



# Diversité et structure de population des levures *Saccharomyces cerevisiae* à l'échelle du vignoble bordelais : Impact de différents facteurs sur la diversité

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Par Marine BÖRLIN

**DIVERSITE ET STRUCTURE DE POPULATION DES  
LEVURES *SACCHAROMYCES CEREVISIAE* A L'ECHELLE  
DU VIGNOBLE BORDELAIS**

**Impact de différents facteurs sur la diversité**

Sous la direction d'Isabelle MASNEUF-POMAREDE

Soutenue le 17 décembre 2015

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**Titre : DIVERSITE ET STRUCTURE DE POPULATION DES LEVURES  
SACCHAROMYCES CEREVISIAE A L'ECHELLE DU VIGNOBLE BORDELAIS -  
Impact de différents facteurs sur la diversité**

**Résumé :** *Saccharomyces cerevisiae* est l'acteur principal de la fermentation du moût de raisin, mais l'influence de facteurs sur sa distribution dans les vignobles est peu connue. La région Bordelaise, par son histoire et ses appellations, est une région d'intérêt pour étudier la diversité de *S.cerevisiae*. Au total, 2422 isolats de *S.cerevisiae* provenant de prélèvements de raisins et de cuves en fermentation spontanées sur deux années consécutives ont été analysés par 15 à 17 marqueurs microsatellites. Une très grande diversité génétique est mise en évidence, supérieure en mode de conduite conventionnel par rapport au mode biologique. Le mode de conduite influence faiblement la structure de la population de *S.cerevisiae* au vignoble. L'appellation et le domaine impactent significativement la structure de population, sans que des gradients de diversité n'apparaissent mais nos analyses révèlent des connections importantes dans le sens Pessac-Léognan vers les autres appellations du Bordelais, en particulier le Médoc. Des flux importants bidirectionnel sont mis en évidence entre les compartiments vigne et chai, illustrés par la présence de 25% de souches apparentées à des levures commerciales au vignoble, retour des souches du chai au vignoble jusqu'alors sous-estimé, alors qu'un flux d'importance similaire est observé entre le vignoble et le chai. La présence de populations ancestrales communes dans des prélèvements anciens (plus de 20 ans) et récents révèle la stabilité des populations sur le long-terme à l'échelle d'une appellation. Une succession temporelle des populations du chai pourrait être favorisée par la mise en œuvre de pied de cuve avec repiquages successifs.

**Mots clés :** *Saccharomyces cerevisiae*, vin, diversité

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**Title: DIVERSITY AND POPULATION STRUCTURE OF YEAST  
SACCHAROMYCES CEREVISIAE AT THE SCALE OF THE VINEYARD OF  
BORDEAUX - Impact of different factors on diversity**

**Abstract:** *Saccharomyces cerevisiae* is the main actor of wine fermentation but still little is known about the factors impacting its distribution in the vineyards. Bordeaux region, by its history and its appellations, is one of the regions of interest to study *S. cerevisiae* diversity. A total of 2422 isolates of *S.cerevisiae* sampled from grapes and spontaneous fermentation tanks during two consecutive years were analyzed by 15 to 17 microsatellite markers. A very high genetic diversity is demonstrated, greater in conventional farming system than in organic one. The type of farming system weakly influences the population structure of *S.cerevisiae* in the vineyard. The appellation and the wine estate significantly impact the population structure, without appearance of diversity gradients, but our analyses reveal important connections from the Pessac-Léognan to other Bordeaux appellations, especially to the Medoc. Bidirectional strong flows are highlight between the vineyard and the cellar compartments as illustrated by the presence of 25% of commercial related strains in the vineyard, due to the unexpected return of strains through cellar to the vineyard, while a flow of similar magnitude is observed between the vineyard and the cellar. The presence of common ancestral populations in old (over 20 years) and recent samples showed population stability over the long-term at an appellation scale. A temporal succession of cellar populations was highlighted that could be link with the implementation of the Pied de Cuve method through successive inoculations.

**Keywords:** *Saccharomyces cerevisiae*, wine, diversity

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## **Liste des abréviations**

ADN: Acide DésoxyriboNucléique

ADY: Active Dry Yeast

CO<sub>2</sub>: Dioxyde de carbone / Carbone dioxyde

DNA: DeoxyriboNucleic Acid

FA: Fermentation Alcoolique

FM: Fermentation Malolactique

LSA: Levure Sèche Active

NS medium: Non-*Saccharomyces* medium

PCR: Polymerase Chain Reaction

PdC: Pied de Cuve

SO<sub>2</sub>: Dioxyde de Soufre / Sulfure Dioxyde

TY medium: Total Yeast medium

YPD: Yeast extract – Peptone – Dextrose

## **Introduction**

### **1) La vinification**

La vinification se rapporte à toutes les étapes allant de la récolte du raisin jusqu'à la fin des fermentations et comprend une étape de macération, qui consiste à extraire sélectivement dans le jus les composés d'intérêt essentiellement des pellicules.

#### **a) En vinification en rouge**

La première étape est la mise en contact des parties solides et liquides de la baie de raisin par foulage de la vendange et juste après sulfitage du moût. L'objectif de cette étape de macération est de favoriser l'extraction en milieu aqueux des constituants de la pellicule (anthocyanes, tanins, polyphénols) par des processus enzymatiques ce qui va contribuer à l'élaboration des particularités aromatiques et gustatives des vins. Le temps de cette étape peut être variable de quelques heures à plusieurs semaines selon le type de vin voulu allant de vins rouge plus légers à des vins plus concentrés. Elle est universellement utilisée et est signe de qualité des vins rouges.

Le départ de la fermentation débute de façon classique spontanément ou après levurage par un réchauffement des moûts et dégagement de CO<sub>2</sub>. Les pellicules forment un chapeau au-dessus du jus représentant 1/3 du volume de la cuve. La technique du remontage est utilisée dans la région du Bordelais afin que le jus soit remonté au sommet de la cuve avec une pompe pour asperger ce chapeau et réaliser une extraction accrue des tanins et des anthocyanes.

Spécifique à la vinification en rouge, en particulier bordelaise, une macération post-fermentaire est réalisée et consiste à laisser le vin en contact avec le marc pendant la période désirée. Encore une fois, cette étape favorise l'extraction des tanins ici en milieu hydroalcoolique à des températures variant de 28°C à 32°C.

### b) En vinification en blanc liquoreux

Une particularité de cette vinification est que l'étape de macération commence directement sur la baie de raisin du fait de la présence souhaitée de *Botrytis cinerea* qui se développe sous forme de pourriture noble. La pellicule de la baie éclate et permet alors un contact entre le jus et l'environnement extérieur.

La fermentation alcoolique se fait de manière classique spontanément ou après levurage à une température inférieur à 20°C. La caractéristique de ces fermentations est qu'elles sont stoppées par un ajout massif de SO<sub>2</sub> afin de garder une teneur en sucre dans le vin supérieur à 40 grammes de sucre non fermenté par litre.

### c) Gestions des fermentations

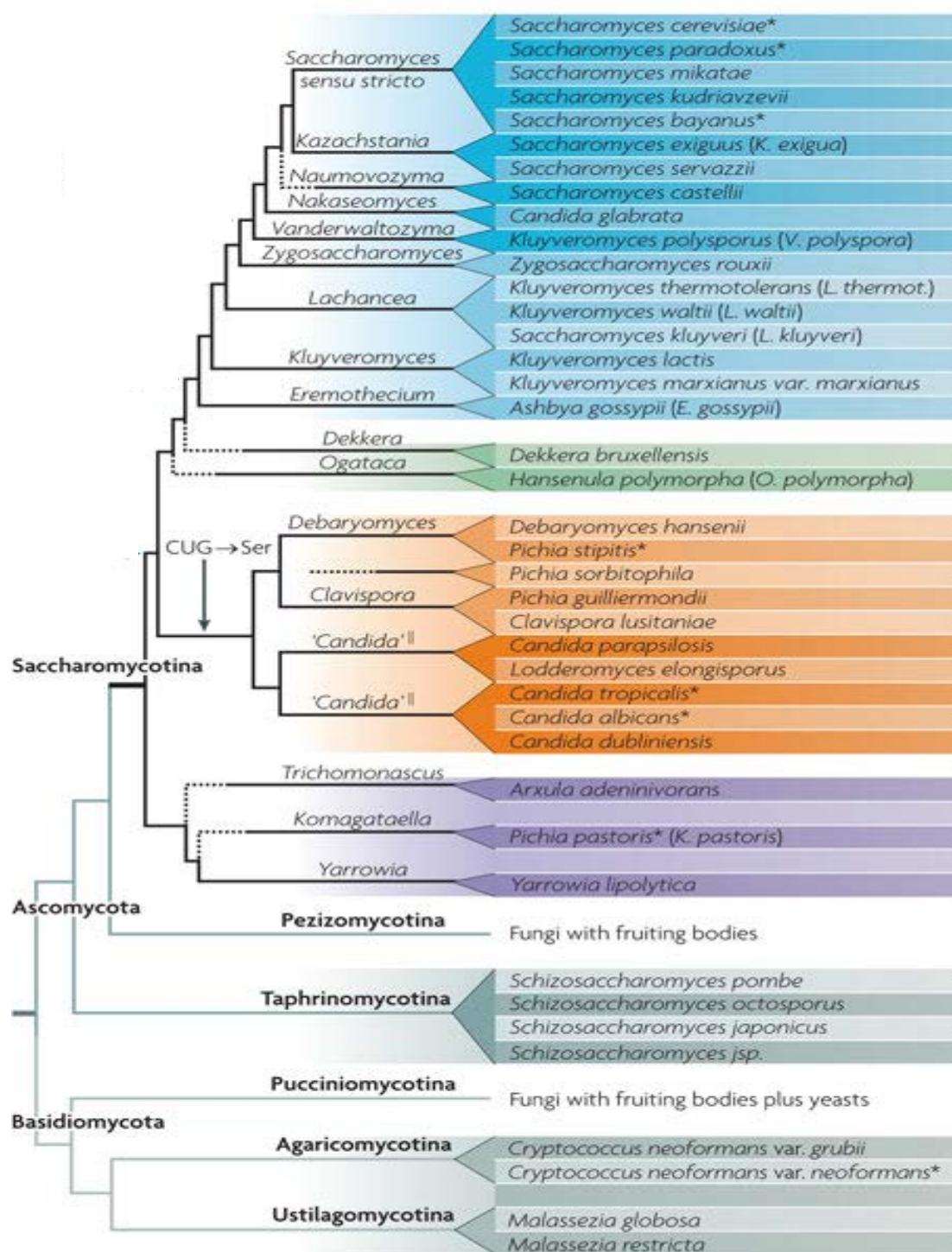
Il existe 2 méthodes différentes de préparation de levain, l'utilisation directe de Levures Sèches Active (LSA) ou l'utilisation de pied de cuve (PdC), permettant de maîtriser le démarrage et le déroulement de la fermentation alcoolique. Chacune d'elles présentent des avantages et inconvénients sur la diversité des levures et les propriétés organoleptiques des vins obtenus (Bely, Masneuf, and Dubourdieu 2005; Santamaría et al. 2005).

L'inoculation directe entre 10 à 20 g/hl de LSA est la méthode la plus communément utilisée, demandant une simple réhydratation du produit. Les souches industrielles proviennent de projets de recherche conduisant à la sélection d'individus ayant des propriétés fermentaires robustes d'un point de vue du déroulement de la fermentation alcoolique mais également d'un point de vue de leur impact organoleptique. Le nombre de souches sélectionnées et commercialisées est très important, plus de 200 souches sur le marché actuellement, offrant aux responsables techniques de domaines viticoles un large choix de souches selon différents critères tels que la sensibilité à la température, la production de

d'acidité volatile ou encore de différents produits issus du métabolisme fermentaires présentant un impact organoleptique sur le vin.

La seconde technique repose sur l'utilisation de PdC, cette méthode consistant en l'utilisation d'un faible volume de moûts en fermentation afin d'ensemencer les lots de jus de raisins constitués au fur et à mesure de l'avancée des vendanges. En amont des vendanges, la technique consiste à récolter 10 à 20 kg de raisins (8 à 10 jours avant la date de récolte) permettant après foulage l'obtention d'une pré-culture de jus de raisin. Il est souvent très utile de réaliser plusieurs prélèvements de raisins, afin de pouvoir sélectionner le lot qui fermente le mieux, sans présenter de défauts olfactifs de type acidité volatile ou acétate d'éthyle. A 50%-75% de la fermentation, ce moût représentant entre 5 à 10% du volume de la cuve sera alors utilisé pour inoculer les premiers lots. La mise en œuvre de PdC peut alors se poursuivre par l'utilisation des premières cuves en fermentation pour ensemencer les suivantes. Ainsi l'inoculation est réalisée en chaîne et chaque fermentation permet l'ensemencement de la suivante jusqu'aux dernières vendanges rentrées. Ce procédé, par l'apport massif de biomasse active et adaptée au milieu, permet de mieux maîtriser le déclenchement et le déroulement des fermentations. Néanmoins, la méconnaissance des microorganismes présents dans ces pieds de cuve ne permet pas toujours d'éviter l'apparition de défauts et la qualité des vins obtenus reste aléatoire. Un protocole d'utilisation général des PdC est alors une bonne façon de gérer au mieux les pré-cultures (Bely, Masneuf, and Dubourdieu 2005).

Il est à noter que certains praticiens combinent les deux approches, levain avec LSA et PdC, en utilisant la première cuve en fermentation inoculée avec des levures sélectionnées pour ensemencer les autres lots de jus de raisin. Il a été montré qu'avec cette méthode, les autres lots de jus de raisins réalisaient une fermentation comparable à une méthode d'inoculation directe et que la LSA sélectionnées restait tout au long des ensemencements la souche majoritaire (Li et al. 2012).



**Figure 1.**

Liste des espèces de levures séquencées, leur désignation initiale au moment du séquençage et nouvelles taxonomies entre parenthèses (Dujon 2010).

## 2) *Saccharomyces cerevisiae*

### a) Les outils pour connaitre sa diversité

Depuis la première proposition de classification des levures de Hansen au début du XX<sup>ème</sup> siècle (Hansen 1904), ce regroupement des levures selon les genres n'a jamais cessé d'évoluer. Le premier critère de différentiation utilisé était la capacité à former des spores, puis sont apparus de nouveau caractères morphologiques et physiologique comme la forme, la taille ou encore la capacité fermentaire ou l'assimilation de différents substrats. Cependant, le fait de la présence d'une forte diversité à l'intérieur même de chaque genre a imposé l'addition de la notion d'espèce. Cependant, l'incapacité de certaines souches à sporuler va malheureusement à l'encontre du terme d'espèce « biologique » (Mayr 1996) qui énonce qu'une espèce est représentée par une communauté d'êtres interféconds, produisant des descendants eux même féconds. Pour y remédier, une classification basée sur les similitudes de composition en base d'ADN des souches de levures (Barnett et al. 2000) a alors été utilisée. L'utilisation de méthode de taxonomie moléculaire par l'amplification et séquençage de la région D1/D2 de l'ADN ribosomique 26S a permis de différentier les différentes espèces levures entre elles (C P Kurtzman and Robnett 1997; C. P. Kurtzman and Robnett 1998). L'étude de levures a par la suite connu de nombreuses avancées depuis le séquençage du tout premier génome de *S.cerevisiae* en 1996 (Goffeau et al. 1996). De plus et en parallèle de l'évolution de la classification des levures (Figure 1), la question de la différentiation des individus à l'intérieur de l'espèce s'est très rapidement posée aux technologues, en particulier pour contrôler la conformité des souches utilisées pour l'espèce *S.cerevisiae*, et pour en maîtriser les propriétés physiologiques. En effet avec les progrès réalisés en séquençage, il est apparu que certaines espèces étaient des hybrides inter-espèces tel que *Pichia sorbitophila* (Louis et al. 2012) ou intra-espèces tel que *Zygosaccharomyces bailii* (Mira et al. 2014) et *Dekkera bruxellensis* (Borneman et al. 2014).

Le polymorphisme présent au niveau de l'ADN mitochondrial a été utilisé pour la première fois pour la caractérisation des levures de bière (Lee, Knudsen, and Poyton 1985), méthode qui a ensuite été appliquée à la caractérisation des levures du vin (Dubourdieu et al. 1987). Cette méthode a été développée et simplifiée de manière à permettre une utilisation à grande échelle (Amparo Querol, Barrio, and Ramón 1992; López et al. 2001) et a été très largement adoptée par la suite pour le typage des levures vinicoles de l'espèce *S.cerevisiae* (Sabate et al. 1998; Vezinhet, Blondin, and Hallet 1990; Amparo Querol et al. 1992; Martínez et al. 1995; Constantí et al. 1997). L'efficacité des résultats des analyses RFLP (Restriction Fragment Length Polymorphism) de l'ADNmt reste très dépendante du type et de la combinaison des enzymes de restriction utilisées, et d'autres techniques de typage lui ont été parfois associées pour augmenter la qualité du résultat (Pramateftaki, Lanaridis, and Typas 2000; Fernández-Espinar et al. 2001).

La méthode d'électrophorèse en champ pulsé ou PFGE (Pulsed Field Gel Electrophoresis) est une variante de l'électrophorèse au cours de laquelle l'orientation du courant électrique est alternée afin de permettre la séparation de molécules d'ADN de grandes tailles (Schwartz et al. 1983; Carle and Olson 1984). La publication de la carte complète des 16 chromosomes de *S.cerevisiae* en 1985 (Carle and Olson 1985) n'a été rendue possible qu'avec l'apparition de cette méthode qui reste toujours utilisée pour la différentiation des souches. Cette méthode reste tout de même contraignante du fait de la nécessité d'un équipement plutôt onéreux et d'un temps de manipulation assez long, ce qui ne permet d'analyser qu'un faible nombre de souches à la fois. L'intérêt de cette méthode est généralement de l'adoindre à d'autres techniques de typage qui associent des résultats d'observations d'anomalies chromosomiques à des observations plus précises à un niveau de variations dans l'ADN (Schuller et al. 2004).

Le génome de *S.cerevisiae* contient un grand nombre de séquences répétées dont les rétrotransposons Ty et les séquences qui leur sont relatives, comme les LTR (ou Long

Terminal Repeats) qui les flanquent. Une cinquantaine de rétrotransposons complets ont été identifiés chez la levure S288C dont une trentaine de rétrotransposons Ty1 entiers et une dizaine de Ty2 (Kim et al. 1998). Les éléments *delta* sont des LTR qui se retrouvent de part et d'autre des rétrotransposons de type Ty1 et Ty2 et ont été utilisés comme cible pour la méthode de PCR *Delta*. Comme les éléments Ty s'insèrent de manière très variable dans le génome des individus, le profil de ces insertions est alors spécifique à chacun. La première version de cette technique utilisant les amorces δ1 et δ2 (Ness and Lavallée 1993) avait été mise au point avant le séquençage du génome de S288C (Goffeau et al 1996). Une seconde version utilisant des régions plus conservées, ciblées par les amorces δ12 et δ21 a été ensuite développée (Legras and Karst 2003) permettant d'obtenir un nombre de bandes plus élevé sur un gel d'électrophorèse.

Les microsatellites font partie de la famille des différents types de séquences répétées présentes dans le génome de la plupart des espèces. Ce sont de courtes séquences d'ADN, de 2 à 10 nucléotides, répétées en tandem et présentes majoritairement dans les régions non-codantes. Elles peuvent aussi, dans une moindre mesure, être retrouvées dans les régions codantes (Li et al. 2004; Tóth, Gáspári, and Jurka 2000). Ces régions présentent la particularité de différer fréquemment d'un individu à l'autre dans le nombre de répétitions. Ceci semble lié à différent mécanismes de recombinaisons inégales induites par le glissement de la polymérase en cours de réPLICATION (Henderson and Petes 1992; Wierdl, Dominska, and Petes 1997). Plusieurs techniques de typages basées sur ce polymorphisme ont été développées pour la levure *S.cerevisiae* (Legras et al. 2005a). Ces marqueurs microsatellites sont fréquemment utilisés comme marqueurs génétiques dans les études de cartographie génétique et pour les études de génétique des populations. La combinaison d'au moins six locus microsatellites très polymorphes se révèle être une technique extrêmement discriminante et reproductible, permettant une différenciation des souches de *S.cerevisiae*.

mais aussi de montrer leurs relations par rapport à leurs origines technologiques (LeGras et al. 2007).

