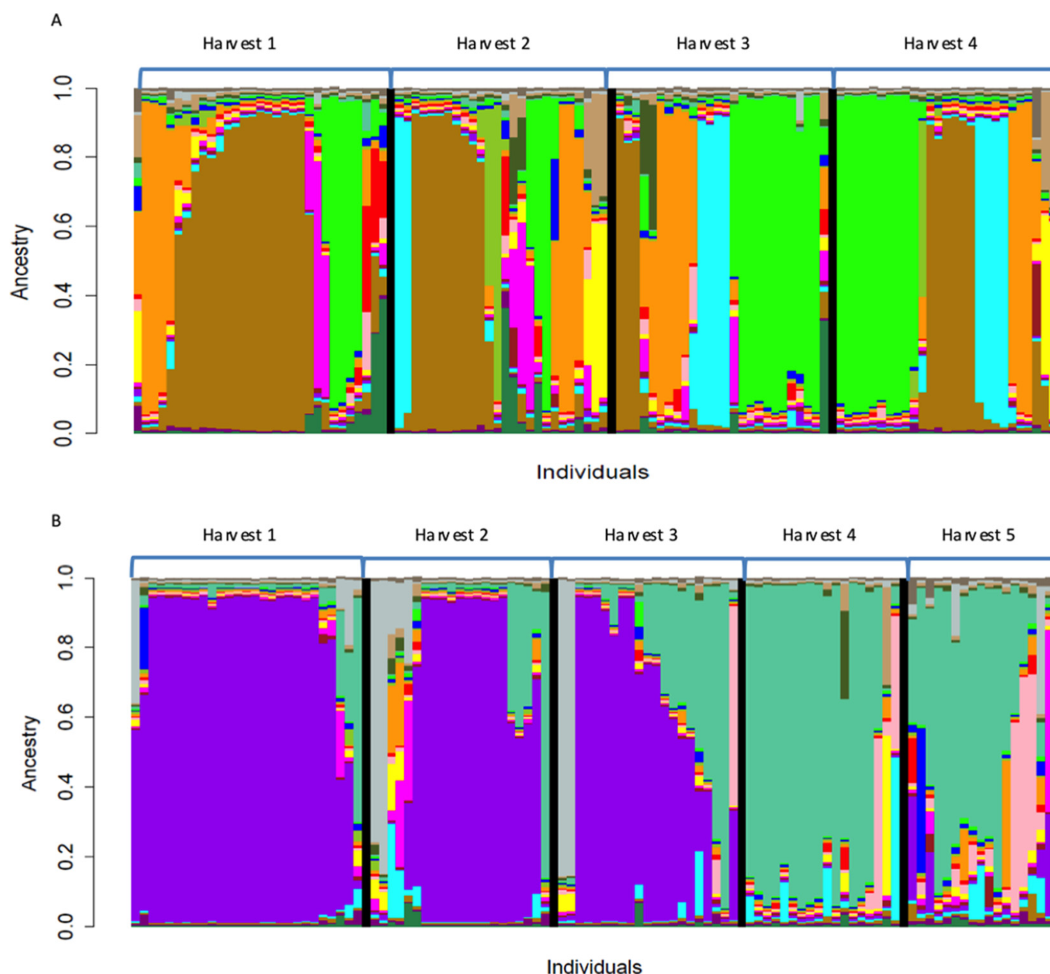

FIG 4 (A) Inference of populations using the InStruct program on the 110 *S. cerevisiae* strains with the optimal *K* value of 19. The strains originated from wine estate A in 2014 and were classified according to the 4 different harvest batches. (B) Inference of populations using the InStruct program on the 105 *S. cerevisiae* strains with the optimal *K* value of 19. The strains originated from wine estate C in 2014 and were classified according to the 5 different harvest batches.

cial strains on the diversity of endogenous wine yeast strains is still controversial, since some authors have shown that the use of active dry yeasts reduced the variability of wine cellar strains (14), whereas other studies did not evidence any impact (6, 12, 53). In this study, only 7% of cellar strains were found to be related to 4 commercial strains usually used in sweet and dry white wine making in the Bordeaux region for over 25 years. Moreover, no significant variation in wine estate pairwise F_{st} values were obtained before and after removing strains genetically related to commercial starters. Despite the past or present use of yeast starters to inoculate dry white wines in the wine estates studied, this practice had a small impact on *S. cerevisiae* diversity and population genetic structure on the winery scale in the Sauternes region.