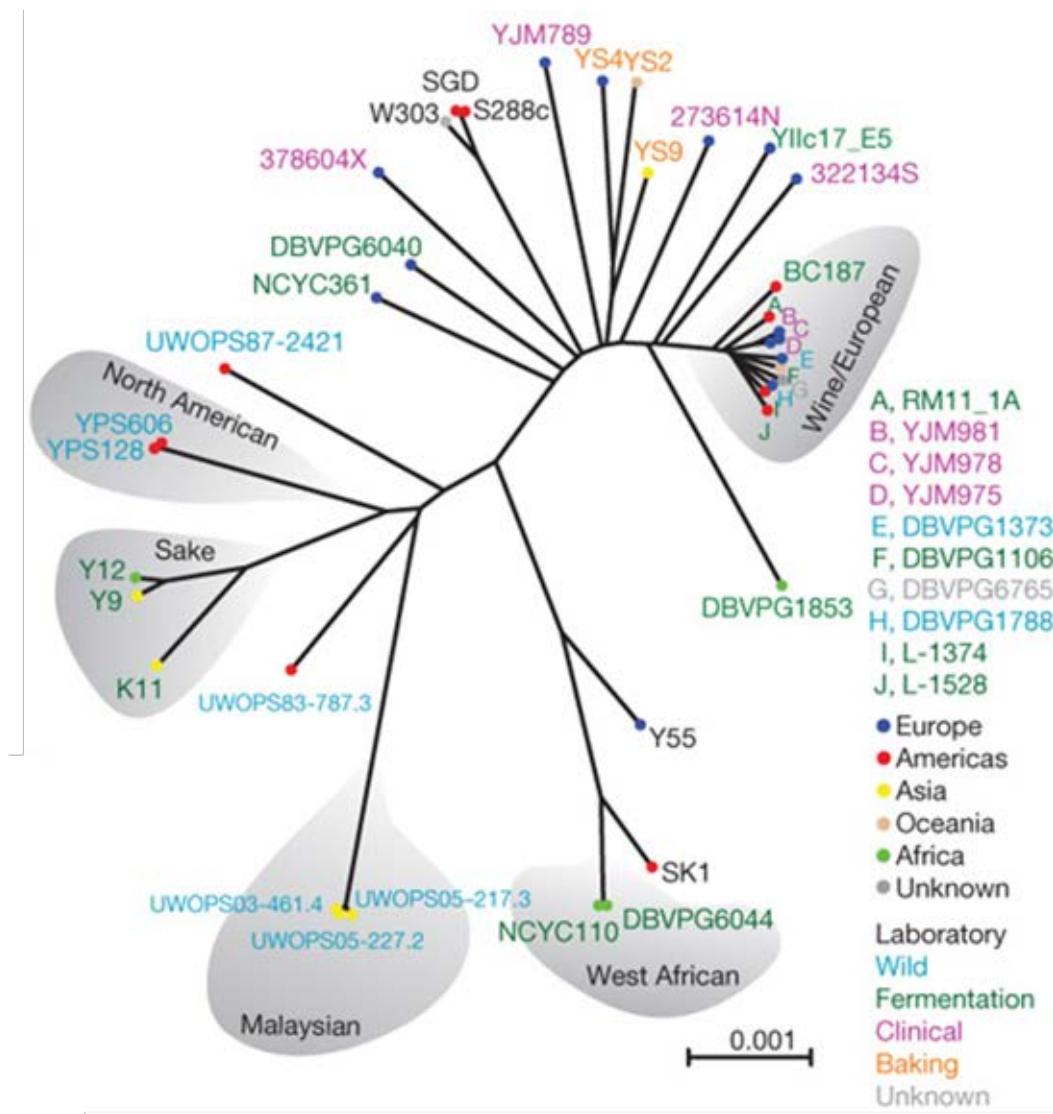
Avec le développement des capacités de séquençage, plusieurs générations de méthodes de caractérisation des levures sont apparues. La première méthode de séquençage nommée MLST (Multi Locus Sequence Typing) est une méthode standardisée d'analyse de séquences entières ou partielles de certains gènes de ménage. Ces gènes sont constitutifs à toutes les espèces et l'accumulation lente de mutations sur ces gènes permet de différencier les individus et donc le typage de souches tout en permettant de déduire des relations phylogénétiques. Depuis sa première description en 1998 (Maiden et al. 1998), cette technique a été largement utilisée au cours d'études épidémiologiques mondiales afin d'identifier des microorganismes pathogènes (Enright et al. 1999; Urwin and Maiden 2003). Des méthodes MLST ont été appliquées très tôt aux levures pathogènes comme *Candida albicans* (Bougnoux, Morand, and d'Enfert 2002; Tavanti et al. 2003; Robles et al. 2004), ou *Candida glabrata* (Dodgson et al. 2003). Bien que cette technique ait été appliquée à *S.cerevisiae*, (Fay and Benavides 2005; Ayoub et al. 2006), les résultats obtenus n'ont pas montré une capacité supérieure de différenciation des levures en comparaisons de l'analyse des PCR *delta* ou du polymorphisme des microsatellites (Ayoub et al. 2006), mais ont en revanche permis de révéler la structure de population particulière des levures de vin ou de saké, indiquant la domestication de ces levures (Fay and Benavides 2005).

Depuis peu, une variante de cette technologie étendue à l'ensemble du génome et permise par les nouvelles générations de technologie de séquençage, a été utilisée dans plusieurs études. Dans cette méthode, les auteurs déterminent les séquences de régions situées entre des sites de restriction choisis de manière aléatoire dans le génome (Restriction site-Associated DNA SEQuencing ou RADSeq). Cette méthode permet d'obtenir de nombreuses positions de variations d'une base, ou « Single Nucleotide Polymorphism » (SNP), et ainsi

plus de 13000 SNP ont été identifiés chez *Neurospora crassa* (Baird et al. 2008) et utilisés pour des analyses génétiques et phylogéniques. Le premier avantage du RADSeq est qu'il peut être utilisé pour réaliser des études de génétique des populations sur les espèces où les données de séquence sont inexistantes ou limitées. Du fait que la plupart des sites de restriction sont communs à toutes les souches d'une même espèce, la méthode RAD-seq a permis l'étude de la diversité et la structure phylogénétique des souches de *S.cerevisiae* provenant de niches écologiques variées et créant ainsi de grandes bases de données ouvertes (Hyma and Fay 2013; Cromie et al. 2013).

b) Sa diversité connue et positionnement des levures œnologiques au sein de l'espèce

La diversité des espèces du genre *Saccharomyces* est très importante et la dernière classification (Cletus P. Kurtzman, Fell, and Boekhout 2011) a défini trois groupes dont celui regroupant toutes les souches de *S.cerevisiae*, qui est donc considérée comme l'espèce fermentaire. De nombreuses études ont analysé son évolution au cours du temps et sa dispersion géographique à un niveau mondial afin de mieux comprendre cette espèce. Elle est généralement associée aux boissons alcooliques (vin, saké, bière), au pain ou encore à des souches utilisées pour la recherche, et les études ont montré qu'elle se subdivise en sous-populations en fonction de leurs origines technologiques sans tenir obligatoirement compte de leurs origines géographiques (Legras et al. 2007). Les premières évidences de breuvages fermentés ont été décrites en Chine en 7000 av. J-C (McGovern et al. 2004) ou encore en Iran 6000 av. J-C (McGovern et al. 1997) et en Egypte 3000 av. J-C (Cavalieri et al. 2003). L'espèce *S.cerevisiae* a subit différents phénomènes de goulots d'étranglement en fonction de leurs utilités pour l'homme, qui a sélectionné de façon consciente ou inconsciente les souches les plus adaptées et performantes (Liti et al. 2009; Schacherer et al. 2009). Plusieurs grands groupes se sont alors formés créant des communautés dépendantes de leur fonctionnalité première (Figure 2), illustrant ainsi la domestication de l'espèce, telle que celle des



**Figure 2.**

Arbre de Neighbor joining basés sur l'analyse des différences de SNP des souches de *S.cerevisiae*. Les lignées sont surlignées en gris, la couleur indiquant la source (nom) et l'origine géographique (points) (Liti et al. 2009).

*S.cerevisiae* œnologiques comme démontré par l'étude de J-L Legras en 2007, où 95% des souches de levures de vin étaient rassemblées en un seul et unique groupe génétique.

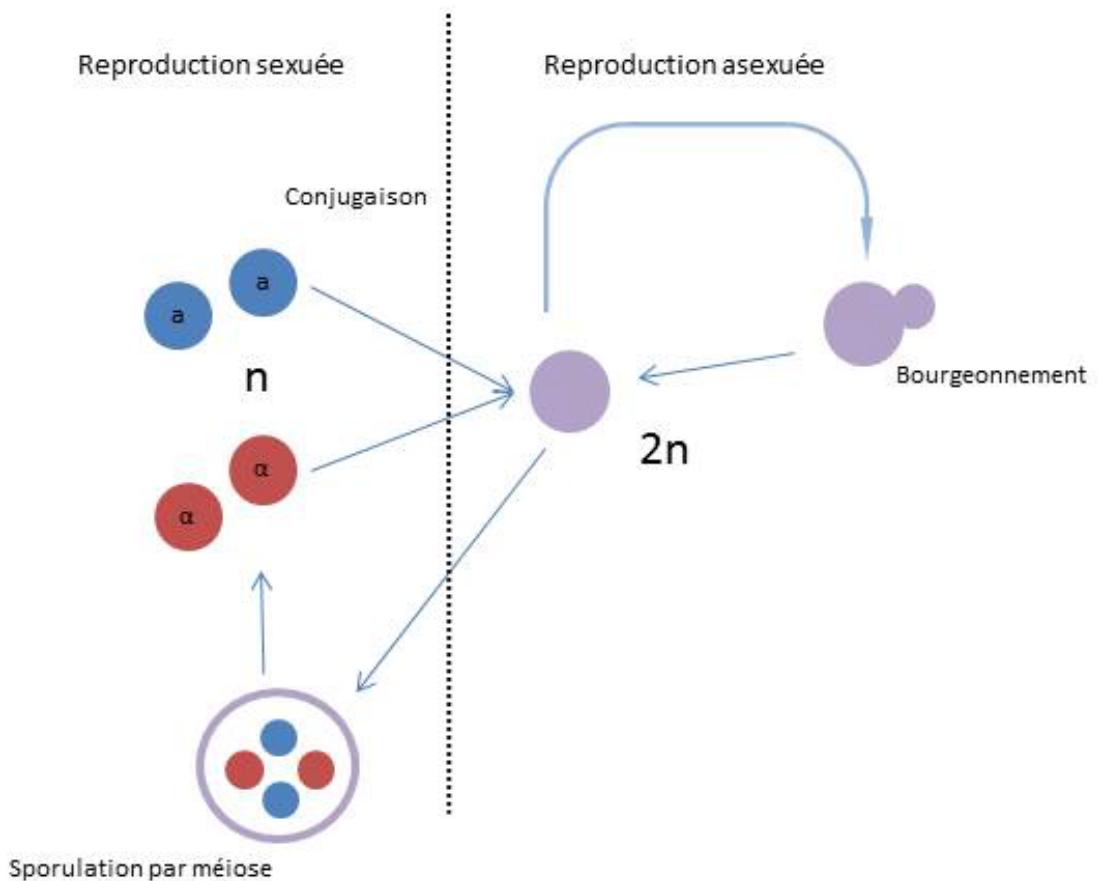
c) Son cycle de vie

La levure *S.cerevisiae* a un cycle cellulaire diplontique, qui comprend deux modes de reproduction asexuée et sexuée. Le premier correspond au processus où une cellule va donner naissance à une autre identique par bourgeonnement, tandis que lors du second, la cellule subit une méiose, et produit des spores haploïdes fusionnant rapidement et principalement dans l'asque (Figure 3) (Goddard 2008; Tsai et al. 2008; Johnson et al. 2004). Selon les conditions environnementales externes, l'un ou l'autre des deux modes de reproduction sera favorisé. Si les levures perçoivent que la quantité de nutriments est suffisante, elles suivent le mode de reproduction asexuée mais à l'opposé, lorsque la quantité de nutriments est insuffisante, les levures sporulent en attendant le retour d'un environnement moins sélectif.

3) La biologie de *Saccaromyces cerevisiae* dans le contexte œnologique

a) L'écosystème vigne

Les levures sont généralement peu nombreuses dans la vigne et sont le plus souvent retrouvées réparties à la surface des serments, des feuilles, sur les rafles ou encore dans le sol. Même si la population levure augmente avec la maturation du raisin allant de  $10 - 10^3$  UFC/g sur raisin immature (Fleet 2003) à  $10^4 - 10^6$  UFC/g pour les grappes mûres (Prakitchaiwattana, Fleet, and Heard 2004; Rosini, Federici, and Martini 1982), *S.cerevisiae* reste peu abondante. En effet, seulement une baie sur mille est positive vis à vis de la présence de *S.cerevisiae* (Mortimer and Polisinelli 1999). Cependant plus une baie sera endommagée plus la présence de levure est probable, mais tout en restant faible (Mortimer and Polisinelli 1999). Ces observations ont été confirmées par de nombreuses études analysant l'effet de la présence de baies endommagées et de l'infection par *Botrytis* sur la communauté levurienne



**Figure 3.**

Représentation schématique du cycle cellulaire sexuel et asexuel de *S.cerevisiae*.

(Nisiotou, Spiropoulos, and Nychas 2007; Nisiotou and Nychas 2007; Loureiro and Malfeito-Ferreira 2003). Cette présence minoritaire de *S.cerevisiae* dans l'écosystème de la vigne, soulève des questions quant à la provenance primaire de l'origine des levures fermentaires dans les moûts. Les environnements créés autour de la vigne par l'homme comme le travail de la vigne peuvent influencer les niveaux de population des levures. Les chais sont également suspectés d'introduire des *Saccharomyces* de façon importante dans les moûts de raisins (Pretorius 2000; Martini 2003; Ciani et al. 2004; Le Jeune et al. 2006; Bokulich et al. 2013).

b) Influence du mode de conduite, (biologique et conventionnel), sur l'écosystème levure

Les traitements phytosanitaires permettent de protéger et prévenir des maladies de la vigne, tel que le mildiou et l'oïdium, mais également de repousser les insectes qui pourraient endommager les baies. Les deux modes de conduites, biologique et conventionnel, s'opposent dans les traitements des vignobles mais également par leur impact négatif, ou non, sur la biodiversité microbienne présente naturellement à la vigne. La différence porte essentiellement sur la nature et la quantité des produits utilisés lors des différents traitements.

Pour le travail de la vigne, le mode de conduite biologique doit suivre les réglementations imposée par les textes RCE 834-2007 et RCE 889-2008. Pour être considéré en agriculture biologique, le vigneron va utiliser uniquement que des produits tels que le soufre et le cuivre pour prévenir les maladies du mildiou et de l'oïdium ainsi que des traitements à base de plantes ou huiles minérales pour la partie insecticide. Il faut également prendre en compte que l'utilisation du cuivre est réglementée (RCE 889-2008 / annexe II) avec un maximum de 6 kg/ha/an. Dans le cas de la vigne, étant une culture pérenne, une dérogation peut être accordée quant à l'utilisation du cuivre. En effet, la limite de 6 kg peut être dépassée au cours d'une année donnée, à condition que la quantité moyenne annuelle utilisée sur une période de 5 ans, comprenant l'année en question et les quatre années précédentes, ne dépasse pas les 6 kg. De

l'autre côté, l'agriculture conventionnelle, qui utilise également le soufre et le cuivre, pourra y associer des produits de synthèses afin de prévenir et traiter les maladies, lutter contre certains insectes et entretenir les sols. Il est à noter que, pour ce mode de conduite, aucune limite dans l'utilisation du cuivre n'est signalée.

Lors de l'étape de fermentation, certaines règles sont à suivre ; en effet, il existe depuis 2012 un cahier des charges européen (R(UE) 203/2012) qui doit être désormais suivi pour pouvoir prétendre à la dénomination de vin biologique. Cette réglementation donne des restrictions ou interdictions sur l'utilisation de certains procédés physiques ainsi qu'une liste restreinte d'additifs et auxiliaires œnologiques en privilégiant pour certains une origine biologique (R(UE) 203/2012 annexe VIII bis). Il est à noter que certains additifs sont pondérés en fonction du type raisin et la concentration en sucre tel que le dioxyde de soufre ( $\text{SO}_2$ ) qui permet de combiner l'effet antioxydant et antiseptique. Les différences sont calculées par rapport à la dose maximale en  $\text{SO}_2$  total dans les moûts, comme le montre ces exemple de dose maximales, 2 à 5 g/hl sur la vendange, 3 à 5 g/hl en fin de fermentation alcoolique pour les blancs, 3 à 5 g/hl en fin de fermentation malolactique et 8 à 10 g/hl lors du mutage des vins doux naturels. Dans le cas des LSA, les producteurs de vin biologique, qui décident de pratiquer le levurage, se doivent dans le meilleur des cas se procurer une souche ayant été produite selon un mode biologique. Si leur attente en matière de spécificité de LSA n'est pas retrouvée dans l'offre commerciale des souches dites « biologique », il leur sera quand même permis d'utiliser une LSA non biologique.

Le concept d'assurance écologique pour les écosystèmes démontre une relation positive entre biodiversité et productivité ainsi qu'entre biodiversité et stabilité des écosystèmes (Loreau 2000). Un certain nombre d'études ont posé le problème de l'effet des différents traitements phytosanitaires sur la biodiversité des écosystèmes microbiens de la vigne, et ont rapporté des résultats contradictoires. D'un côté, il a été montré des effets négatifs de

l'utilisation de pesticides sur la diversité de la communauté levurienne de la vigne et des moûts (Cus and Raspor 2008; Setati et al. 2012). Ainsi, pour deux traitements vis à vis du mildiou, l'un de synthèse et le second naturel, il a été montré que, pour une même efficacité, les souches de levures étaient plus sensibles au traitement de synthèse, notamment *S.cerevisiae* avec des concentrations minimales inhibitrices de soufre 200 fois inférieur ( $>10\text{mg/l}$ ) à celles pour le penconazole ( $<0.05\text{mg/l}$ ) (Cordero-Bueso, Arroyo, and Valero 2014). De plus, les traitements appliqués à la vigne peuvent être transférés, via les résidus sur les baies de raisin, au moût lors de la fermentation alcoolique (Caboni and Cabras 2010) et ainsi influencer la sélection des souches de levures, dont *S.cerevisiae*, et la qualité du produit final . Mais d'un autre côté, des études comparant la biodiversité des levures sur raisins et moûts, en agriculture conventionnelle et biologique, ont montré que malgré un écart faible, le mode de conduite conventionnelle, conduisait à un nombre d'espèces de levures sur la baie et une diversité de *S.cerevisiae* dans le moût plus importante (Oliva et al. 2007) avec 2 génotypes contre 6, respectivement dans les moûts biologique et conventionnel (Milanović, Comitini, and Ciani 2013).

### c) L'écosystème chai

Différents aspects du chai peuvent être pris en compte ici tel que le matériel vitivinicole, l'air circulant à l'intérieur des bâtiments et les bâtiments eux-mêmes ou encore la diversité de *S.cerevisiae* présente au cours des fermentations spontanées.

L'apport des levures par le matériel vitivinicole débute dès le début des vendanges. La surface des équipements utilisés lors de la récolte, manuelle ou mécanique, est une source potentielle de levures. De plus, si la vendange est stockée avant traitement au chai ou s'il y a un trajet important entre le site de vendange et le chai, cet apport peut-être d'autant plus important (Pretorius 2000). En plus du matériel vitivinicole, il est suspecté que *S.cerevisiae*

soit introduite dans les moûts via la surface des chais. En effet, *S.cerevisiae* a souvent été décrite comme étant présente à la « surface de chais » (Santamaría et al. 2005; Sangorrín et al. 2007) et ainsi comptée parmi les souches de levures résidentes du chai. Une étude a montré sa présence dans les différentes zones du chai (pressurage, fermentation ou stockage) avant, pendant et après la période de vendange (Bokulich et al. 2013). *S.cerevisiae* étant devenue résidente, elle est sélectionnée au cours des années et sur plusieurs générations. Elle peut alors être introduite dans les moûts au cours des différentes étapes entre la réception de la vendange et l'obtention des jus telles que la sélection sur table de trie, l'éraflage, le foulage ou pressurage des moûts ( Ciani et al. 2004). Il faut cependant tenir compte que cette introduction de levure est totalement indépendante des souches inoculées lors du levurage par les œnologues. Pour analyser la présence des levures dans le chai, il faut également prendre en compte les fûts en bois, qui sont des surfaces irrégulières et non polies pouvant héberger des levures tel que *S.cerevisiae* (Pretorius 2000; Sangorrín et al. 2007), contrairement aux cuves en acier qui sont composées de structures lisses. Les conditions hygiéniques des chais rentrent donc en compte dans la composition en levures des moûts.

En plus de tous ces éléments techniques, l'air lui-même peut influencer la distribution des levures dans le chai et les moûts. L'industrie alimentaire a montré que les levures pouvaient se déplacer dans l'air en se fixant à des particules de poussières mais également seules (Curiel, Van Eijk, and Lelieveld 2000). Une étude a également montré la présence de levures dans l'air de chais de Rioja en Espagne (Garijo et al. 2008). La population de levure présente dans l'air étant limitée en début de fermentation et augmentant à mesure de son avancement. Les systèmes d'aération des chais tels ceux de la climatisation ou du dégagement de CO<sub>2</sub> au cours de la fermentation peuvent être comptés parmi les causes de ce mouvement perpétuel des levures dans l'air environnant des chais.

L'écosystème du chai est donc également très dépendant de l'évolution de la diversité en *S.cerevisiae* au cours des fermentations spontanées, comme expliqué précédemment, l'un inoculant l'autre et inversement. La fermentation des jus de raisin est représentée par un mélange complexe et de multiples d'interactions entre de nombreuses espèces et souches de levures. Ce mélange peut varier selon les caractéristique des moûts telles que la quantité initiale en sucre et l'acidité du jus (Blanco, Mirás-Avalos, and Orriols 2012). Même si ce sont les levures non-*Saccharomyces* (Fleet 1993), comme *Hanseniaspora spp.*, *Pichia spp.*, *Candida spp.* et *Metschnikowia spp.*, qui sont prédominantes en début de fermentation (Torija et al. 2001; Zott et al. 2008), elles déclinent en milieu de fermentation. *S. cerevisiae* devient alors dominante (Fleet 2003) tout le long de la fermentation alcoolique. Ce phénomène est dû à la capacité de *S.cerevisiae* à créer un environnement qui lui est favorable tout en étant délétère pour les autres espèces. Par l'effet Crabtree il va donc y avoir production d'éthanol mais également de chaleur, qui vont à eux deux permettre à *S.cerevisiae* de passer de souche minoritaire à majoritaire au cours de la fermentation, passant de 0.1% à 99.9% de la population globale en quelques jours (Goddard 2008). En effet, *S.cerevisiae* semble être la seule espèce ayant évoluée vers une adaptation à ces conditions de croissance (Merico et al. 2007), l'effet de l'alcool, augmenté par son association à la chaleur, étant un antimicrobien général des levures. La croissance des levures *S.cerevisiae* est accompagnée par le développement de plusieurs souches différentes, (conduisant à des fermentations polyclonales), mettant en place des étapes de substitutions séquentielles au cours de la fermentation (A. Querol, Barrio, and Ramón 1994; Sabate et al. 1998)

d) Lien entre les deux écosystèmes?

De nombreuses études se sont questionnées sur les relations qui pouvaient exister entre la vigne et le chai, les transferts de souches et les variations de conditions subies par les souches.

Nous avons expliqué précédemment que les quelques souches de *S.cerevisiae* présentes sur les baies de raisins étaient transférées dans la partie chai au moment des vendanges. Elles ne sont pas nombreuses et sont directement mises en contact avec un milieu acide, riche en sucre et en compétition pour les nutriments avec d'autres espèces présentes en plus grand nombre. Cette étape fait alors subir aux populations de *S.cerevisiae* de la vigne une sélection rude d'où vont émerger une ou plusieurs souches capables de survivre et de se développer rapidement, au détriment des autres espèces. Une seconde étape de sélection est réalisée peu après lorsque la fermentation commence, le sucre laissant la place à des teneurs en alcool croissantes en plus d'une température du moût qui augmente. Cette étape va aboutir à la diminution des populations non-*Saccharomyces* mais également permettre une sélection de souches de *S.cerevisiae* les plus tolérantes aux forts degrés d'alcool.

La question se pose également à propos du retour des souches de *S.cerevisiae* présentes dans le chai vers le vignoble. Plusieurs études ont montré la présence de souches commerciales dans le vignoble environnant du chai. Mais la détection de ces souches n'était possible que dans les 10 à 200 mètres autour du chai d'utilisation de la souche en question et dans des domaines utilisant celle-ci depuis une longue période (Valero et al. 2005; Schuller et al. 2007; Schuller and Casal 2006). Dans le cas de ce retour de souches commerciales au vignoble, une adaptation à ce nouveau milieu est nécessaire, passant, d'un milieu à fort degré d'alcool à un milieu pauvre en nutriment sur la baie de raisin. Ces changements adaptatifs, principalement liés à des variations de taille chromosomique, surtout pour les petits chromosomes, des pertes d'hétérozygotie, une expansion de certains marqueurs microsatellites ou encore des différences d'amplification de séquences *delta* ont été montrés sur des souches trouvées au vignoble et liées à la levure commerciale VL1 (Schuller et al. 2007). Une étude plus poussée, sur ces mêmes souches apparentées à VL1, a montré des modifications d'expression de certains gènes d'adaptation tel que *ASP3-2*, qui est surexprimé lors d'absence

d'azote, mais également la présence de SNPs absents de la souche commerciale et présents sur des souches du vignoble (Franco-Duarte et al. 2015). D'autres études, utilisant une dissémination volontaire dans le vignoble, ont étudié le maintien des souches commerciales sur le court terme (3 années) (Cordero-Bueso, Arroyo, Serrano, and Valero 2011). Or, il n'y a pas eu d'implantation de ces souches commerciales au vignoble, signifiant alors que, pour qu'un retour de souches commerciales vers le vignoble ait lieu et soit maintenu dans le temps, ces souches doivent être présentes et persistantes dans le chai depuis une longue période et non pas ponctuellement.

## **Chapitre 1**

# **Evaluation of the main factors impacting ancestry *S.cerevisiae* diversity in the Bordeaux vineyards**

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## **Abstract**

*Saccharomyces cerevisiae* is the main actor of wine fermentation but still little is known about the factors impacting its distribution in the vineyards. In this study, 23 different vineyards and 7 cellars were sampled during 2 consecutive years. A total of 1374 *S.cerevisiae* merlot grape isolates and 330 cellar isolates were analyzed thought 17 microsatellites markers to investigate the diversity and population structure. Different parameters were tested on grape isolates population and comparison between the 2 compartments, grape and cellar, was also investigated. A very high genetic diversity was demonstrated, that was higher in conventional farming system than in organic one. The type of farming system weakly influences the population structure of *S.cerevisiae* in the vineyard whereas the appellation and the wine estate significantly impact the population structure, without appearance of diversity gradients. Connections from the Pessac-Léognan to other Bordeaux appellations, especially to the Medoc, were showed. Bidirectional strong flows were highlighted between the vineyard and the cellar compartments as illustrated by the presence of 25% of commercial related strains in the vineyard, due to the unexpected return of strains through cellar to the vineyard, while a flow of similar magnitude was observed between the vineyard and the cellar.

**Keywords:** Population structure, *Saccharomyces cerevisiae*, microsatellites, wine, commercial strains

## **Introduction**

The vineyard and the winery are the two main ecological habitats that house many species of yeast and bacteria. The microbial community present on the grape berry is very different comparing to the cellar microorganisms associated with the fermentation process. Indeed, the species naturally present on the grape berry which are those of a fruit microflora have to face drastic changes during wine making such as high concentration of sugar in the must, low pH, presence of sulfites and increase in alcohol concentration and temperature, and have to adapt to these very different physicochemical conditions during the alcoholic fermentation. Wine yeast are assembled in two main groups, with the *Saccharomyces sp* that initiate the alcoholic fermentation and non-*Saccharomyces* which dominate the grape berry community but will then underwent a negative selection during the fermentation through the increase in alcohol and temperature (Goddard 2008; Salvadó et al. 2011). The species *S. cerevisiae* is the main actor in the alcoholic fermentation during the manufacture of many fermented beverages and foods such as beer, sake, palm wine and bread (Fay and Benavides 2005; Knight and Goddard 2015; Legras et al. 2007; Liti et al. 2009). In agricultural areas, this species was well studied in order to better know its diversity and population structure. It has been repeatedly shown that wine *S. cerevisiae* strains were totally differentiated compared to other strains isolated from other substrates (Fay and Benavides 2005; Legras et al. 2007; Liti et al. 2009; Schacherer et al. 2009). Because of the key role of *S. cerevisiae* in the production of high quality wines, its genetic diversity has been widely analyzed along with technological advances in the field of microbial ecology. The first molecular methods used to describe the strains diversity of wine *S. cerevisiae* was the mtDNA restriction analysis (Dubourdieu et al. 1987), followed by pulsed field electrophoresis (Frezier and Dubourdieu 1992b; Versavaud et al. 1995; Valero et al. 2007b; Vezinhet, Blondin, and Hallet 1990). Then restriction map analysis of mtDNA was used by different authors (Cappello et al. 2004b; Schuller et al. 2005;

Cubillos et al. 2009; Amparo Querol et al. 1992), as well as inter-*delta* analysis (M Ciani et al. 2004; Le Jeune et al. 2006; I. Vigentini et al. 2015) and finally microsatellite analysis (Schuller and Casal 2006; Schuller et al. 2012; Legras et al. 2005a).

Multiple parameters that could influence the diversity and population structure of wine *S. cerevisiae* were highlighted by different authors. The most often studied environmental parameter is the geographical distance between the sampling sites and the influence of the varieties of grapes. The influence of geographical distance has been studied, often covering large area, comparing different regions in a given country (Knight and Goddard 2015). These studies have shown that the diversity of *S. cerevisiae* was undergoing significant change between distant areas. Indeed, on smaller distances, many settings can go into account to create a homogenization of diversity and structure through insects, such as wasps, bees and fruit flies (Stefanini et al. 2012; Goddard et al. 2010; Buser et al. 2014), or by the presence of migratory birds (Francesca et al. 2012). Finally, grape berries varieties can influence *S. cerevisiae* diversity (Schuller et al. 2012), even if that effect seems to be low (Schuller et al. 2012; V Gayevskiy and Goddard 2012).

Vine and cellar management, while impacting the global microflora environment might as well influence yeast diversity. At the vineyard level, winemakers could follow different farming systems in the choice of organic or conventional. The different use of phytosanitary products in both farming systems against fungal pathogens could impact the endogenous yeast populations present on the grape berry (Cordero-Bueso, Arroyo, and Valero 2014). However, the impact of organic farming system on the yeast diversity (organic/versus conventional) is not clearly defined till now, with studies reported controversial results (Setati et al. 2012; Cordero-Bueso, Arroyo, Serrano, Tello, et al. 2011; Oliva et al. 2007; Milanović, Comitini, and Ciani 2013). In the winery, commercial *S. cerevisiae* strains used to ensure the alcoholic fermentation process were found in grapes samples collected in 200m vineyards surrounding

the winery buildings (Valero et al. 2005). Integrating the endogenous population of the vineyard, the commercial strains would then change the diversity and population structure of *S. cerevisiae* (Franco-Duarte et al. 2015; Schuller et al. 2007). Yet the propagation of commercial yeasts and their persistence in the environment was showed to be not a continuous and persistent process (Cordero-Bueso, Arroyo, Serrano, and Valero 2011).

In addition, few research studies have worked on the possible relationships between the vineyards and winery *S. cerevisiae* diversity, thus raising the question on the origin of wine yeast (Ciani et al. 2004; Le Jeune et al. 2006; Mortimer and Polsinelli 1999). Strains involved in spontaneous fermentation originated both partly from vineyard and winery (Le Jeune et al. 2006). As described above, possible return of commercial strains used in the winery to the population present on the vine was shown. Indeed, a large and diverse yeast population is present mainly in winery surfaces, including *S. cerevisiae* prior to harvest (Bokulich et al. 2013). This population could act as potential reservoir to inoculate the grape musts during spontaneous fermentations. Links between the population from vineyards and wineries are important to study from an ecological point of view but is still unclear so far.

France is one of the largest wine producers in the world and Bordeaux is one of the most famous winemaking regions. Many grapes varieties are cultivated in the Bordeaux region such as Cabernet Sauvignon, Cabernet Franc, Carmenere, Malbec, Petit Verdot, but the most widespread one is the Merlot representing over 50% of the Bordeaux vineyards and mainly used during the wine assembly. The Aquitaine region has the third rank in France for the extent of organic farming system and in 2010, 5% of the global vineyard of the region was in this operating system (AGRESTE).

In this work, the diversity and population structure of *S. cerevisiae* in the different appellations of Bordeaux and Bergerac regions was studied. In order to avoid variability that

may be caused by the different grape varieties present in the region, only Merlot grape samples were collected during two consecutive years, both in vineyards following organic or conventional farming system. The aim of this study was to test for structural differentiation within this *S. cerevisiae* population achieved in relation to farming system and the different appellations at the regional level. For that purpose, genetic diversity assessed from the polymorphism at 17 microsatellite markers. In addition, the impact of commercial strains on the *S. cerevisiae* diversity and population structure was investigated. Finally, relations between vineyard and cellars *S. cerevisiae* population were evaluated.

## **Materials and methods**

### **Samples collection and processing**

Five wine producing areas in Aquitaine region of the south west of France were selected corresponding to 4 Bordeaux appellations Medoc, Pessac Léognan, Entre-Deux-Mers, Saint Emilion and to one in Bergerac (figure 1). Within an appellation, one to nine different wine estates were collected (table 1), (Supplementary data, table S1). For each wine estate, 5 times 2 kg of healthy and mostly undamaged grapes were collected few days before the harvest. For all the wine estates sampled, the grape variety cultivated was Merlot, the dominant grape variety of the different Bordeaux appellations. In 2012, 23 wine estates were sampled, 11 conducted in organic and 12 in conventional farming system as well as in 2013, where 6 organic and 6 conventional wine estates were selected (table 1). Moreover, some wineries in organic farming system were sampled for must at 75% of the fermentation from the vats in cellars. In 2012, 6 wineries were collected whose 5 were sampled for grapes, and in 2013, 3 vineyards were collected whose 2 were also sampled for grapes (table 1).

## Fermentation and strain isolation

An enrichment method by extracted juice fermentation was used to ensure the presence of *Saccharomyces* strains. For each fruit samples, the grapes were crushed, macerated for 2 hours with skin and seeds and after addition of 50mg/l of SO<sub>2</sub>, the extracted juice was fermented at 21°C in small glass-reactors (500ml). Fermentation progress was monitored through the amount of CO<sub>2</sub> released by a daily weighing measurement of glass-reactor to assess the weight loss. When fermentation reached about 2/3 of the sugar consumption or just stopped, different dilutions (10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup>) were plated onto YPD (yeast extract, 1% w/v, peptone, 1% w/v, glucose, 2% w/v, agar 2% w/v) with 100µg.ml<sup>-1</sup> of chloramphenicol and 150µg.ml<sup>-1</sup> of biphenyl to delay bacterial and mold growth. A maximum of 30 randomly chosen colonies were collected after incubation (2 days at 26°C). After two sub-cloning on YPD plates, each colony was stored in (30%, v/v) glycerol at -80°C. For the fermenting must samples from vats, same dilutions were made and a maximum of 40 randomly chosen colonies were collected after incubation (2 days at 26°C). After two sub-cloning on YPD plates, each colony was stored in (30%, v/v) glycerol at -80°C.

In addition to the collected samples, 33 sequenced yeasts strains from diverse origins associated or not to fermentation (Liti et al. 2009; Schacherer et al. 2009), (Supplementary data, table S2) and 35 commercial wine strains (Supplementary data, table S3) wildly used in Bordeaux wine estates were added to the collection. To also analyze the diversity of the commercial wine strains throughout production steps, 6 of frequently used commercial yeasts were selected and analyzed for two different years of production batch, except for one, for which only one production batch was available. A micromanipulator was used to isolate 10 single yeast cell clones from each production batch, thus resulting in 110 additional commercial wine yeast isolates into the collection.

## Molecular methods

Yeast colonies of all grape and vats samples were cultivated on differential WL nutrient agar medium (2 days at 26°C) which generated a specific coloration depending of their genus (Medina et al. 2012) and 2 of each type of colonies were filed on a FTA™ cards for DNA transferred. The PCR amplification of the ITS region with primers ITS1 and ITS4 (White et al. 1990) was used to identified *Saccharomyces* colonies and selected them. Each colony on WL medium corresponding to *Saccharomyces* were suspended in 20µl of MilliQ water and analyzed by optical density at 660nm. A readjustment of the amount of MiliQ water was made to obtain a final OD in the suspension cell of 10. All of these cell suspensions were then genotyped using 2 multiplex PCR reaction of 9 (unlinked/neutral) hyper-variable microsatellites loci each (Supplementary data, table S4) (John E. Bradbury et al. 2005; Dawn Field and Wills 1998; González Techera et al. 2001a; Hennequin et al. 2001b; Legras et al. 2005b; Pérez et al. 2001a). The 2 multiplex PCR contained (for 8 samples) a total of 15,5µl multiplexed primers, 50µl of QIAGEN Multiplex PCR kit Master Mix and 18,5µl water MiliQ. The PCRs were run in a final volume of 12µl containing 2µl of cell suspension. The following PCR program was used in routine: initial denaturation at 95 ° C for 15 minutes followed by 35 cycles of 95 ° C for 30 seconds, 57 ° C for 2 minutes, 72 ° C for 1 minute and finally a final extension at 60 ° C for 30 minutes. PCR products were sized on a capillary electrophoresis ABI3730 (Applied Biosystems) using size standard 600LIZ® (GeneScan™). Locus YLL049W providing non reproducible amplification was removed for the following structures and diversity analyzes.

## Data analyses

ABI3730 genotyping results were read using GeneMarker (V2.4.0, Demo) and the sizes of microsatellites amplicons were kept to investigate the genetic relationships between strains.

The presence of missing value was authorized to a maximum of 3 markers per samples and they were taken into account in the analyses considering that they could reflect a part of the diversity. Estimation of population diversity by rarefaction of 10000 individuals repeated 10 times, Shannon index ( $H'$ ) and Simpson diversity index (D) with them equitability indexes (reciprocally  $J'$  and  $1-D$ ) were calculated using EstimateS (V9) (Colwell 2004) using the sample-based abundance data.  $H'$  was determined with the following equation:

$$H' = - \sum_{i=1}^S P_i \ln(P_i)$$

and D following the equation:  $D = \frac{\sum_{i=1}^S \frac{N_i(N_i-1)}{N(N-1)}}{\sum_{i=1}^S \frac{N_i}{N}}$ . With S the total number of genotypes in the population, the term  $P_i$  calculated as follows:  $P_i = \frac{N_i}{N}$ ,  $N_i$  the number of individuals for a specific genotype and N the total of unique genotypes. GenClone software (V2.0) (Arnaud-Haond and Belkhir 2007) was used to remove from our dataset strains with exact similar profiles resulting from potential clonal expansion. AMOVA and genetic differentiation tests (pairwise Fst distance and the corresponding p-value) were performed with GenAlEx (Genetic Analyses in Excel) Version 6.501 (Peakall and Smouse 2012). Dendograms were constructed using Bruvo's distance (Ružica Bruvo et al. 2004) and neighbor-joining clustering by means of the R program (R Development Core Team 2013a) and the following packages: ape (Paradis, Claude, and Strimmer 2004a) and poppr (Kamvar, Tabima, and Grünwald 2014a). In order to assess the robustness of trees nodes, bootstrap resampling was performed by means of R and the pvclust package (Suzuki and Shimodaira 2014a) and inferred with MEGA6, all bootstraps lower than 25 were not showed in the trees. Population structure was evaluate using a Bayesian clustering method with the software InStruct that does not take account the Hardy-Weinberg Equilibrium (Gao, Williamson, and Bustamante 2007). 5 chains of 150000 iterations with a burn-in of 5000 were run for K=1 to K=25. The most likely number of ancestry's populations was selected choosing the lowest DIC (Deviance information criterion). Analyses of these ancestry profiles were conducted with first a color label bar plot for each profile using a script under R command, calculating

the percentage of the K profiles attribution for each individual. Spanning trees were made by BioNumerics V5.1 software (Applied Maths, Belgium) entering microsatellite amplicons sizes as character values and constructing dendograms using categorical coefficients and the ward algorithm (Cormack 1971). Spanning tree analyses were the only ones where missing value were not taken into account to minimize the differences between individuals and regarding the fact that only very close individuals were analyzed here. To analyze the relationship between geographic distance and genetic divergence, we used the Mantel test (Diniz-Filho et al. 2013; Mantel 1967) that is the most commonly used method and the contribution of niche and geographic region to population structure was also conducted with ObStruct software (Velimir Gayevskiy et al. 2014).

## Results

### **Sampling from grapes and *S.cerevisiae* diversity**

In order to perform this study, we collected 193 healthy grapes samples from Merlot grape variety in both organic and conventional wine estates, few days before harvest. Five sampling points were selected per wine estate (table 1). During 2012 sampling campaign, 134 samples were collected and 107 samples (80%) initiated a spontaneous fermentation, 56 and 51 in organic and conventional farming system, respectively. The 134 fermentation kinetic curves (Supplementary data, figure S1.a) were then grouped according to the length of the lag phase and the % of CO<sub>2</sub> consumption reached when the fermentation stopped. Depending on these 2 parameters 7 groups were obtained, with 3 groups of 34 partial alcoholic fermentations, less than 50% of CO<sub>2</sub> consumption, and 4 groups comprising 101 fermentations that were able to over pass the 50% of CO<sub>2</sub> consumption. Among these 101 fermentations, 50 were from 11 different organic farming system wine estates and 51 from 13 different conventional farming system wine estates. These fermentations that reached at least the 50% of CO<sub>2</sub> consumption

were selected for sampling and 2143 colonies were collected including a total of 755 *S.cerevisiae* strains after identification on WL medium culture and ITS-PCR-RFLP analysis (Granchi et al. 1999). In 2013, 59 grapes samples were collected, 26 in organic and 33 for conventional farming systems. All of them initiated a spontaneous fermentation and reached more than 50% of CO<sub>2</sub> consumption (Supplementary data, figure S1.b). This differences between the two harvests might originate by the high presence of *Botrytis cinerea* leading to higher proportion of damaged berries containing very likely more *S. cerevisiae* strains (Mortimer and Polzinelli 1999). Over these fermentations, 10 in organic and 24 in conventional farming permitted to isolate 619 *S.cerevisiae* isolates after WL medium culture and ITS-PCR-RFLP characterization among 1226 the colonies analyzed. To summarized, from a total of 3369 yeasts colonies isolated in 2012 and 2013, 1374 *S. cerevisiae* strains were collected, corresponding to 41% of the global yeast population analyzed. These 1374 *S. cerevisiae* strains were genotyped at 17 microsatellite loci, and only 1002 individuals presented less than 4 loci with missing data.

In order to compare the diversity of the yeast populations obtained from the different wine estates, we calculated three diversities index using EstimateS: the Shannon (H') index that measure the diversity within a population and take into account both richness and evenness, the Simpson index (D) with its opposite Simpson's index of diversity (1-D) which gives more weight to common or dominant species, and Pielou evenness index (J'). The different indexes were evaluated on the basis of the number of different genotypes and regarding the standard deviation of H' and D, all results were different. Despite a similar number of sampling sites for organic and conventional wine estates (13 and 14 respectively), the number of *S.cerevisiae* grapes isolates was approximately twofold higher in conventional farming: 662 grapes isolates, in comparison to organic: 340 grapes isolates. As a consequence, the 3 diversity indexes of *S. cerevisiae* (table 2.a) were higher for the samples collected in conventional

farming when compared with organic, but the gap between the 2 Simpson's indexes of diversity (1-D) was smaller indicating that the diversity regarding common isolates was alike. The comparison of rarefaction curves for possible population diversity in both farming systems confirmed these variations in *S. cerevisiae* isolation frequencies (Supplemental data, figure S2) as 6169 and 3620 strains were inferred over 1000 samples, for conventional and organic farming respectively. When looking at a region scale, without taking into account the farming system, we obtained high diversity index for 3 out of the 5 sampling regions, but for 2 of them, we could only recover low diversity results (table 2.b). These variations observed for the different diversity indexes can be explained by the disparity in the number of *S.cerevisiae* isolates in each appellation. As 27% of global *S.cerevisiae* population was not taken into account because of a too high proportion of missing values, same analyzes were made keeping the 7 markers giving the less missing values which increased the total number of strains to 1213 but led to equivalent results (data not shown). Our sampling strategy did not provide enough unique strains to exhaust the diversity of the whole region, as shown with the rarefaction analyses estimating that these genotypes at the whole region scale were sampled from an underlying population containing 6777 different genotypes (with 95% confidence limits of 3194.26 - 10351.73).

### **Global grapes population structure and diversity**

As a first step, in order to limit the impact of clonal expansion caused by the fermentation enrichment step, isolates with identical genotypes from the same wine estate were removed after GenClone analyzes. This reduced again the data set from 1002 to 402 grape isolates, corresponding to 398 unique genotype profiles.