AMOVA, pairwise F_{st}, and ancestry profile and ObStruct analyses showed contrasting results concerning genetic differentiation between populations originating from different wine estates. While population differentiation between wine estate A and wine estates B and C was high, a much smaller differentiation was observed between wine estates B and C. Ancestry profile analysis provided evidence that wine estate B and C populations are mixed to a certain degree. Taking into account the short geographic distance between wine estates A, B, and C (less than 10 km), it is not realistic to postulate that the various degrees of genetic differentiation between wine estate populations are linked to their respective geographic distances. However, one of the possible explanations of the small differentiation between wine estates B and C in comparison to A is the short distance between B and C, which have juxtaposed vineyard plots. At such a short distance, insects like bees, wasps, and fruit flies, as well as birds, which are known to be vectors for yeasts, could have homogenized these yeast populations (51, 54, 55). Humans can also influence the yeast population structure and promote dispersal (49). Wine estates B and C shared seasonal staff and wine-growing equipment during the harvest and fermentation periods, which may also have facilitated exchanges between the *S. cerevisiae* populations of the two estates. On the very small scale of the appellation, this is an illustration of possible *S. cerevisiae* dispersion.

During a period of 23 years, strains from wine estate C were collected, and we could observe the systematic persistence of specific ancestral populations that were never dominant on wine estate C. The ancestral populations observed in 1992 and 1993 at winery C were also detected in the sampling performed during the 2012-2014 period on wine estate B but were absent on wine estate A. This result demonstrates, on the small scale of two wine estates,

the existence of a local and stable group of strains with shared ancestry over 20 years, as well as the occurrence of multiple yeast population exchanges between the two wine estates over time. The phenotypic traits of this local and long-term stable group of strains would be interesting to investigate, in order to better understand to what extent those ancestral *S. cerevisiae* populations may contribute to the characteristics and typicality of the wine produced in this area.

Previous consecutive-year follow-up studies reported contrasting results concerning the possible establishment of strains as resident at a given winery (8, 14). The comparison by AMOVA of samples obtained from wine estate C over a long period revealed that the variation between the most distant years (1993 and 2014) provided more differences than a comparison of different samples from the same year or from successive years (1992 and 1993 or 2013 and 2014). From this preliminary analysis, we could hypothesize that time, over the long term, may be a key factor for genetic differentiation between cellar-resident *S. cerevisiae* populations at a given winery.

Finally, a cellar-associated *S. cerevisiae* population during the harvest period of 2014 for wine estates A and C was more closely explored. Ancestry profile analysis revealed a clear temporal succession of two main ancestral populations for wine estate C and, to a lesser extent, for wine estate A during the harvest campaign. A characteristic of both wine estates compared to wine estate B was the use of fermented batches to inoculate the others. This method, named "pied de cuve," was shown to better acclimatize the yeast inoculum to the high sugar content of the fermentation medium. Such a stress factor provokes the upregulation of structural genes involved in glycerol synthesis and intracellular accumulation by *S. cerevisiae* in response to external osmolarity (56, 57), which results in the formation of acetic acid from acetaldehyde (58). The use of yeasts collected from already fermenting wine is advantageous, since the yeast cells had had the opportunity to acclimate to the high sugar content of the musts and produced less acetic acid than selected starters inoculated directly (19, 59). Our data indicated that the selection of specific ancestral *S. cerevisiae* populations through successive fermentations may also be favored by the use of subculture on wine estates A and C. Still, the factors that explain the selection of given ancestral populations remain to be elucidated. In the case of Sauternes winemaking, the sugar content of the musts, which is dramatically increased during harvest, with concentrations as high as 40 to 45% (wt/vol) at the end of the campaign, is probably a key parameter. In the case of wine estate C, this selection of one ancestral population during the harvest period was highlighted but raises the underlying question, to what extent does the increase of the must's sugar content explain this temporal succession?

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