The genetic relationship between these 402 *S.cerevisiae* grapes isolates, 33 commercials yeast starters as well as a set of 33 strains isolated from various resources whose genome has been

sequenced (Liti et al. 2009) (Supplementary data, table S2 and S3) was first analyzed through a bootstrap consensus tree constructed (figure 2). A set of strains from non-wine origin was used as a midpoint rooting for the tree. Different clusters were revealed by the neighbor-joining tree. Recently sequenced strains from non-wine origins clustered together, and strains related to wine strains whose genome has been sequenced were found mixed with Bordeaux grape strains (1). In contrast and surprisingly, some of our grapes isolates were close to Sake or bakery isolates strains (2). For the grape isolates group, some clusters contained isolates from different area (3) whatever the type of farming system or the year of sampling campaigns considered. With some exceptions it can be noticed that some grapes isolates are clustered according to the wine estate where they had been isolated as for wine estate B2 (4) in Medoc and wine estate C1 (5) in Pessac Léognan, for which all individuals came from a unique grape sample fermentation and for wine estate C2 in Pessac Léognan, for which individuals are from different grape sample fermentations and years (6). Grape strains were also clustered depending to their appellation, including different wine estates (7). Finally, some clusters contained commercials strains as well as many isolates from different appellations (8).

In order to infer population structure from ancestry profile, we used the Bayesian clustering method implemented in InStruct (Gao, Williamson, and Bustamante 2007) to the microsatellites data set including grapes and commercial strains. This method takes into account selfing, and does not make any assumption on the location where the strains have been obtained. Given the microsatellite data set, the optimal number of ancestral populations inferred was K=14 and the percentage of ancestry identified for yeast starters or grape strains is presented in figure 3 a and b, respectively. Ancestry profile of commercial strains, 522D and F33 were in green, VL1 and X5 in light green, F15 in red and FX10 in blue (figure 3.a), were shared with numerous grape strains (figure 3.b) and suggested, as observed in the

neighbor joining tree, a close relation between some grape isolates and several commercial yeast starters widely used in the Bordeaux area. Besides the presence of isolates related to yeast starters, the comparison of the ancestry profile per region indicate that each regional population results from a complex mix of the different ancestral profile in different proportion. Pessac Léognan and Medoc appellations appear to also have a small proportion of specific and unique ancestral population linked with a precise wine estate origin.

### **About grape isolates and industrials yeast starters**

Given the frequency of share ancestry of some grapes isolates and some yeast starters, it was necessary to further investigate this genetic relatedness in order to better understand the impact of the use of commercial yeast on the population structure of grape-associated *S. cerevisiae* population. The most representative commercial yeasts starter used in Aquitaine, (522D, FX10, F15, VL1, F33 and X5) were selected for further analysis.

The selection of grapes isolates with a minimum of 75% of common alleles with the main commercial yeast starters provided a group of 100 isolates. Spanning trees were constructed to evaluate the genetic relationship between these grape isolates and their closest yeast starters (figure 4). For each commercial yeast, 4 to 5 grapes isolates had exactly the same microsatellite profile and in addition 2 to 16 grape isolates had allelic differences at one or two loci with their nearest commercial strain suggesting clonal variants (figure 4.a1/b2/c2/e2). The group of grape isolates related to F15 commercial strain only contained individuals with more than two different alleles compare with commercial one (figure 4.d2). The yeast starter VL1 gathered with numerous grapes isolates particularly from Pessac Léognan (figure 4.e2) that is the main white wine production area from Bordeaux. Interestingly this strain was selected in 1987 from this appellation. VL1 is mainly used for white winemaking so that we cannot assess if the grapes isolates are from the ancestral population from which VL1 has

been isolated or if they derived from the frequent use of VL1 yeast starter. A similar situation can be pointed out for yeast starter F15 isolated from Medoc area in 1995 while numerous grapes isolates from this appellation cluster with those two commercial strains.

As the variation in the grapes isolates genotype might derive from variation during the production of yeast starter, the homogeneity of the microsatellite genotypes of clones isolated from several industrial productions was assessed. For that purpose, 10 clones per industrial batch of two different production years were isolated using a micromanipulator for further microsatellites analysis and compare to the original isolates of F15, X5, F10 and VL1 that have been selected in the laboratory for yeast starter production. Spanning trees based on microsatellite profiles of clones and original isolates of each commercial strain were constructed (figure 4). For FX10, F15 and VL1, all starter isolates gave identical profiles (figure 4.b1/d1/e1) showing that there was no clonal variation during manufacturing and over time. F15 and VL1 were also similar to their respective original strains, and FX10 was different from the original strain F10 at one locus, which is very likely due to the fact that FX10 was created after genetic improvement by a backcross approach (Marullo et al. 2009) from the original strain F10. For X5, 3 strains of 10 clones presented differences at one locus, giving two additional profiles to the main one (figure 4.c1). Finally, the majority of 522D and F33 starter isolates were united in one central cluster showing very close relation between the two commercial yeasts, but 7 out of 20 and 2 out of 20 isolates presented variations of F33 and 522D respectively, thus creating 9 different profiles in addition to the main one with one or two divergent allele for each (figure 4.a1). Identical karyotype profiles were obtained for the commercial strain FX10, F10, X5, F15 and VL1 and their respective vineyard clonal variant based on microsatellites patterns whereas different karyotypes were obtained for vineyard isolates for the 522/F33 genetic background but similar to clonal variant isolated from different industrial batches. All together, microsatellite and karyotype analysis

confirmed the close genetic relationships between industrial starters and grapes isolates (Supplementary data, figure S3 and table S5).

These results showed that the clonal variations observed among grape isolates related to yeast starters did very likely not result from the industrial scale multiplication during starter production. Indeed, those results were more probably resulting from the multiplication on a longer period, which indicate first that these strains were part of the cellar and then went to the vineyard ecosystem and underwent variations.

### **Testing for population differentiation**

To further analyze the diversity of the grape sample populations, the pattern and degree of divergence among areas were estimates by Fst over all 17 loci after removing strains with at least 75% of alleles shared with commercial strains yeast starters, thus compressing the data set from 402 to 302 grape individuals, meaning that 1/4 of the grape isolates collected were considered close to commercial strains.

When comparing the two farming system (table 3.b), the estimation of population by Fst 0.036 indicated a low but significant differentiation ( $p\text{-value} < 0.001$ ), similar to what could be estimated when taking into account all strains, including commercial related strains (table 3.a). These two farming systems were thus taken simultaneously in further analysis. When comparing the different appellations (table 4.b), the pairwise Fst values showed low to moderate differentiation (0.058 to 0.172), again similar to what could be estimated when taking in account all strains (0.051 to 0.124), including commercial related strains (table 4.a).

In order to confirm the differentiation between the different appellations, we first re-analyzed ancestry profile of the reduced grape isolate dataset (without strains related to yeast starter) with InStruct, and performed ObStruct analysis (Velimir Gayevskiy et al. 2014) (figure 5), (Supplementary data, table S6). Differentiation and importance in shaping the population

structure were stronger for three populations, Médoc, Bergerac and Saint Emilion appellations. More divergence can be observed for the two first appellations which present the highest contribution to population structure. But it had to be noticed that, for 2 of these 3 appellations, they were mostly represented by one predominant wine estate, B2 for Médoc appellation and A2 for Bergerac. The other 2 appellations of Entre-Deux-Mers and Pessac Léognan, contributed uniformly to the population structure.

This geographic differentiation in the population structuration might result from a spacial pattern of genetic variation. We thus tested for the significance of the correlation between the Fst matrix distance and the geographic distance with a Mantel test. The correlation between the matrixes of genetic and geographic distances was low (0.130) and not significant ( $p$ -value = 0.754). At the same time, an estimation of the gene flow between the different appellations, from 2012 sampling years, was made with Migrate software (figure 6). Inferred mean rates of movement between appellations was ranging from 10 to 60 migrants per generation (Supplementary data, table S7), and show different incoming and outgoing movements between the appellations along the different rivers Garonne in the south and Dordogne in the north. The greatest migration movements were between Médoc and Pessac Léognan with rates higher by 2.5 to 4 times compared to the mean of the others rates. Pessac Léognan appellation seemed to have a central position, with medium rates in outgoing migration. For Saint Emilion and Entre-Deux-Mers, the migration rates with the others appellations were equivalent and low for incoming and outgoing movements (around 14 migrants per generation).

### **Relation between grape and cellar diversity**

Grapes are one source of *S. cerevisiae* strains involved in the winemaking process, but the link between grape and cellar *S. cerevisiae* populations were poorly investigated till now.

Thus, we aimed to evaluate connections and possible genetic exchange between grape and cellar yeast metapopulation.

We collected *S. cerevisiae* strains from 11 spontaneously fermenting must of 7 cellars in 2012 and 2013 (table 1). Among these wine estates, 6 out of 7 were following organic farming system with little commercial starter inoculation used and 5 out of them were both sampled for grapes and cellars, with wine estates E1 and D1 collected during the two consecutive years for both Merlot and white grape varieties (Sauvignon blanc and Semillon), and the 2 others were only collected for Merlot must samples. Sampling was not possible, for other wineries associated with vineyard sampling as they used yeast starters. A total of 330 colonies were collected and identified as *S. cerevisiae*. After genotyping with microsatellite markers and filtered for clonality with GenClone, only 243 unique genotypes remained and after removing all strains presenting more than 75% of similarity with commercial yeast starters, this data set decreased to 225 cellar-associated *S. cerevisiae* unique profiles.

As dispersion of yeast starters from the cellar has already been reported (Valero et al. 2007b), we evaluated if the distance between the sampling site and the winery could explain the frequency of *S. cerevisiae* encountered in grapes samples fermentation, and as well as the frequency of strains related to commercial starters. These analyses were made only on the 2012 harvest season. The distances between grape sampling sites and the closest cellars varied between 28 to 389 meters. No clear relation had been seen when comparing the number of grapes samples harboring *S. cerevisiae* for each wine estate with distance (figure 7.a). When analyzing the percentage of grape isolates related to commercial strains, this percentage seems apparently greater within the first 200 meters distance from the cellar, than for higher distance (figure 7.b).

The relation between cellar and grape isolates can be analyzed from their phylogenetic relatedness. If cellar and grape isolates share the same origin, they should be found in similar phylogenetic clusters. A tree combining grape and cellar isolates (figure 8) presented a strikingly contrasted picture with grape and cellar strains separated in two main clusters: group 1 containing almost only cellar isolates, whereas group 2 contained almost only grapes isolates. A third cluster of strains mixed 48 isolated from grape, cellar, and commercial starters as well as 4 strains whose genome has been sequenced. For 4 out of the 5 wine estates for which we could both sample grapes and cellars, (A2 in Bergerac, C1 in Pessac Léognan, E2 in Saint Emilion and D1 in Entre-Deux-Mers), the cellar isolates were separated from the grape isolates of the same wine estate. For the 5th wine estate (E1 in Saint Emilion), the cellar isolates included in a cluster 2 grape isolates from this wine estate. In addition, some grape strains from wine estates C4, E4 and E5 were found mixed with multiple cellar strains. In the same manner, some cellar strains from wine estates D1 and E1 were mixed with the grape strains group. Last, all of the cellar strains from wine estate A1 and E10 were found in the same 3rd group.

A population analysis is another manner to compare the diversity of grape and cellar strains, and pairwise Fst between populations enable to measure population differentiation. We therefore calculated pairwise Fst between grapes and cellar populations. The comparison of cellar population to the appellation corresponding grape isolates populations indicated a significant differentiation with high Fst values between 0.09 to 0.22 (table 5.a). In contrast, the comparison of these cellar populations to that of the vineyards at the whole Aquitaine region level (table 5.b) indicated a lower differentiation.

Our previous results showed that the grape and cellar *S.cerevisiae* strains had their diversity and population structure well differentiated, but we decided to search for gene flow between the 2 compartments. MIGRATE analyses between the whole regional grape and cellar

metapopulation showed that there were still important gene flow migrations between the 2 environments (figure 9). The migrations were also not balance with 28% of number of additional migrants per generation from the grape to the cellar flow direction (268 vs. 193).

## Discussion

In this study, the diversity and population structure of *S.cerevisiae* were analyzed in the Bordeaux and Bergerac region, which are world well-known wine producing areas. Different studies have considered the whole yeast community and *S.cerevisiae* population diversity and structure at a country scale (Ayoub et al. 2006; V Gayevskiy and Goddard 2012; Pramateftaki, Lanaridis, and Typas 2000; Schuller et al. 2012), but less often at a region scale (Mercado et al. 2007; Nemcová et al. 2014; Schuller et al. 2005). Multiple parameters were often considered such as different grape varieties, farming systems, technological practices and geographical localization (Cordero-Bueso, Arroyo, Serrano, Tello, et al. 2011; Pramateftaki, Lanaridis, and Typas 2000; Schuller et al. 2012). To our knowledge, the only study describing the diversity of *S.cerevisiae* associated with grapes in the Bordeaux region was conducted in 1992 by Frezier, based on karyotype analysis, which reported that a small number of strains were dominant during non-inoculated alcoholic fermentation, whatever the variety or the time of harvest considered (Frezier and Dubourdieu 1992b). In this study, five wine producing appellations representative of the Bordeaux's vineyards were considered. Merlot grape variety couple samples were collected originated from close geographical areas characterized by similar microclimate conditions and from two vineyards conducted in organic and conventional farming system. This approach with such high numbers of wine estates (25) including 2 farming systems is unique comparing to other studies that only considered 2 wine estates for each farming system (Cordero-Bueso, Arroyo, Serrano, Tello, et al. 2011; Milanović, Comitini, and Ciani 2013). Most of previous studies relied on molecular analysis not or less appropriate for population analysis: such as PFGE (Pulsed Fieald gel

Electrophoresis), mitochondrial DNA, inter-*Delta* analysis (Schuller et al. 2005; Valero et al. 2007b; I. Vigentini et al. 2015). Within the last 14 years, microsatellites markers which are codominant markers and much more appropriate for population analysis, have been more frequently used (Goddard et al. 2010; Hennequin et al. 2001a; Schuller et al. 2012; Settanni et al. 2012), but with a small number of markers. In this study, we aimed to conduct an in-depth genetic diversity analysis of *S.cerevisiae* population structure, based on the robustness of microsatellite markers with a higher number of loci (17 loci).

During two consecutive years, 2012 and 2013, a total of 193 grape samples were collected in the Bordeaux appellation vineyards and from them, 101 did ferment and enabled us to isolate *S.cerevisiae*, representing 52% of fermentations. It is important to notice that in 2013, due to the specific climatic conditions during the ripening season, the grapes were partly damaged. The presence of damaged berries on vineyards are well known to be a greater source of microorganisms such as *S.cerevisiae* which have been shown to be harbored and disseminated by fruit-flies e.g. *Drosophila* sp. (Barata et al. 2012; Mortimer and Polsinelli 1999). Even if only healthy grapes were collected for our sampling, this situation could explain the extreme high success fermentation this year as all fermentations were effective. In contrast, in 2012, only 31% of fermentations enabled us to isolate *S.cerevisiae* (42 over 134). This data is in accordance with the 28% and 38% of positive fermentations obtained in 2 Portuguese wine-making regions by Schuller (Schuller et al. 2012). When comparing samples originated from organic and conventional farming systems in 2012, results were also similar with 29% and 33% of positive fermentations, respectively. Over all these fermentations, a total of 389 genotypes were revealed from an initial population of 402 *S.cerevisiae* isolates (96% of different genotypes), thus indicating a very high genetic diversity. However, our estimate of yeast diversity, suggest that Bordeaux and Bergerac region is expected to contain a larger diversity of *S.cerevisiae* strains, with an underling population of more than 6670

unique genotype strains. This result, based on a region scale, is nearly four time higher than the estimate of 1700 inferred in the all NZ country vineyard (Knight and Goddard 2015), which is expected considering that the New Zealand wine yeast population can be seen has a population deriving separated from European wine yeast.

The principal goal of this study was centered on the evaluation of vineyard-associated *S.cerevisiae* diversity and population structure in the Bordeaux and Bergerac regions with the aims to test for factors that may explain the resulting population structure. Different parameters were assessed, among which the use of organic or conventional farming systems. The influence of agronomic management system, including the use of pesticides on vineyard-associated yeast biodiversity is a key issue for the wine industry in the context of sustainable agriculture but is still a controversial ecological topic. The residual fungicides on leaves and grapes should not only be considered, since the impact of systemic pesticides and herbicides treatments on grape yeast diversity was questioned recently by Mandl (Mandl et al. 2015) who showed that soil yeast could be taken up by vine roots and transported via vine to the grapes and stems by an endophytic way. Previous studies reported contradictory positions on this point. In one hand, some authors showed that the use of phytosanitary treatments in the vineyards would negatively influence the yeast population diversity (Cus and Raspor 2008; Setati et al. 2012), especially the *S.cerevisiae* yeast, reducing the number of yeast isolated and their diversity (Cordero-Bueso, Arroyo, and Valero 2014). But on the other hand, recent studies showed that higher *S.cerevisiae* strains diversity was showed in conventional must fermentation in comparison to organic ones and that fungicides had no impact on yeast counts on grapes and during the alcoholic fermentation (Milanović, Comitini, and Ciani 2013; Oliva et al. 2007). In our study, based on a large numbers of wine estates (25), the global estimation of the number of genotypes from a rarefaction curve, indicate an approximately two fold increase of the number of *S.cerevisiae* grapes isolated from grapes in vineyards under

conventional farming system when comparing to organic ones. This higher diversity index of *S. cerevisiae* observed for conventional farming systems could be explained by a lower fungal microbial community due to repeated fungicide applications. As consequence, lower competition for available nutrients could offer more ecological space for *S. cerevisiae*. In addition, strains isolated from organic and conventional farming systems, were mixed in the individual trees, which is also confirmed by the low pairwise Fst values, which indicates that in our experimental conditions, farming system is not a main driver on the *S. cerevisiae* population structure.

The persistence of commercial *S. cerevisiae* starters in the vineyard and its impact on autochthonous yeast diversity is another topic that was investigated by many authors in different wine producing areas. The studies reported concordant results concerning the almost absence of dissemination of commercial yeast in the vineyard surrounding the vineyard and that the dissemination is restricted to short distances (maximum distance of 100 m around the dissemination area and in limited period of time) (Cordero-Bueso, Arroyo, Serrano, and Valero 2011; Valero et al. 2007b; Valero et al. 2005). In New Zealand, by comparing indigenous *S. cerevisiae* genotypes with a database of 79 commercial wine strains commonly used by the wine industry, Gayevskiy showed that only few isolates shared one microsatellite allele with commercial strain, thus supported the concept that a diverse natural population resides in New Zealand (V Gayevskiy and Goddard 2012). This study presented the results of a region scale approach, including 25 vineyards and 7 cellars using or not commercial strains. Our data that reported numerous strains isolated from grapes (approximately 25%) presenting a close genetic relationship with the commercial starters was a novelty. These grape isolates sharing more than 75% identical alleles with those of commercial strains were not only obtained in conventional farming system but also in organic farming one. The distance separating the closest cellars and the sampling area ranged from 1 to 350 meters, supporting

here the fact that commercial strains, even if they are used or not, can be transferred through the vineyard at a longer distance than previously reported, mixing with the grape endogenous strains population. Dispersal of commercial strains could be mediated by water run-off, macerated grape skin at dumping site (Valero et al. 2005) but also by drosophila (Buser et al. 2014), or even by the air through CO<sub>2</sub>-extraction system (Garijo et al. 2008). The clonal variations observed among grape isolates related to industrial starters could be an indication of a long-term dissemination of yeast starters in the environment. This hypothesis is reinforced by the fact that the use of industrial yeast to inoculate grape juice has been widespread in the Bordeaux wine producing area for over 40 years. However, we could not exclude for some grape isolates related to industrial strains initially isolated from the same Bordeaux region (e.g. VL1 in 1987 or F10 in 1991), that they derived from pre-existing endogenous population. In both cases, those results suggested the possible existence of long-term *S. cerevisiae* population (over 25 years) in a given wine-producing region. Cellars could contribute to the vineyard diversity enrichment by enological relevant *S. cerevisiae* strains that were previously selected for their fermentative properties. However, the transition from nutrient-rich musts to nutritionally scarce natural environments was recently shown to induce adaptive responses for the clonal variants that diminished capacities related with winemaking in comparison with the reference strain (Franco-Duarte et al. 2015). It will be interesting to extend this study at the genomic and phenotypic level to the biological material provided by this study.

The question of regional differentiation is still an open question for wine makers and wine microbiologists. It was recently showed that within region (encompass a radius of 100 km) in New Zealand, there is no compelling evidence for genetic differentiation between niches managed and native ecosystem and within managed ecosystems (Knight and Goddard 2015), however, regional delineations of natural *S. cerevisiae* populations were evidenced

(Gayevskiy and Goddard 2012; Knight and Goddard 2015). In this study, we aimed to test for geographic differences in *S. cerevisiae* population at the appellation scale in the Bordeaux and Bergerac wine-producing region. After removing strains related to commercial strains, which may blur local genetic structure, a first comparison of individuals suggested that strains from different appellation are mixed together. Population-based analysis revealed some variable differentiation between appellations, indicating some population structure. Direct geographic distance did not explain this diversity pattern. However, inference of gene flows by the maximum likelihood approach provided by MIGRATE revealed unequal transfer between regions. It is now well established that insects like bees, wasps and fruits flies, or even birds, could disseminate *S.cerevisiae* especially when the different regions are apart less than 100km (20km to 80km) (Francesca et al. 2012; Goddard et al. 2010; Stefanini et al. 2012) and thus be responsible in the homogenization of *S. cerevisiae* within regions (Knight and Goddard 2015). In the case of Bordeaux wine producing area, our results suggest higher migrations between appellations along the same riverbanks, like Pessac Léognan and Médoc, which are consistent with low pairwise Fst between these two appellations. Pessac-Léognan seemed to have a central position, with the higher migration rates in outgoing migration compared to outgoing ones.

A critical feature of the relevance of yeast diversity is the correspondence between cellar population and wine estates grape populations. The presence of specific clusters grouping cellar strains was highlighted. Those strains were not necessarily clustered with strains from the same appellation, indicating that wine estates contribute as well to the global population structure. Indeed the joined analysis of cellar and grapes isolates reveals a clear population structure differentiating most grapes from cellar isolates, with some exceptions as for wine estate E1. Population analysis, confirmed this differentiation between grape and cellar at the region and global scale. The differentiation measured by Fst statistic was low to intermediate,

when comparing cellar isolates populations to grapes isolates population from the whole Aquitaine region, in accordance with what could be expected since grapes are supposed to be one of the first sources of fermentative *S. cerevisiae* strains. However, genetic differentiation was higher when comparing grape and cellar strains within the appellation. The dynamics of grape *S. cerevisiae* dissemination in the environment on one side and the dynamics of native cellar-resident strains on the other side, might be two divergent factors explaining the lack of connection between grape isolates populations at the wine estate and appellation scales and cellar populations.

This study provided new knowledge on the diversity and population structure of *S.cerevisiae* within an historical wine making region. At the appellation scale, the populations were structured and significantly differentiated. The concept of strain originated from a given appellation was not relevant in this study, but a specialization of genotypes by wine estate could be highlighted, that however had not been assessed in the long-term. The comparison between grape and cellar metapopulation showed low to intermediate differentiation. The high frequency of commercial related strains isolated from grape samples in both conventional and organic vineyards with 1/4<sup>th</sup> of the global grape population, is a new indication of a long-term dissemination of yeast starters in the environment. However, the differentiation of cellar and grapes isolates suggests that despite the yeast potential release into the environment by wineries, attested by the identification of commercial starter related strains, the impact of the release is low and does not lead to the homogenization of both populations. This suggests two potential divergent life styles inside the cellars or in the vineyards. Last, the comparison of organic *versus* conventional farming systems, revealed little impact on yeast population structure, but an impact on the diversity of grape isolates, attested by a higher number of genotypes on grapes under conventional farming system.

All these results draw a new perspective of the strong inter-connection between vineyard and cellar population and of their limits. Winemakers should be aware that this connection has not a unique direction, from grapes to cellars, but also from cellars to grapes. More efforts have to be performed to decipher the causes of these population structures.

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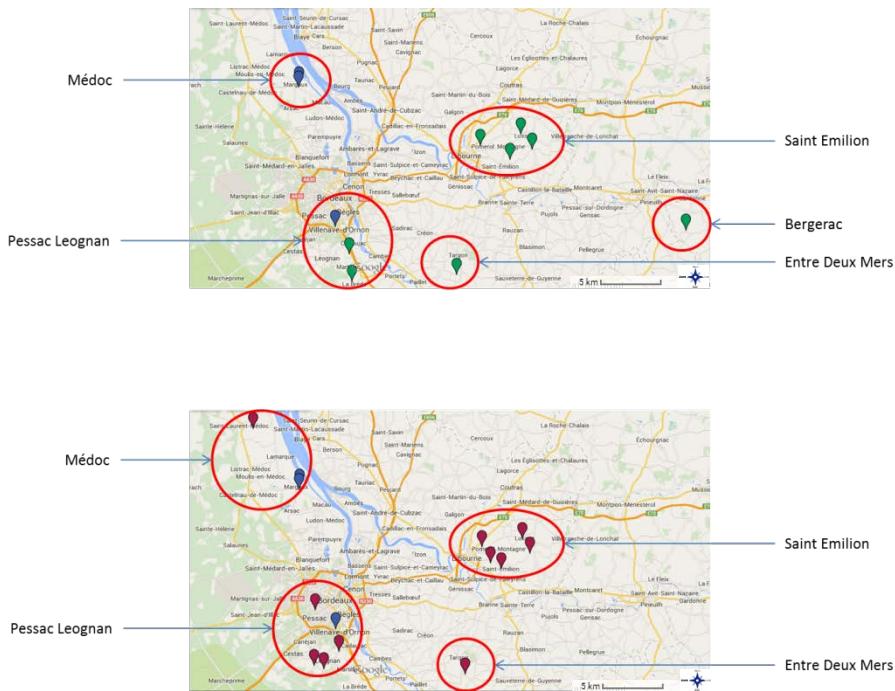
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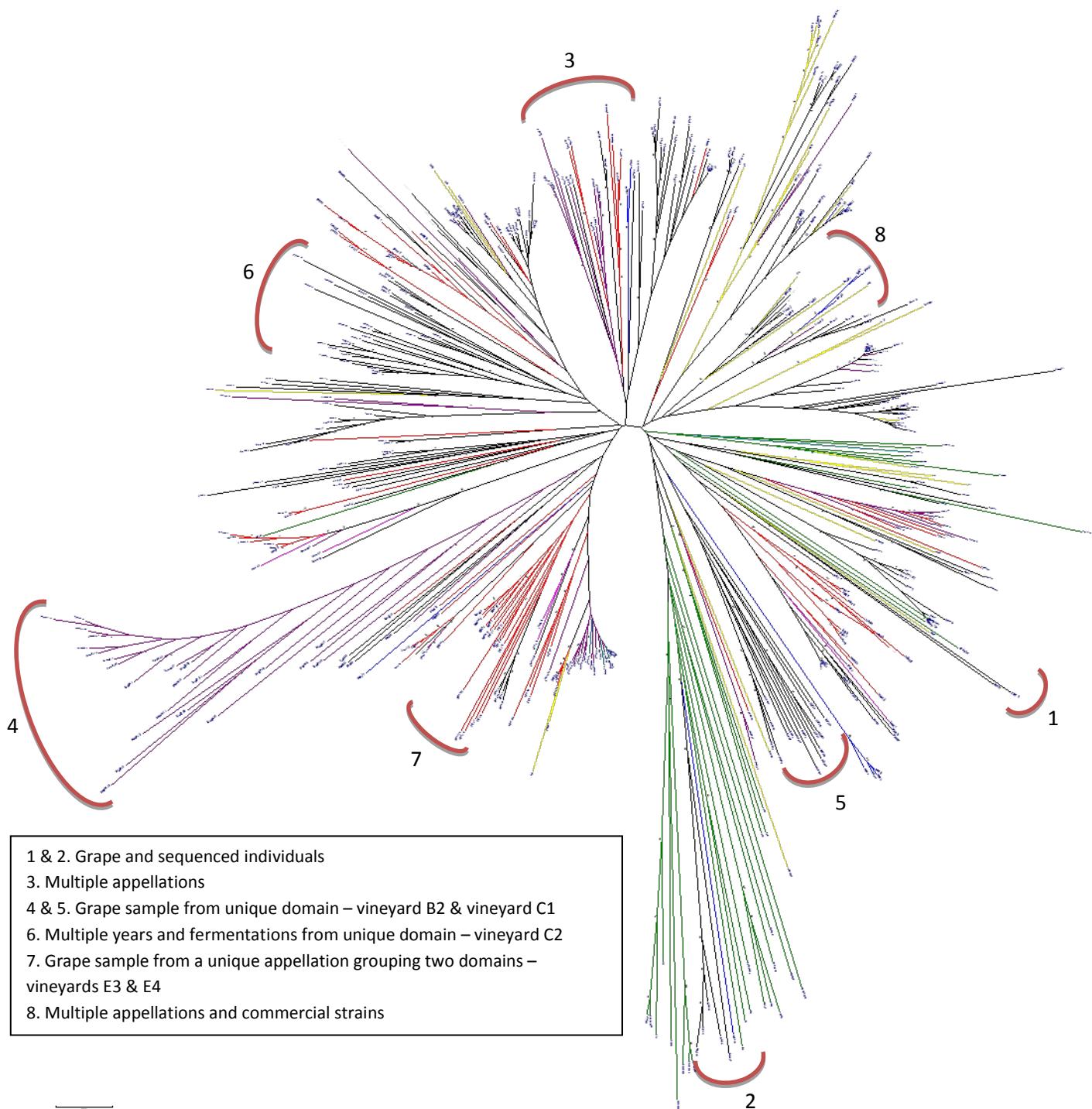
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## FIGURES



**Figure 1.**

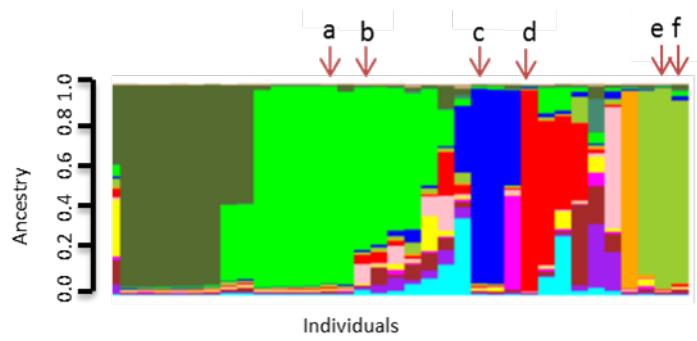
Geographic localization of the wine estates in the appellations of Bordeaux and Bergerac regions. Green labels represent vineyards in organic farming system, red in conventional one and blue, vineyards managed with both organic and conventional farming system.



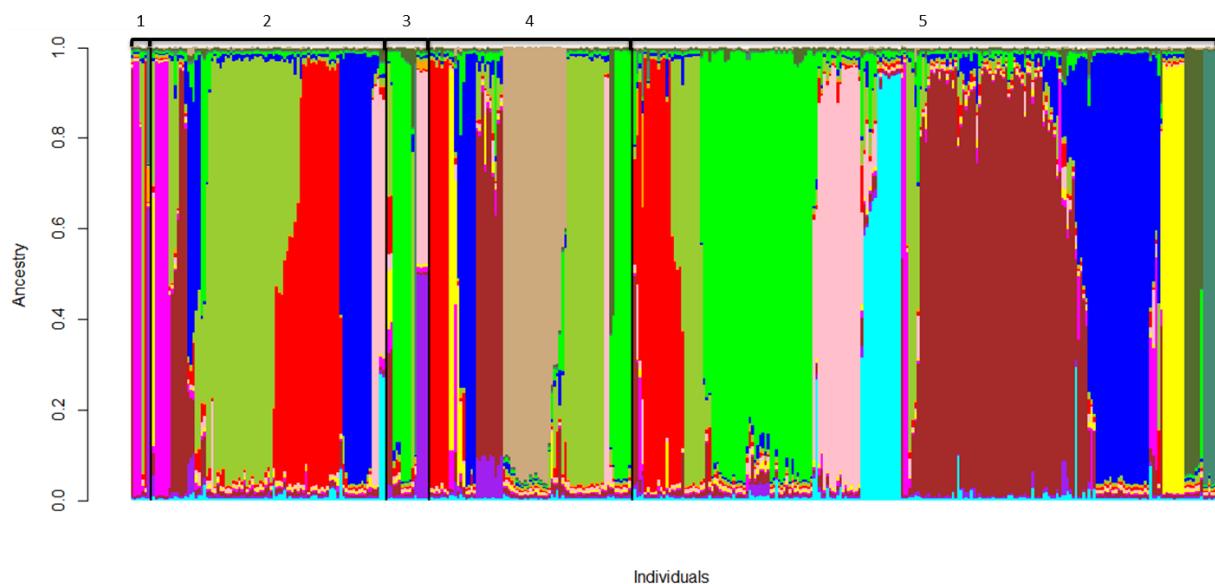
**Figure 2.**

Neighbor joining tree clustering 402 strains from grapes of Bordeaux and Bergerac regions 33 commercial strains and 33 strains from the *S.cerevisiae* sequenced data base. The tree was constructed from Bruvo's distance between strains based on the polymorphism at 17 loci. Color code: Sequenced strains, green; commercial strains, yellow; Médoc, purple; Saint Emilion, red; Entre Deux Mers, pink; Pessac-Léognan, black; Bergerac, blue.

a.

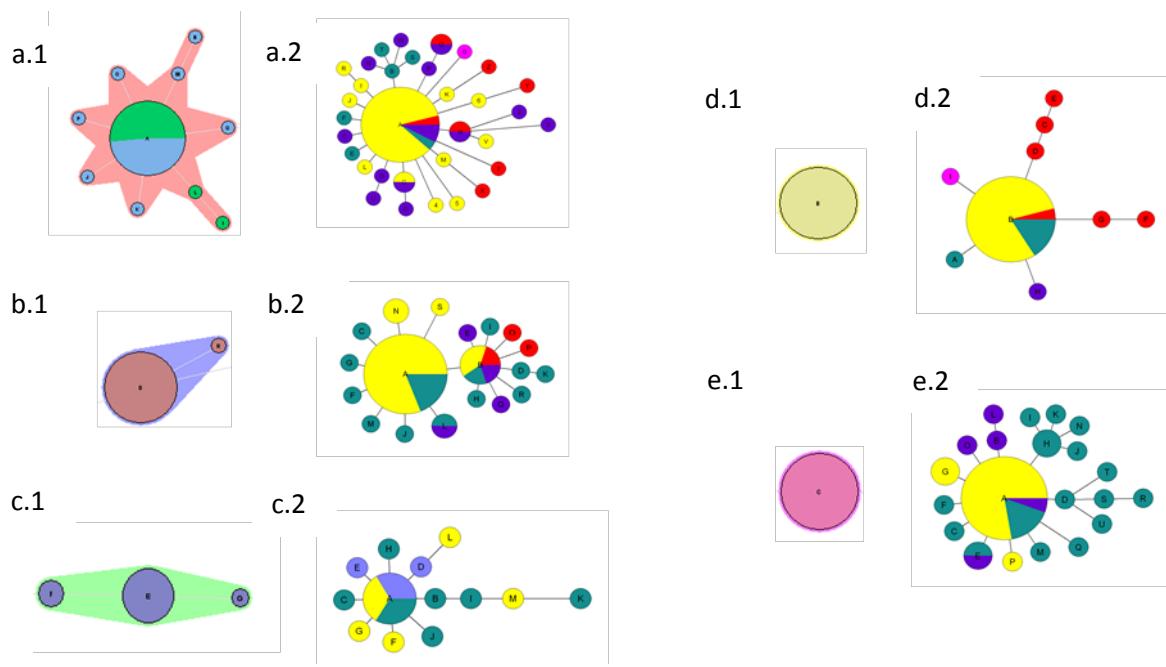


b.



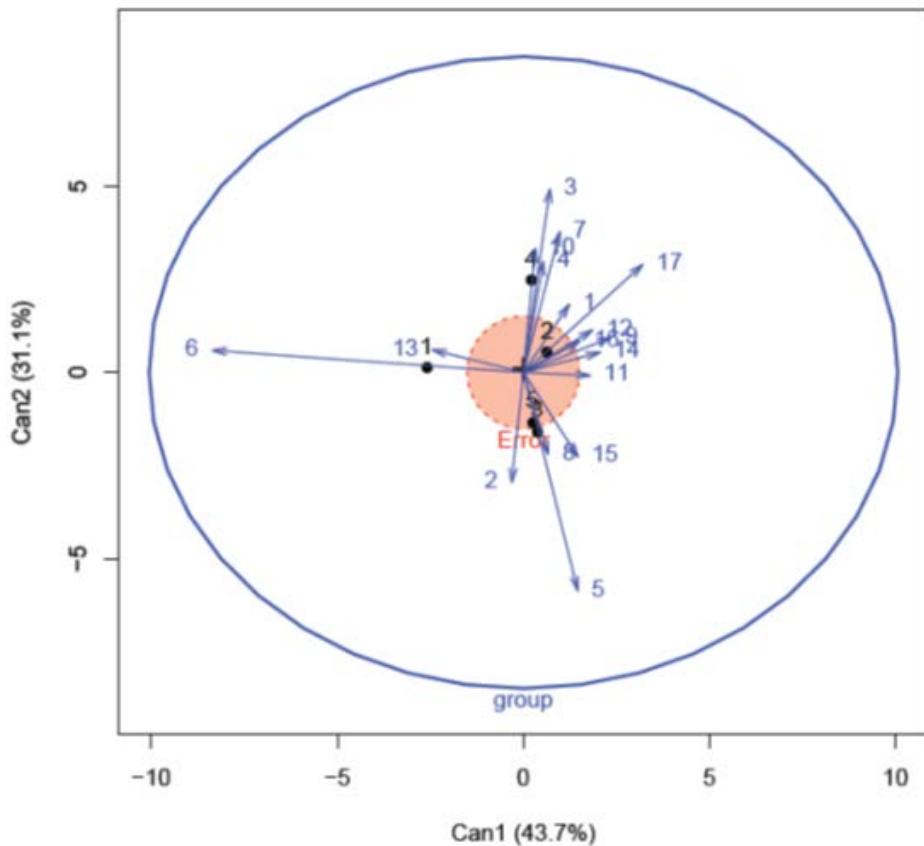
**Figure 3.**

Inference of population using InStruct program on the a. 33 commercial strains and b. 402 grape sample strains with the optimal  $K = 14$ . Commercials selected strains: a. VL1; b. X5; c. FX10; d. F15; e. F33 and f. 522D. Appellations code : 1. Entre Deux Mers; 2. Saint Emilion; 3. Bergerac; 4. Médoc and 5. Pessac Léognan.



**Figure 4.**

Spanning trees constructed with sampling of commercial strains only (1) and association of commercial and individuals from grapes strains (2). Color code (1) a. 522D/F33 (green and light blue); b. FX10; c X5; d.1 F15; e.1 VL1. Color code (2): commercial strains, yellow; Médoc, purple; Saint Emilion, red; Entre Deux Mers, fuchsia; Pessac-Léognan, green; Bergerac, dark blue



**Figure 5.**

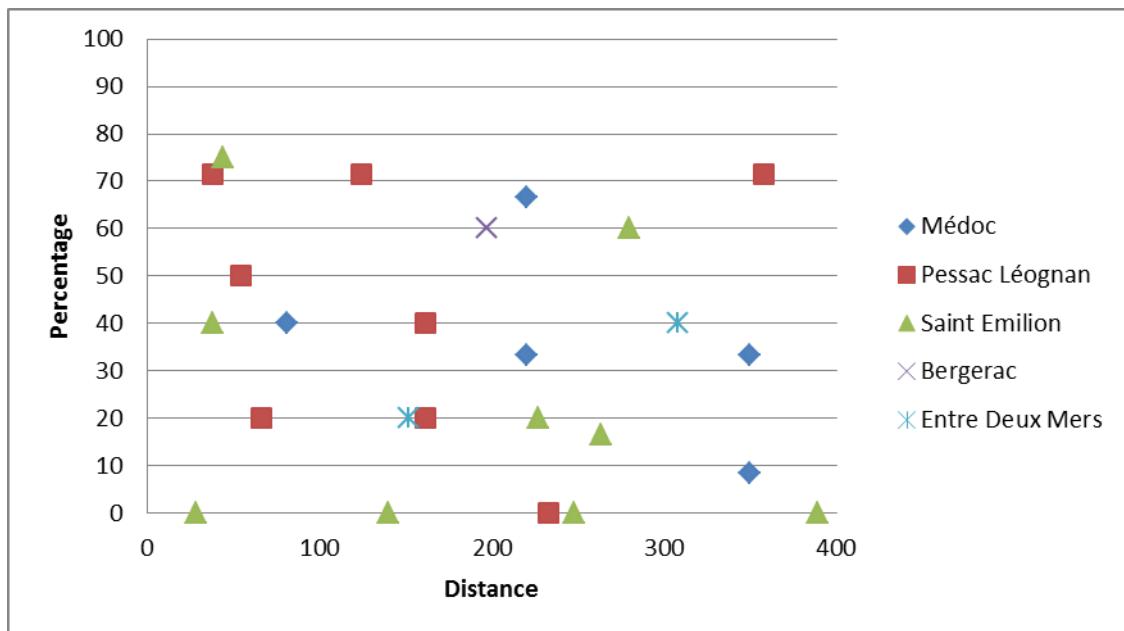
Canonical discriminant analysis on the *Saccharomyces cerevisiae* dataset. The HE plot shows the relation of variation in the group means on two variables relative to the error variance. The arrows indicate the position of the inferred populations relative to the axes obtained by the canonical discriminant analysis. The black points indicate predefined populations (1 = Médoc; 2 = Pessac Léognan; 3 = Saint Emilion; 4 = Bergerac; 5 = Entre Deux Mers) while numbers at the arrows indicate inferred populations.



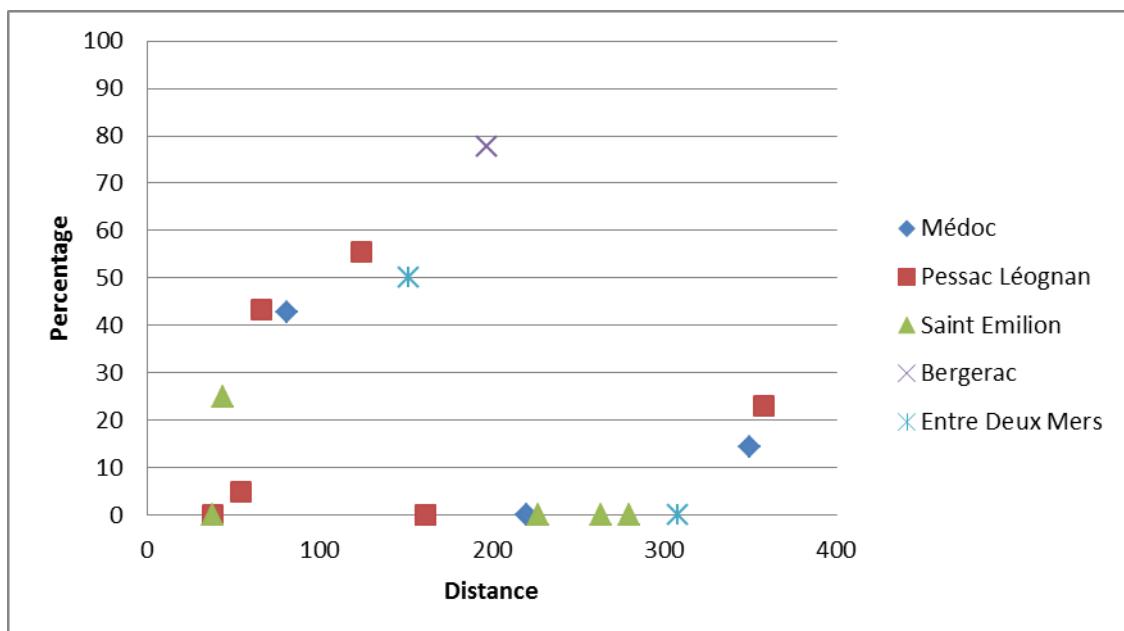
**Figure 6.**

Directional gene flow migration between the 4 appellations where *S.cerevisiae* strains were found in 2012. The size of the arrows were equivalent to the number of migrants per generation that were calculated in MIGRATE. The absolute values are in supplementary table S6. Appellations code: 1 – Médoc, 2 – Pessac Léognan, 3 – Saint Emilion, 4 – Entre Deux Mers.

a.

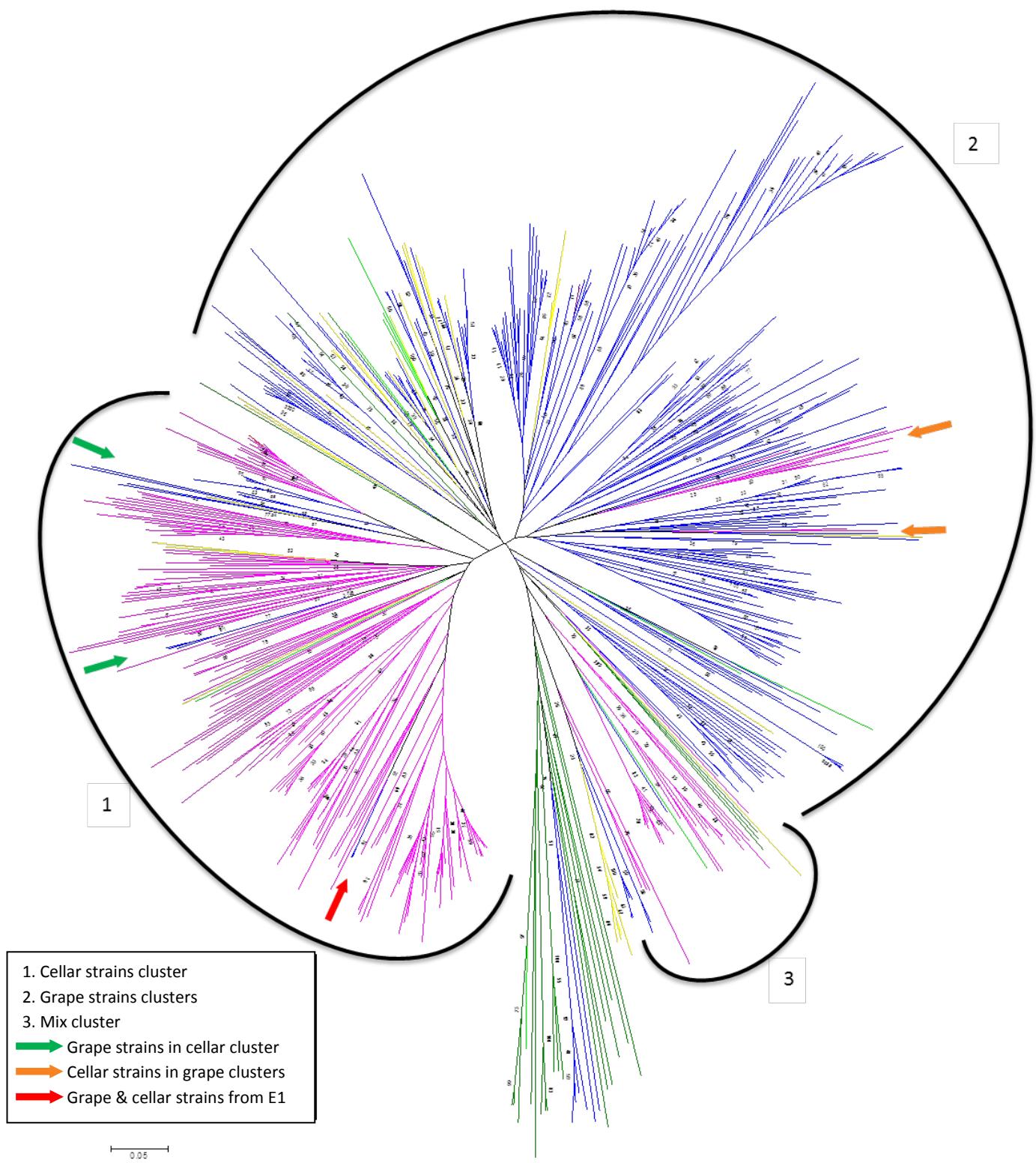


b.



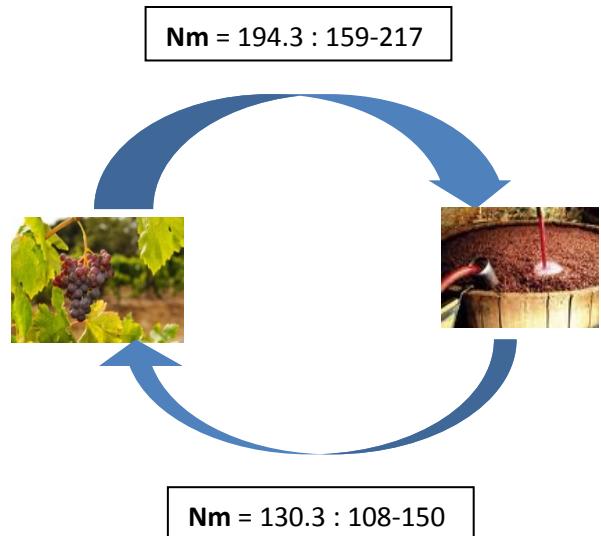
**Figure 7.**

Distance relation between the closest cellar and the grape sampling site for each appellation **a.** on the percentage of grape fermentations that provided *S.cerevisiae* strains and **b.** on the percentage of commercial related grape strains found.



**Figure 8.**

Neighbor joining tree clustering 302 strains from grapes (blue) and 173 stains from cellars (pink) of Bordeaux and Bergerac regions, 33 commercial strains (yellow) and 33 strains from the *S.cerevisiae* sequenced data base (green). The tree was constructed from Bruvo's distance between strains based on the polymorphism at 17 loci.



**Figure 9.**

Directional gene flow migration of *S.cerevisiae* strains were found in the grape and the cellar ecological niches in 2012. The size of the arrows were equivalent to the number of migrants per generation (Nm) that were calculated in MIGRATE with the absolute values.

## TABLES

**Table 1.**

Summary of grape and must samples collected in Bordeaux and Bergerac regions with indication of wine estate, sampling years, number of fermentations and number of *S.cerevisiae* profiles types

\*Also corresponding to the total number of fermentation for each domains \*\*Started fermentations that ended between 28 and 100% of the alcoholic fermentation \*\*\*Number of distinct microsatellite profiles after GenClone analyzes

Appellation	Wine estate	Farming system	2012				2013				2012		2013	
			Grape samples*	Number of started fermentation**	Number of fermentation giving S.c isolates	Number of S.c unique profiles***	Grape samples*	Number of started fermentation**	Number of fermentation giving S.c isolates	Number of S.c unique profiles***	Must sample	Number of S.c unique profiles***	Must sample	Number of S.c unique profiles***
Bergerac	A1	Organic	-	-	-	-	-	-	-	-	1	32	-	-
Bergerac	A2	Organic	5	5	3	9	5	5	2	6	1	22	-	-
Medoc	B1	Organic	12	8	1	7	6	6	2	12	-	-	-	-
Medoc	B1	Conventional	3	1	0	0	3	3	2	13	-	-	-	-
Medoc	B2	Organic	3	2	2	12	-	-	-	-	-	-	-	-
Medoc	B2	Conventional	3	3	1	24	-	-	-	-	-	-	-	-
Medoc	B3	Conventional	5	4	2	7	-	-	-	-	-	-	-	-
Pessac – Leognan	C1	Organic	5	5	2	30	-	-	-	-	1	17	-	-
Pessac – Leognan	C2	Conventional	4	4	2	21	5	5	4	46	-	-	-	-
Pessac – Leognan	C3	Conventional	5	5	0	0	-	-	-	-	-	-	-	-
Pessac – Leognan	C4	Conventional	10	5	3	18	5	5	4	13	-	-	-	-
Pessac – Leognan	C5	Organic	7	7	5	26	5	5	-	0	-	-	-	-
Pessac – Leognan	C6	Organic	5	5	1	5	-	-	-	-	-	-	-	-
Pessac – Leognan	C6	Conventional	5	5	2	9	6	6	1	4	-	-	-	-
Pessac – Leognan	C7	Conventional	7	6	5	28	4	4	4	18	-	-	-	-
Entre deux Mers	D1	Organic	5	5	1	2	-	-	-	-	2	37	1	26
Entre deux Mers	D2	Conventional	5	3	2	5	-	-	-	-	-	-	-	-
Saint Emilion	E1	Organic	5	4	0	0	5	5	1	6	2	52	1	23
Saint Emilion	E2	Organic	5	5	2	2	5	5	-	0	1	22	-	-
Saint Emilion	E3	Conventional	5	1	0	0	5	5	4	33	-	-	-	-
Saint Emilion	E4	Conventional	5	5	3	8	5	5	2	17	-	-	-	-
Saint Emilion	E5	Conventional	4	4	3	12	-	-	-	-	-	-	-	-
Saint Emilion	E6	Organic	6	4	1	2	-	-	-	-	-	-	-	-
Saint Emilion	E7	Organic	5	4	0	0	-	-	-	-	-	-	-	-
Saint Emilion	E8	Conventional	5	2	1	7	-	-	-	-	-	-	-	-
Saint Emilion	E9	Conventional	5	5	0	0	-	-	-	-	-	-	-	-
Saint Emilion	E10	Conventional	-	-	-	-	-	-	-	-	-	-	1	12
Total			134	107	42	234	59	59	26	168	8	182	3	61

**Table 2.**

Shannon index ( $H'$ ), equitability index ( $J'$ ) and Simpson index ( $1/D$ ) and his complement index ( $1-D$ ). Analyses of the 1374 *S.cerevisiae* obtained after microsatellites analyzes depending of a. the type of farming system, organic or conventional and b. the different appellations of Bordeaux and Bergerac.

a.

	Organic farming	Conventional farming
<b>Number of individuals</b>	340	662
<b><math>H'</math> (Shannon Index)</b>	4.09	4.86
<b><math>J'</math> (Equitability Index)</b>	0.68	0.80
<b><math>1/D</math> (Simpson index)</b>	32.47	66.83
<b><math>1-D</math> (Simpson complement)</b>	0.97	0.99

b.

	Médoc	Pessac Léognan	Saint Emilion	Bergerac	Entre Deux Mers
<b>Number of individuals</b>	149	524	235	73	21
<b><math>H'</math> (Shannon Index)</b>	3.78	4.69	3.73	1.9	1.22
<b><math>J'</math> (Equitability Index)</b>	0.62	0.77	0.62	0.31	0.20
<b><math>1/D</math> (Simpson index)</b>	24.26	56.17	22.51	4.49	2.15
<b><math>1-D</math> (Simpson complement)</b>	0.96	0.98	0.96	0.78	0.53

**Table 3.**

Pairwise  $F_{st}$  values between the type of farming system, organic or conventional a. before and b. after removing all grape strains associated to commercial wine strains. All values are significant ( $P<0.001$ ).

a.

	Conventional	Organic
Number of individuals	283	119
Conventional	0,000	0,001
Organic	0,031	0,000

b.

	Conventional	Organic
Number of individuals	138	66
Conventional	0,000	0,001
Organic	0,036	0,000

**Table 4.**

Pairwise Fst values between appellations of Bordeaux and Bergerac regions a. before and b. after removing all grape strains associated to commercial wine strains. All values are significant ( $P<0.001$ ) except for some values comparing Entre-Deux-Mers and the others appellations (\*).

a.

	Médoc	Pessac Léognan	St Emilion	Bergerac	Entre deux Mers
<b>Number of individuals</b>	75	218	87	15	7
<b>Médoc</b>	<b>0,000</b>	0,001	0,001	0,001	0,004*
<b>Pessac Léognan</b>	0,059	<b>0,000</b>	0,001	0,001	0,001
<b>St Emilion</b>	0,074	0,051	<b>0,000</b>	0,001	0,005*
<b>Bergerac</b>	0,108	0,075	0,124	<b>0,000</b>	0,001
<b>Entre deux Mers</b>	0,087	0,080	0,054	0,106	<b>0,000</b>

b.

	Médoc	Pessac Léognan	St Emilion	Bergerac	Entre deux Mers
<b>Number of individuals</b>	51	164	71	11	5
<b>Médoc</b>	<b>0,000</b>	0,001	0,001	0,001	0,001
<b>Pessac Léognan</b>	0,104	<b>0,000</b>	0,001	0,001	0,001
<b>St Emilion</b>	0,127	0,058	<b>0,000</b>	0,001	0,001
<b>Bergerac</b>	0,151	0,089	0,139	<b>0,000</b>	0,001
<b>Entre deux Mers</b>	0,172	0,134	0,120	0,163	<b>0,000</b>

**Table 5.**

Measurement of population differentiation between wine estates and grapes populations, from pairwise Fst statistics. Fst have been estimated between a. cellar and grape population of the same appellation and b. between cellar population and grape from the whole Aquitaine region. All values are significant ( $P<0.001$ ).

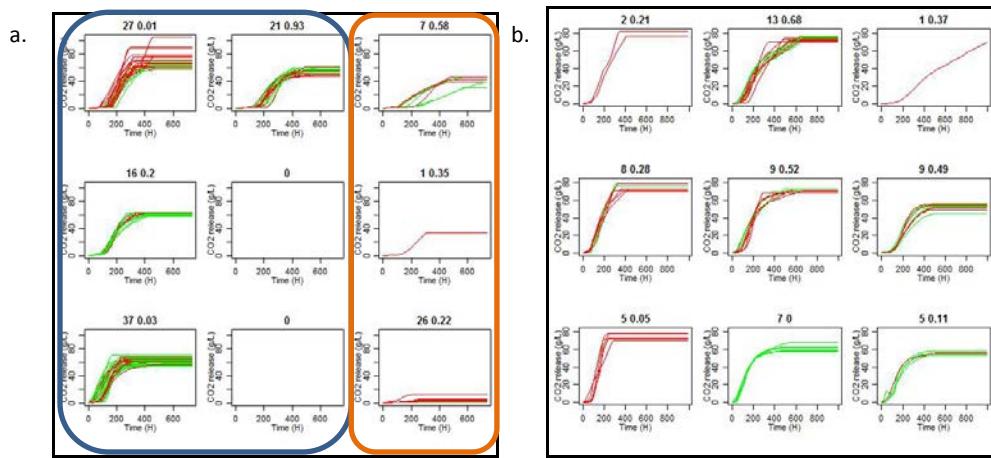
a

Cellars	A1	A2	C1	D1	E1	E2	E10
<b>Number of grape strains</b>	11	11	164	5	71	71	71
<b>Number of cellar strains</b>	28	20	12	61	75	20	9
<b>Fst</b>	0.215	0.136	0.214	0.158	0.129	0.087	0.092
<b>P-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.001

b

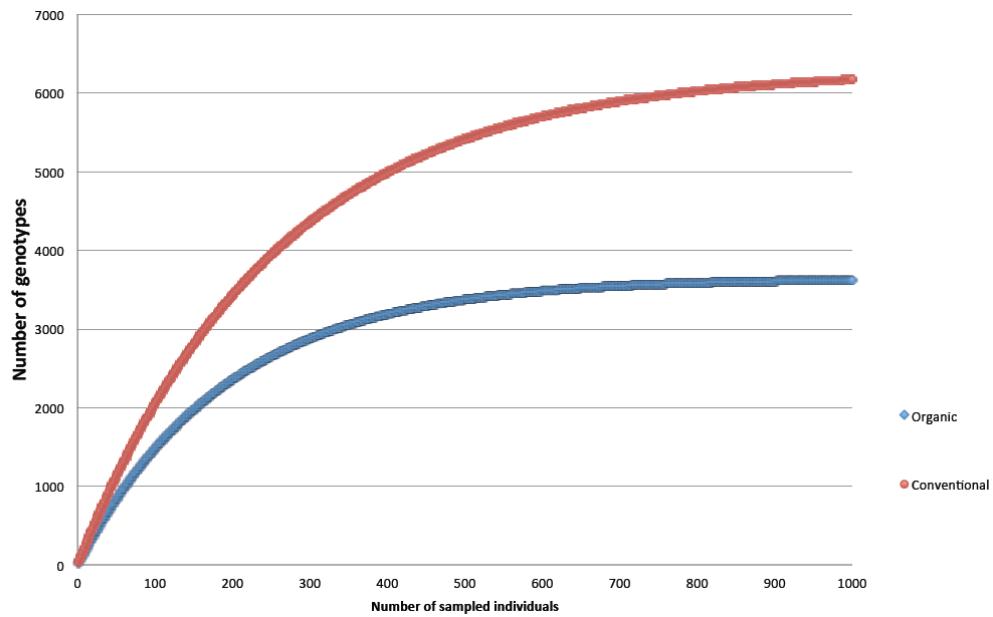
Cellars	A1	A2	C1	D1	E1	E2	E10
<b>Number of grape strains</b>	302	302	302	302	302	302	302
<b>Number of cellar strains</b>	28	20	12	61	75	20	9
<b>Fst</b>	0.133	0.067	0.201	0.057	0.109	0.042	0.04
<b>P-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.002

## SUPPLEMENTAL DATA



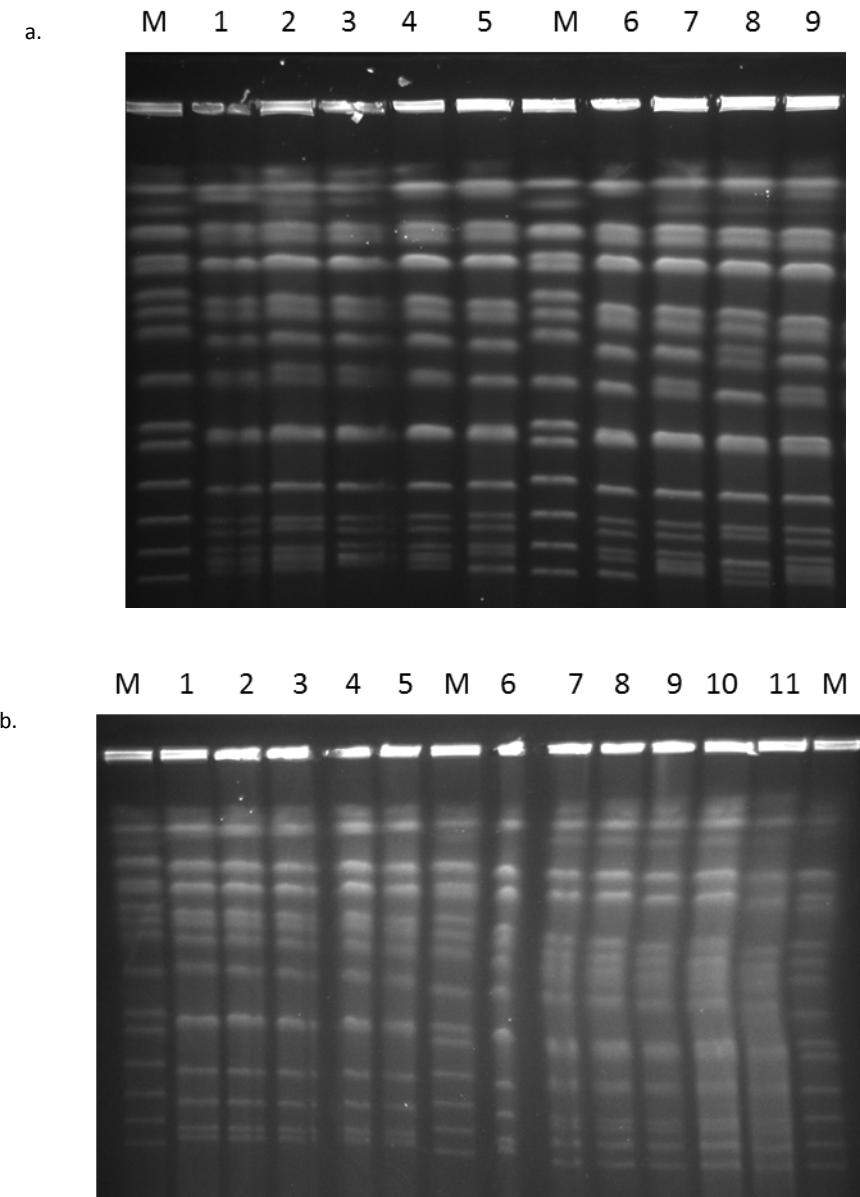
**Figure S1.**

Grape sample fermentation curves showing the consumption of CO<sub>2</sub> in function of time in hours. **a.** 2012, 134 fermentations in which blue selection represent fermentation that did reach at least 50% of the alcoholic fermentation and in orange fermentation that stopped before the 50% of alcoholic fermentation. **b.** 2013, 59 fermentations. Curves clustered depending on the CO<sub>2</sub> consumption on the vertical axis and the lag time on the horizontal axis. Samples from organic farming system were in green and from conventional one in red.



**Figure S2.**

Comparison of genotypes rarefaction curves obtained for grapes sampled vineyards managed in organic (blue) and conventional (red) farming system. All calculations were performed using EstimateS v9.10.



**Figure S3.**

Example of karyotypes analysis of commercial strains and associated microsatellites patterns clonal variants isolated from the commercial lots and vineyards. a. M: molecular marker, 1-5: clonal variants of the commercial lot of F33, 6: clonal variants of the commercial lot of 522; 7: 13caMconv4\_3, 8: 13maMconv3\_12, 9: 12yfMconv1\_9. b. M: molecular marker, 1: F15, 2: 13hpMconv2\_4, 3: 13caMconv4\_9, 4: 12fz1Mconv5\_11, 5: 12fz1Mconv5\_24, 6: 12fz1Mconv5\_29, 7: VL1, 8: 12bcMbio1\_16, 9: 12bcMbio1\_10, 10: 12bcMbio1\_14, 11: 13maMbio1\_10.

**Table S1.**

Assembly of the different site selected with the given code, the region, the farming system, the domains names, domain abbreviations, GPS data for cellars, site of sampling and distance between them. \*Those samples were closer to others cellars than the one from the wine estate. The distances to a cellar were 350m for B1, 220m for B2 and 280m for E4.

Code	Sub-region	Farming system	Name	Abbreviation	Cellar GPS data	Sampling GPS data	Distance (m)
A1	Bergerac	Organic	Domaine Des Costes	cos	44°51'33.0"N 0°30'35.0"W	-	
A2	Bergerac	Organic	Château Richard	ri	44°46'45.4"N 0°18'56.3"E	44°46'39.2"N 0°18'59.4"E	197
B1	Medoc	Organic	Château Margaux	ma	45°02'39.9"N 0°40'07.5"W	45°02'47.2"N 0°41'10.1"W	1380*
B1	Medoc	Conventional	Château Margaux	ma	45°02'39.9"N 0°40'07.5"W	45°02'49.8"N 0°41'11.6"W	1440*
B2	Medoc	Organic	Château Palmer	pa	45°02'12.2"N 0°40'10.8"W	45°01'45.8"N 0°40'58.3"W	1360*
B2	Medoc	Conventional	Château Palmer	pa	45°02'12.2"N 0°40'10.8"W	45°01'46.3"N 0°40'58.2"W	1350*
B3	Medoc	Conventional	Château Camensac	cm	45°08'47.8"N 0°47'11.4"W	45°08'49.2"N 0°47'07.8"W	81
C1	Pessac – Leognan	Organic	Château Baulos Charmes	bc	44°44'09.3"N 0°32'26.3"W	44°44'07.5"N 0°32'27.4"W	66
C2	Pessac – Leognan	Conventional	Château Carbonnieux	ca	44°44'41.9"N 0°34'07.9"W	44°44'42.5"N 0°34'05.9"W	54
C3	Pessac – Leognan	Conventional	Domaine De Chevalier	ch	44°43'06.9"N 0°37'57.7"W	44°43'02.2"N 0°38'06.7"W	233
C4	Pessac – Leognan	Conventional	Château Fieuzal	fz	44°42'50.8"N 0°36'23.5"W	44°42'54.6"N 0°36'23.1"W	124
C5	Pessac – Leognan	Organic	Château Bichon Cassignol	bi	44°41'10.3"N 0°32'02.2"W	44°41'17.3"N 0°32'15.0"W	358
C6	Pessac – Leognan	Organic	INRA	in	44°47'18.6"N 0°34'41.3"W	44°47'23.0"N 0°34'43.2"W	161
C6	Pessac – Leognan	Conventional	INRA	in	44°47'18.6"N 0°34'41.3"W	44°47'23.0"N 0°34'43.2"W	161
C7	Pessac – Leognan	Conventional	Château Luchey Halde	lh	44°49'12.5"N 0°37'49.5"W	44°49'13.3"N 0°37'50.5"W	38
D1	Entre deux Mers	Organic	Domaine Du Bourdieu	du	44°41'54.4"N 0°16'23.2"W	44°41'58.5"N 0°16'27.0"W	152
D2	Entre deux Mers	Conventional	Château Ducourt	dc	44°42'16.5"N 0°14'51.8"W	44°42'09.9"N 0°14'41.3"W	308
E1	Saint Emilion	Organic	Château Bellevue	be	44°57'07.8"N 0°06'18.3"W	44°57'15.0"N 0°06'12.9"W	28
E2	Saint Emilion	Organic	Château Moulin de Lagnet	ml	44°54'23.7"N 0°07'58.4"W	44°54'24.3"N 0°07'57.4"W	38
E3	Saint Emilion	Conventional	Château Haut Piquat	hp	44°56'55.1"N 0°06'04.1"W	44°56'57.0"N 0°06'15.4"W	248
E4	Saint Emilion	Conventional	Couvent des Jacobins	cj	44°53'40.8"N 0°09'18.6"W	44°53'27.0"N 0°09'42.0"W	661*
E5	Saint Emilion	Conventional	Château Yon Figeac	yf	44°54'17.8"N 0°10'59.7"W	44°54'17.8"N 0°11'01.3"W	44
E6	Saint Emilion	Organic	Château Gombard Guillot	gg	44°55'55.1"N 0°12'28.7"W	44°55'53"N 0°12'17"W	263
E7	Saint Emilion	Organic	Château Beauséjour	beau	44°55'28.4"N 0°04'33.6"W	44°55'26"N 0°04'39"W	140
E8	Saint Emilion	Conventional	Château Clinet	cli	44°56'02.7"N 0°12'17.2"W	44°55'56"N 0°12'13"W	227
E9	Saint Emilion	Conventional	Château Soleil	so	44°55'19.9"N 0°04'54.7"W	44°55'26"N 0°04'39"W	389
E10	Saint Emilion	Conventional	Montagne	mo	44°57'27.2"N 0°10'23.1"W	-	

**Table S2.**

33 *S.cerevisiae* strains of Sanger data base from wine and divers origins. OS column is the accession number in the internal collection at the University of Nottingham. OSNNN/A means a single spore was isolated from the original diploid and OSNNN/A/A indicates this process was repeated. The absence of any /A indicates that either the strain was haploid or that a monosporic culture was provided.

\*Strains related to wine fermentation

OS	Strain	Geographic, Isolated by, Year and references	Source
288/A	273614X	Royal Victoria Infirmary, Newcastle UK, Galloway A	Clinical isolate (Fecal)
287/A	378604X	Royal Victoria Infirmary, Newcastle UK, Galloway A	Clinical isolate (Sputum)
181	BC187*	Napa Valley, Bisson L, USA <sup>Ref</sup>	Barrel Fermentation
150/A	DBVPG1106*	Australia, 1947, Fornachon J	Grapes
91/A	DBVPG1373	Netherlands, Capriotti A, 1952 <sup>Ref</sup>	Soil
84/A	DBVPG1788	Turku, Finland, Capriotti A, 1957 <sup>Ref</sup>	Soil
92/A	DBVPG1853	Ethiopia, Rossi, 1959 <sup>Ref</sup>	White Teff
155/A	DBVPG6040	Netherlands, 1947 <sup>Ref</sup>	Fermenting fruit juice
60/A	DBVPG6044	West Africa, Guillermond A, 1925 <sup>Ref</sup>	Bili wine, from <i>Osbeckia grandiflora</i>
3/A	DBVPG6765	Unknown <sup>Ref</sup>	Unknown
251/A/A	K11	Japan, 1981 <sup>Ref</sup>	Shochu sake strain
220/A	L-1374*	Cauquenes, Chile, Ganga A, 1999	Fermentation from must País
21/A	L-1528*	Cauquenes, Chile, Ganga A, 1999	Fermentation from must Cabernet
247/A	NCYC110	West Africa, Guillermond A, pre-1914 <sup>Ref</sup>	Ginger beer from <i>Z.officinale</i>
96	S288c	Merced, California, USA, Mrak E, 1938 <sup>Ref</sup>	Rotting fig
17/A	SK1	USA, Kane S, pre-1974 <sup>Ref</sup>	Soil
278/A	UWOPS03-461.4	Telok Senangin, Malaysia, Wiens F, 2003 <sup>Ref</sup>	Nectar, Bertram palm
280/A	UWOPS05-227.2	Telok Senangin, Malaysia, Lachance M, 2005	<i>Trigona spp</i> (Stringless bee) collected near Bertam palm flower
279/A	UWOPS05-217.3	Telok Senangin, Malaysia, Lachance M, 2005	Nectar, Bertram palm
270/A	UWOPS83-787.3	Great Inagua Island, Bahamas, 1983, Lachance M	Fruit, <i>Opuntia stricta</i>
271/A	UWOPS87-2421	Puhelu Road, Maui, Hawaii, Lachance M, 1987	Cladode, <i>Opuntia megacantha</i>
281	W303	Created by Rothstein R by multiple crossing <sup>Ref</sup>	NA
253/A/A	Y12	Ivory Cost, pre-1981 <sup>Ref</sup>	Palm wine strain
97/A	Y55*	France, Winge Ö, between 1930-60 <sup>Ref</sup>	Grape
252/A	Y9	Indonesia, pre-1962 <sup>Ref</sup>	Ragi (similar to sake wine)
174	YIIC17_E5*	Sauternes, France	Wine
308/A	YJM975*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
303/A	YJM978*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
304/A	YJM981*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
182	YPS606	Pennsylvania, USA, Sniegowski P, 1999 <sup>Ref</sup>	Bark of <i>Q.rubra</i>
258/A/A	YS2	Australia <sup>Ref</sup>	Baker strain
259/A/A	YS4	Netherlands, 1975, Barnett J <sup>Ref</sup>	Baker strain
262/A/A	YS9	Singapore <sup>Ref</sup>	Baker strain

**Table S3.**

33 *S.cerevisiae* strains from industrial yeast production commonly used in Aquitaine region, in organic and conventional farming.

Commercial name	Strain	Distributor
Actiflore cerevisiae	522D	Laffort
Excellence B2		Lamothe Abiet
Lalvin BM 45		Lallemand
Levuline CHP	CIVC8130	Oenofrance
Lalvin CY 3079	BourgoBlanc	Lallemand
Excellence C1 Val oeno		Lamothe Abiet
Zymaflore F10	FZ 182	Laffort
Zymaflore F15		Laffort
Actiflore F33	F33	Laffort
Fermol Arome plus	PB2010	Spindal
Fermivin	7013	Littorale
Zymaflore FX10		Laffort
ICV-GRE	138 grenache	ICV
K1	Killer non marquée	Lallemand
L.A. L13	L13	Lamothe Abiet
Rhône L2226		Lallemand
Lallferm bio		IOC / Lallemand
Lalvin QA23	QA23	Lallemand
Lalvin 71B	71B	Lallemand
Levuline ALS	EG8	Oenofrance
Levuline BRG	UP 30Y5	Oenofrance
Oenoferm Bio		Littorale
Vitilevure KD	R2	Martin Vialatte
Zymaflore RX60	rx60	Laffort
SP organic		Martin Vialatte
Uvaferm CEG	CEG - Epernay 2	Lallemand
Achor vin 13	vin 13	Littorale
Vitilevure quartz		Martin Vialatte
Zymaflore VL1	vl1	Laffort
Zymaflore VL3	lv3	Laffort
Zymaflore X16	x16	Laffort
Zymaflore X5	x5	Laffort
Zymaflore 011 organiq		Laffort

**Table S4.**

Microsatellite loci for *Saccharomyces cerevisiae* genotyping with, repeated motif, ORF, primer sequence, fluorescence dye, mix number and concentration used for 8 samples PCR mix preparation for each marker

Site name	Motif and type	ORF or coordinates	Primers	Fluorescent dye	Multiplex	Quantity (nM)	Author
ScAAT2	TAA	YBL084c	FW: CAGTCTTATTGCCCTGAAACGA RV: GTCTCCATCCTCCAACAGCC	PET	1	100	4
ScAAT3	TAA	YDR160w	FW: TGGGAGGAGGGAAATGGACAG RV: TTCAGTTACCCGCACAATCTA	NED	1	200	1, 3
C5	GT	VI-210250/210414	FW: TGACACAATAGCAATGCCCTCA RV: GCAAGCGACTAGAACAAACATACA	VIC	1	50	5
C3	CAA	YGL139w	FW: CTTTTATTACGAGCGGGCAT RV: AAATCTCATGCCGTGAGGGGTAT	NED	1	100	5
C8	TAA	YGL014w	FW: CAGGTCGTTCTAACGTTGGAAAATG RV: GCTGTTGCTGTTGGTAGCATTACTGT	6FAM	1	25	5
C11	GT	X-518870/519072	FW: TTCCATCATAACCGCTCTGGGATT RV: TGCCCTTTCTTAGATGGCTTTC	6FAM	1	50	5
YKR072c	GAC	YKR072c	FW: AGATACAGAAAGATAAGAACGAAAA RV: TTATTGATGCTTATCTATTATACC	PET	1	50	1, 2
ScAAT6	TAA	IX-105711/105883	FW: TTACCCCTCTGAATGAAAACG RV: AGGTAGTTAGGAAGTGAGGC	PET	1	100	1, 3
SCYOR267c	TGT	YOR267c	FW: TACTAACGTCAACACTGCTGCCAA RV: GGATCTACTTGCAGTATACGGG	VIC	1	100	1, 4
YKL172w	GAA	YKL172w	FW: CAGGACGCTACCGAAGCTCAAAAG RV: ACTTTGGCCAATTCTCAAGAT	6FAM	2	25	2
ScAAT1	TTA	XIII-86902/87140	FW: AAAGCGTAAGCAATGGTGTAGACTT RV: CAAGCCTCTCAAGCATGACCTTT	VIC	2	100	1, 3, 4
C4	TAA+TAG	XV-110701/110935	FW: AGGAGAAAATGCTGTTATTCTGACC RV: TTTCCCTCCGGGACGTGAAATA	NED	2	200	5
C9	TAA	YOR156c	FW: AAGGGTTCGAACATATAACTGGCA RV: TATAAGGGAAAAGAGCACGATGGC	NED	2	100	5
ScAAT5	TAA	XVI-897051/8970210	FW: AGCATAATTGGAGGCAGTAAAGCA RV: TCTCGTCTTTTGACTCGTG	NED	2	100	5
C6	CA	XVI-485898/485996	FW: GTGGCATCATATCTGTCATTTTAC RV: CAATCAAGCAAAAGATCGGCCT	VIC	2	50	5
YPL009c	CTT	YPL009c	FW: AACCCATTGACCTCGTTACTATCGT RV: TTCGATGGCTCTGATAACTCCATT	6FAM	2	50	1, 4
YLR	TC	XII-823393/823562	FW: CTGGAATGAAATTAAACAAAGC RV: TCTTCCTTTCTACTATCTTC	PET	2	100	2
YLL049W	TA	XII-40666/41205	FW: GCAACATAATGATTTGAGGT RV: GTGTCTTGTGAGCATAGTGGAGAA	PET	2	50	6

Authors: (1) Field and Wills (1998), (2) Hennequin et al. (2001), (3) Perez et al. (2001), (4) Gonzalez Techera et al. (2001), (5) Legras et al. (2005), (6) Bradbury et al. (2006)

**Table S5.**

Pulsed field electrophoresis profiles obtained for the commercial strains and the associated microsatellites patterns clonal variants isolated either from the commercial lots or vineyards. In each group of strain, the commercial strains of reference are the first of the group and is indicated in bold.

\*Strains used for diversity and populations structure analysis

Strains	Wine estate	Profiles ECP
<b>FX10</b>		I
<b>F10</b>		II
13caMconv3_9	C2	I
13lhMconv3_10	C7	I
13lhMconv3_15	C8	I
13lhMconv3_22	C9	I
13lhMconv2_26	C10	I
12caMconv3_12	C2	II
13beMbio3_10	E1	I
12cmMconv5_10	B3	I
<b>X5</b>		III
12piMbio3_1		III
12piMbio3_9		III
12riMbio2_28		III
12riMbio3_24		III
<b>F15</b>		IV
13hpMconv2_4	E3	IV
13caMconv4_9	C2	IV
12fz1Mconv5_11	C4	IV
12fz1Mconv5_24	C4	IV
12fz1Mconv5_29	C4	IV
<b>VL1</b>		V
12bcMbio1_16	C1	V
12bcMbio1_10	C1	V
12bcMbio1_14	C1	V
13maMbio1_10	B1	V
<b>F33_16_1*</b>		VI
<b>F33_16_7</b>		VI
<b>F33_16_8</b>		VI'
<b>F33_18_2</b>		VI''
<b>F33_18_5</b>		VI
<b>F33_18_8</b>		VI
<b>F33_18_10</b>		VI'''
<b>Acti522D*</b>		VII
<b>522_16_1</b>		VII''
<b>522_17_6</b>		VI'''
<b>522_17_8</b>		VI'''
13caMconv4_3	C2	VI''
13maMconv3_12	B1	VI''''
12yfMconv1_9	E5	VI''
12yfMconv1_9	E5	VI''

**Table S6.**

Pairwise matrix of R2 values between the 5 appellations of Bordeaux and Bergerac regions, after removing all related to commercial grape strains (with P.value).

	Pessac Léognan	Entre deux Mers	Médoc	Bergerac	Saint Emilion
Pessac Léognan	<b>NA</b>	0.02 (0.02)	0.08 (<0.001)	0.02 (<0.001)	0.06 (<0.001)
Entre deux Mers	0.02 (0.02)	<b>NA</b>	0.07 (0.01)	0.23 (0.02)	0.04 (0.16)
Médoc	0.08 (<0.001)	0.07 (0.01)	<b>NA</b>	0.10 (<0.001)	0.12 (<0.001)
Bergerac	0.02 (<0.001)	0.23 (0.02)	0.10 (<0.001)	<b>NA</b>	0.08 (<0.001)
Saint Emilion	0.06 (<0.001)	0.04 (0.16)	0.12 (<0.001)	0.08 (<0.001)	<b>NA</b>

**Table S7.**

Mean directional estimates of migration rate (Nm) as calculated by MIGRATE with 95% confidence intervals (mean estimate: lower 95% confidence interval – upper 95% confidence interval). All estimation goes from the appellation on the left to the appellation of the top line.

	Médoc	Pessac Léognan	Saint Emilion	Entre Deux Mers
Médoc		39.0: 5-62	15.0: 0-29	15: 0-30
Pessac Léognan	60.3: 37-81		22.3: 3-41	20.3: 0-37
Saint Emilion	13.0: 0-27	19.0: 1-35		15.7: 0-31
Entre Deux Mers	11.7: 0-25	14.3: 0-29	10.3: 0-22	

## Chapitre 2

# Cellar-associated *Saccharomyces cerevisiae* population structure revealed high diversity and perennial persistence in Sauternes wine estates

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## **Abstract**

Three wine estates (A, B and C) were sampled in Sauternes, a typical appellation of Bordeaux wine area producing sweet white wine. Over those wine estates, 551 yeasts strains were collected between 2012 and 2014 adding 102 older strains from 1992 to 2011 from wine estate C. All strains were analyzed through 15 microsatellites markers resulting in 503 unique *Saccharomyces cerevisiae* genotypes, thus revealing a high genetic diversity, and a low presence of commercial yeast starters. Population analysis performed from Fst statistics, or from 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 together than with wine estate A indicating exchange between estates. The characterization of isolates collected 23 years ago in wine estate C related to recent isolates obtained in 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 to the selective picking of noble rotted grapes was highlighted.

**Keywords:** Population structure, *Saccharomyces cerevisiae*, microsatellites, wine, *Botrytis cinerea*

2    **Introduction**

3    *Saccharomyces cerevisiae* is widely distributed and associated with human-related  
4    fermentations, as well as with those from the natural environment (e.g., oak trees and fruits).  
5    The population genetic structure of *S. cerevisiae* was shown to correlate with its ecological  
6    differentiation (Fay and Benavides 2005; Legras et al. 2007; Liti et al. 2009; Schacherer et al.  
7    2009), as well as geographical distance (Legras et al. 2007; Cromie et al. 2013). Strains  
8    isolated from vineyard and wine-related environment constitute a genetically well-  
9    differentiated homogeneous group. In the last 20 years, many studies described the genetic  
10   diversity of *Saccharomyces cerevisiae* isolated from different grape varieties. Molecular  
11   methods such as pulsed field gel electrophoresis (Valero et al. 2007a; Versavaud et al. 1995;  
12   Frezier and Dubourdieu 1992a), mtDNA RFLP analysis (Cappello et al. 2004a; Schuller et  
13   al. 2005; Cubillos et al. 2009), Inter-*delta* analysis (Maurizio Ciani et al. 2004; Le Jeune et al.  
14   2006; Ileana Vigentini et al. 2015), and microsatellite analysis (Schuller and Casal 2007;  
15   Schuller et al. 2012) were used to describe the genetic diversity of vineyard-associated *S.*  
16   *cerevisiae*. Numerous factors such as climate conditions, geographical location of the  
17   vineyard, fungicides management, grape varieties, winemaking practices impact the natural  
18   yeast population's diversity (Combina et al. 2005; Cordero-Bueso, Arroyo, Serrano, Tello, et  
19   al. 2011; Schuller et al. 2012). Grapes are supposed to be the first source of *S. cerevisiae*  
20   strains involved in the winemaking process and then, the winery surfaces are probably the  
21   main microbial reservoir to carry out the must spontaneous fermentation (Maurizio Ciani et  
22   al. 2004; Le Jeune et al. 2006; Bokulich et al. 2013; Martini 2003). Gayevskiy and Goddard  
23   were the first to show evidence for region-specific *S. cerevisiae* populations associated with  
24   vines and wines in New Zealand using microsatellite markers, and they also pointed the  
25   exchanges of strains among these regions (Velimir Gayevskiy and Goddard 2012; Knight et  
26   al. 2015). The presence of specific fermentative profiles with a perennial persistence over

27 different successive years in a given wine producing area was highlighted by different authors  
28 (Frezier and Dubourdieu 1992a; Versavaud et al. 1995; Torija et al. 2001). However, a recent  
29 study inferred that view of a stable population in a wine environment over time since no *S.*  
30 *cerevisiae* strain was isolated in the same vineyard or cellar during different three consecutive  
31 years (Ileana Vigentini et al. 2015). Till now, very few studies reported long-term  
32 observations of the changes in the *S. cerevisiae* population over time (Beltran et al. 2002).

33 Botrytized wines are natural sweet wines, produced from grapes that are affected by *Botrytis*  
34 *cinerea* under a rare and special form of fungal infection called “noble rot”. Sauternes region  
35 in France, similar to the Tokaj wine region in Hungary, is one of the most famous and highly  
36 estimated areas for noble rot sweet wines. The development of noble rot includes complex  
37 enzymatic conversions and concurrent dehydration of the grape berry and results in a highly  
38 concentrated final product (Magyar 2011). Grapes are picked when they reach their optimum  
39 maturation state and must are obtained from noble rotted grapes cluster selective pickings.  
40 The noble rot development is subject to weather conditions that dictate the number of  
41 selective pickings each year, typically up to three or four. The resulting grape musts have  
42 specific characteristics with high sugar, acid, glycerol and mineral contents, nitrogen  
43 deficiency, special polysaccharides and aroma composition (Ribereau-Gayon et al. 1979).  
44 This specific composition provides extremely difficult nutritional and environmental  
45 conditions for yeast growth and fermentative metabolism. As consequence, yeasts produce  
46 high level of acetic acid during the alcoholic fermentation, ranging from 0.56 to 1.50 g/L  
47 depending on the must (Bely, Masneuf, and Dubourdieu 2005) and the fermentation can be a  
48 slow process. Taking into account the difficult fermentation conditions, the use of selected  
49 yeast starters is generally recommended (Ribereau-Gayon et al. 2000). Another alternative is  
50 the use of subcultures or “pied de cuve” produced from fermenting must that were proven to  
51 limit acetic acid production in wine (Bely, Masneuf, and Dubourdieu 2005). Finally,

52 fermentations are prematurely stopped, generally by a massive addition of sulphur dioxide,  
53 thus producing sweet wines with residual sugars.

54 Many previous studies reported the population dynamic on the surface of botrytized grapes  
55 and revealed a complex microbiota. *Botrytis* infection stimulates a high diversity level of  
56 yeasts, and the community is likely enriched with fermentative and/or spoilage species (A. a.  
57 Nisiotou and Nychas 2007). The significant presence of *Candida stellata*, later renamed as  
58 *Starmerella bacillaris* (synonym *Candida zemplinina*) species (Sipiczki 2003), was reported.  
59 In the fresh botrytized must (Sauternes), *C. pulcherrima* were also detected at a significant  
60 population (Fleet, Lafon-Lafourcade, and Ribéreau-Gayon 1984). These two non-  
61 *Saccharomyces* species are both strong competitors for *B. cinerea*, and their presence has  
62 been suggested as a biocontrol agent against postharvest fungal pathogens. The presence of *S.*  
63 *cerevisiae* and *S. uvarum* was described on Tokaj grapes (Naumov et al. 2002). The yeast  
64 microbial community of the grape must mirror the grapes microbiota and is highly diverse  
65 when compared to traditional dry wines. *Candida zemplinina* could dominate fermentation  
66 during the first stages and later *Kluyveromyces*, *Hanseniaspora* and *Pichia* were frequently  
67 isolated from mi-fermentation (Fleet, Lafon-Lafourcade, and Ribéreau-Gayon 1984; Mills,  
68 Johannsen, and Cocolin 2002; A. a. Nisiotou and Nychas 2007). Thus non-*Saccharomyces*  
69 yeast may contribute significantly to the fermentation of botrytized wines at early stages, but  
70 *S. cerevisiae* still dominates the fermentation process frequently associated with *S. uvarum*  
71 (Fleet, Lafon-Lafourcade, and Ribéreau-Gayon 1984; Naumov et al. 2002; Naumov et al.  
72 2000; Antunovics, Csoma, and Sipiczki 2003). The damaged grape berries' state may impact  
73 the *Saccharomyces* yeast diversity and population level since they may be very rich  
74 depositories of *S. cerevisiae*, comparing with sound berries (Mortimer and Polsinelli 1999).  
75 However the *S. cerevisiae* population associated with the winemaking process of botrytized  
76 must was poorly investigated until now. In a survey concerning wine estates in the south

77 region of Bordeaux, Frezier (1992) described the existence of dominant *S. cerevisiae* profiles  
78 whenever white, red and botrytized wine spontaneous fermentations were studied, during two  
79 consecutive years. Latter, Masneuf and Dubourdieu (2000) using PGFE method, established  
80 the karyotypes of 199 *S. cerevisiae* strains isolated from indigenous fermentation of  
81 botrytized must and reported a high diversity of the profiles, with no dominant ones.

82 The occurrence of local and resident *Saccharomyces cerevisiae* populations in a given  
83 viticultural region, and at a smaller scale in a given winery, is a recurring issue asked by the  
84 scientific community and the winemakers. In viticulture, the terroir effect is a complex  
85 concept and can be explained by agronomic interactions between the vine and its environment  
86 (soil, climate, and landscape) (Van Leeuwen et al. 2010). Climate and soil act on vine  
87 behavior through their impact on precocity, water status, and nutrient status. Alongside these  
88 key factors, the microbial aspect to terroir was recently illustrated by different studies that  
89 suggested a link between vineyard environmental conditions and microbial inhabitation  
90 patterns and revealed the importance of microbial populations for the regional identity of  
91 wine (Bokulich et al. 2014; Knight et al. 2015). At the winery scale, the stability of a given *S.*  
92 *cerevisiae* population over different vintages could be an indication of its possible impact on  
93 the local wine style and thus its contribution to the characteristic wine's feature.

94 The objective of this study was to establish the population genetic structure of *S. cerevisiae* at  
95 a spatial (region/winery) and temporal scale (over 20 years) in the case of a fermentative  
96 system characterized by a highly complex microbiota and difficult nutritional and  
97 environmental conditions for yeast growth. For that purpose, we used a robust molecular  
98 method based on the analysis of 15 microsatellite markers. *S. cerevisiae* isolates were  
99 collected from spontaneous fermented must samples of three wine estates in the Sauternes  
100 Appellation from 2012 to 2014. We aim to gain deeper knowledge on cellar-associated *S.*  
101 *cerevisiae* ecology and possible exchanges between populations in the same appellation. We

102 took advantage of having a large collection of *S. cerevisiae* isolates collected in grape  
103 spontaneously fermenting grape must since 1992 in one of the wine estates to survey the long  
104 term diversity and population structure of cellars-associated *S. cerevisiae* and to test the  
105 hypothesis of the presence of specific wine cellars populations with a perennial persistence in  
106 a given region or wine estate.

107

## 108 **Materials and methods**

### 109 **Samples collection and processing**

110 A total of 3 wine estates were selected to conduct this study in the Sauternes appellation,  
111 which is one of the sweet wine producing areas in Gironde, part of the Aquitaine region in  
112 southwest France. The distance between wine estate A and B/C is 10 km, whereas distance  
113 between wine estate B and C is 1.8 km. The three wine estates produce sweet wines from  
114 botrytized Sauvignon and Semillon grape varieties (Figure 1). The initial sugars content of the  
115 grape must were between 350 and 450 g/L. Wine estates A and B are managed according to  
116 organic practices, whereas wine estate C is managed according to conventionally. Briefly,  
117 sulfur and copper are both used in organic and conventional farming systems whereas  
118 synthetic fungicides are also used in the conventional one. Alcoholic fermentation was  
119 stopped thanks to a massive addition of sulfur dioxide (20-30 g/hL). Samples were taken at  
120 75% of the alcoholic fermentation. Different numbers of lots from different years were  
121 sampled in those wine estates (Table 1). In wine estate A, sampling was performed for 2  
122 years, in 2012 and 2014, from 2 and 4 different lots respectively. In wine estate B, sampling  
123 was performed for 3 years, 2012, 2013 and 2014, with 3 vats in 2012 and 2013, and 2 in  
124 2014. Finally, sampling was performed in wine estate C in 2014 in 5 different vats.

125

126

127 **Strain isolation**

128 Different dilutions ( $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$ ) of the collected samples were plated onto YPD (yeast  
129 extract, 1% w/v, peptone, 1% w/v, glucose, 2% w/v, agar 2% w/v) with  $100\mu\text{g.ml}^{-1}$  of  
130 chloramphenicol and  $150\mu\text{g.ml}^{-1}$  of biphenyl to delay bacterial and mold growth. A maximum  
131 of 40 randomly chosen colonies were collected after incubation (2 days at  $26^\circ\text{C}$ ). After two  
132 sub-cloning on YPD plates, each colony was stored in (30%, v/v) glycerol at  $-80^\circ\text{C}$ .

133 **Additional isolates**

134 For wine estate C, *S.cerevisiae* isolates collected since 1992 that were kept in the laboratory  
135 collection at  $-80^\circ\text{C}$  were added to the dataset collection, increasing the original dataset  
136 sampled by 102 new isolates (Table 1).

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

141 In addition to cellar samples, 33 yeasts strains from diverse origins whose genomes have been  
142 sequenced (Liti et al. 2009; Schacherer et al. 2009) (Supplementary data, table S1) and 35  
143 commercial wine strains (Supplementary data, table S2) widely used as yeast starters were  
144 added to the dataset.

145 **Molecular methods**

146 Considering that all yeasts collected at 75% of wine must fermentations would very likely  
147 belong to the *Saccharomyces cerevisiae* species, and that this technique will provide complete  
148 genotypes only for *S. cerevisiae* strains (Legras et al. 2005b), all colonies were directly  
149 analyzed by microsatellites. For each of them, a small amount of fresh colony was suspended  
150 in  $50\mu\text{l}$  of MilliQ water and  $7\mu\text{l}$  of this suspension was dropped on FTA card for DNA  
151 preservation. Those samples were then genotyped using 2 multiplex PCR reaction of 8 and 7

152 microsatellites loci, respectively for mix 1 and 2, (Supplementary data, table S3) (J.E.  
153 Bradbury et al. 2005; D Field and Wills 1998; González Techera et al. 2001b; Hennequin et  
154 al. 2001a; Legras et al. 2005b; Pérez et al. 2001b). Mixes were prepared for at least 8 samples  
155 for a volume of 100 $\mu$ l with in both 50 $\mu$ l of 2X QIAGEN Multiplex PCR Master Mix. Mix 1  
156 had 8 multiplexed primers and mix 2 the 7 others, each of them had specific concentration  
157 specified in the supplementary data, table S3. The PCRs were run in a final volume of 12 $\mu$ l  
158 containing 2 $\mu$ l of cell suspension. The following PCR program was used in routine: initial  
159 denaturation at 95 ° C for 15 minutes followed by 35 cycles of 95 ° C for 30 seconds, 57 ° C  
160 for 2 minutes, 72 ° C for 1 minute, and finally a final extension at 60 ° C for 30 minutes. PCR  
161 products were sized on a capillary electrophoresis ABI3730 (Applied Biosystems) using size  
162 standard 600LIZ® (GeneScan™).

163 **Data analyses**

164 ABI3730 genotyping results were read using GeneMarker (V2.4.0, Demo). The presence of a  
165 missing value was allowed to a maximum of 3 markers per sample. Estimation of population  
166 diversity by rarefaction of 10000 individuals repeated 10 times, Shannon index ( $H'$ ) and  
167 Simpson diversity index ( $D$ ) with their equitability indexes (reciprocally  $J'$  and  $1-D$ ), were  
168 calculated using EstimateS (V9) (Colwell 2004) using the individual-based abundance data.  
169  $H'$  was determined with the following equation:  $H' = -\sum_{i=1}^S P_i \ln(P_i)$ , and  $D$  following the  
170 equation:  $D = \sum \frac{N_i(N_i-1)}{N(N-1)}$ . With  $S$  the total number of genotypes in the population, the term  $P_i$   
171 calculated as follows:  $P_i = \frac{N_i}{N}$ ,  $N_i$  the number of individuals for a specific genotype and  $N$  the  
172 total of unique genotypes. GenClone (V2.0) software was used to remove from our dataset  
173 strains with exact similar profiles resulting from potential clonal expansion (Arnaud-Haond  
174 and Belkhir 2007). Observed and expected heterozygosity, Fst and AMOVA analyses were  
175 performed using Arlequin (V3.5.2.2) software (Excoffier and Lischer 2010).

176 SplitsTree v4.12.6 (Huson and Bryant 2006) was used to reconstruct a neighbor-net  
177 phylogenetic network for *S.cerevisiae* using Bruvo's distance (R Bruvo et al. 2004) calculated  
178 from the R program (R Development Core Team 2013b) with the following packages: ape  
179 (Paradis, Claude, and Strimmer 2004b) and poppr (Kamvar, Tabima, and Grünwald 2014b).  
180 Population structure was evaluated using a Bayesian clustering method with the software  
181 InStruct that does not account for the Hardy-Weinberg Equilibrium (Gao, Williamson, and  
182 Bustamante 2007). 5 chains of 150000 iterations with a burn-in of 5000 were run for K=1 to  
183 K=25. The most likely number of ancestry's populations was selected choosing the lowest  
184 DIC (Deviance information criterion). Ancestry profiles were drawn as barplots from the  
185 Instruct output, using a different color for each inferred ancestral population under R  
186 statistical environment. The contribution of each population was then evaluated with ObStruct  
187 software (V Gayevskiy et al. 2014).

188

## 189 **Results**

### 190 **Cellar samples diversity**

191 To investigate *Saccharomyces cerevisiae* population diversity in the typical appellation of  
192 Sauternes in the Bordeaux region, 3 wine estates were selected, A, B and C. Samples of  
193 spontaneously fermenting must were taken before mutage and at different times of the harvest  
194 corresponding to selective pickings. A total of 653 colonies were collected in the wine estates  
195 between 1992 and 2014 and analyzed by 15 microsatellites markers. Isolates with genotypes  
196 with missing values at more than 3 markers were removed from the dataset. The summary of  
197 the sampling, years and number of *S. cerevisiae* colonies collected with completed  
198 microsatellite genotype are provided in Table 1. For the wine estate C, *S. cerevisiae* strains  
199 from the laboratory collection that were isolated between 1992 and 2011 were included in the  
200 study (Masneuf and Dubourdieu 2000). After microsatellite analyzes, 43 additional *S.*

201 *cerevisiae* with full microsatellite genotypes were kept, giving a final data set of 604 *S.*  
202 *cerevisiae* isolates for further analysis (Table 1).

203 In order to compare the diversity of the yeast populations obtained from the three wine  
204 estates, we calculated three diversities index using EstimateS: the Shannon ( $H'$ ) index that  
205 measures the diversity within a population and takes into account both richness and evenness,  
206 the Simpson index ( $D$ ) with its opposite Simpson's index of diversity ( $1-D$ ), which gives  
207 more weight to common or dominant species, and the Pielou evenness index ( $J'$ ). The  
208 different indices were evaluated on the basis of the number of different genotypes (Table 2)  
209 and on the standard deviation of  $H'$  and  $D$ : all results were significantly different. The  
210 Shannon index ( $H'$ ) showed strong diversity in all 3 wine estates (over 4.50) with a slight  
211 decrease of diversity for wine estate B. The Pielou index ( $J'$ ) was close to 1, thereby  
212 suggesting that genotypes have similar abundance within the population. The Simpson  
213 complement index ( $1-D$ ) results are in accordance with the  $H'$  and  $J'$  indices, with high values  
214 over 0.9. When considering wine estates as a whole group of the Sauternes appellation, the  
215 Pielou index value was even higher, reaching 98% of diversity and again confirming the  
216 results from the Simpson and diversity indices. The diversity index of Sauternes *S. cerevisiae*  
217 population was similar to the diversity index obtained for Merlot red wine cellars *S.*  
218 *cerevisiae* population (270 individuals) with the  $H'$  and  $J'$  indices of 5.38 and 0.96  
219 respectively (data not shown). The whole appellation diversity, evaluated by the rarefaction  
220 analyses, estimated a number of unique genotype at the whole appellation scale greater than  
221 4895 (with 95% confidence limits of 4320-5470), and evaluated with a sampling design  
222 including more than 10000 *S. cerevisiae* isolates throughout the region to achieve full  
223 diversity.

224

225

226 **Strains genetic relationships**

227 As alcoholic fermentation results into a clonal expansion, it was necessary to remove all  
228 identical genotypes within each sampling site before accessing the genetic relationships. From  
229 the initial 604 *S.cerevisiae* isolates, GenClone software inferred 503 unique genotypes,  
230 grouping a total of 517 isolates from all 3 wine estates with 14 genotypes shared between the  
231 3 wine estates (Table 1). 37 industrial *S.cerevisiae* strains widely used in the Bordeaux region  
232 and in Sauternes appellations specifically were then added to this data set in order to detect  
233 the potential presence of yeast starters within cellar populations and 49 *S. cerevisiae* isolates  
234 from Bordeaux region Merlot must fermentation were also included in the analysis (Table 1).  
235 Finally, 33 *S. cerevisiae* strains from various origins whose genome have been recently  
236 sequenced were also included as an out-group in our dataset.

237 The relationship between cellars and commercial strains, the 636 isolates were accessed from  
238 a phylogenetic network built from the Bruvo's pairwise distance matrix (Figure 2). As  
239 expected, one cluster gathered the sequenced strains of different origins (group A), and most  
240 of the sequenced strains originated from a wine environment clustered with our wine isolates,  
241 except for clinical strains (YJM978, 981, 975) and baker strains (YS2, YS4, YS9), which  
242 were grouped in the same branches including Sauternes strains and commercial strains  
243 (respectively arrows 2 and 1). Note that the sequence strain YIIc17\_E5, the genome for  
244 which has been sequenced, was isolated from the Sauternes region in 1992 and clustered with  
245 strains isolated in wine estate C in 1992 (arrow 3). Concerning Sauternes cellars *S. cerevisiae*  
246 population, some branches clustered isolates from one wine estate with very close genetic  
247 relationships (group B), suggesting clonal variants. Those branches were mostly observed for  
248 wine estate A and C, and to a less extent for wine estate B. Others branches were composed of  
249 clusters mixing wine estates C and B isolates and with only few rare isolates from wine estate  
250 A. Finally, there also appeared possible links between cellar's strains and commercial strains

251 (arrow 4), whichever the wine estate considered. Concerning Merlot isolates, all from wine  
252 estates D and most from wine estate F clustered apart from the others (group D), whereas few  
253 isolates of wine estate E and F were spread over the network.

254 To further compare the population by wine estate,  $F_{st}$  statistics were calculated between all  
255 Sauternes and Merlot wine estates (Table 3). All population comparisons indicated a  
256 significant differentiation ( $p<0.001$ ). As suggested by the individual network, there was  
257 higher differentiation between wine estates A and B (0.109) than between wine estates B and  
258 C (0.038), this last being more than twice lower than comparing A and C (0.145, the highest  
259 Sauternes pairwise  $F_{st}$ ). Pairwise  $F_{st}$  between Sauternes wine estates and Merlot wine estates  
260 ranged from 0.103 to 0.165 indicating a moderate but still notable differentiation. However,  
261 pairwise  $F_{st}$  between Sauternes wine estates (A and C) could be higher than pairwise  $F_{st}$   
262 between Sauternes and Merlot wine estates (e.g., A and E or B and F), whereas the  
263 geographical distance was greater in these cases. Surprisingly, the pairwise  $F_{st}$  distances  
264 between Merlot wine estate D and any others Sauternes or Merlot wine estates was high  
265 (0.222-0.399) indicating a strong differentiation, indicated by the external position of  
266 individuals in the network, which might be explained also by the lower number of strains  
267 from that sample.

268 As highlighted in the network, some cellar's isolates appeared very close to commercial  
269 strains. Cellar isolates were further considered genetically related to the industrial strains  
270 when sharing at least 75% of alleles. For example, one isolate from wine estate B was related  
271 to commercial strain VL3 and 4 isolates to strain X5. For commercial strains UvaFerm BC,  
272 20 cellar isolates were related to this starter strain, one obtained from wine estate A, 18 from  
273 wine estate B and one from wine estate C. Finally, 4 cellar isolates from wine estate A were  
274 related to commercial strains Levuline BRG. Commercial strains VL3, X5, UvaFerm BC and  
275 Levuline BRG have been frequently used in the Sauternes region during the last 30 years,

even for wine estates following organic agricultural practices. Nevertheless, only 7% of all Sauternes strains were considered as genetically related to specific commercial strains, thus indicating a minor but substantial relation between cellar and commercial strains (Table 1). In order to limit the potential impact of yeast related to commercial starters on the detection of yeasts population structure, these were removed from the dataset 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 variation at these 15 microsatellites loci is 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 year of sampling or wine estate. The contributions of variation among individuals within groups (AIWP) always explained most of the global variation, greater value, ranging from 60 to 94% of the total variance. The percentage comparison of variation Among Group (AG) and Among Population Within Group (APWG) indicates different patterns. The comparison of genetic diversity from the different wine estates indicates that wine estate has the highest impact on genetic diversity as this factor explains from 11.7 to 31 % of genetic variability according to the comparison. Wine estate A appeared as 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 B C comparison, with 14.1 %) and low among individuals variation (AIWP). On the contrary, vintage contributed less to the global variation for close vintages in wine estate, with 0 to 6% of global variation, with a low inter-sample variation (5 to 7 % of variation in APWG) and the highest values for among

301 individuals variation (88.6 to 94.5% for AIWP). However, winery C presents an original  
302 picture, as the differences between the most distant vintage explains the highest part of  
303 genetic variation (13.1 to 19.3%), whereas the differences between the 1992 and 1993  
304 vintages were the lowest, in a similar manner to what can be observed for 2012-2013 and  
305 2013-2014 for wine estate B. Interestingly, this wine estate shows as well the highest sample-  
306 to-sample variations (APWG explains 12 to 16% of global variation).

307 We used InStruct to evaluate population structure from shared ancestry. Deviance Information  
308 Criterion indicated the most likely population number to be K=19 (Figure 3). In an overall  
309 view, whatever the year or wine estate considered, numerous strains were composed by  
310 mosaic ancestral subpopulations. Unique ancestral populations associated with a given wine  
311 estate were highlighted (D and F, A in 2014). Strains from the wine estate E presented mainly  
312 a mosaic ancestry which may explain their dispersion over the individual network. Wine  
313 estate A shared few ancestral populations with wine estates B and C, whereas one of the main  
314 ancestral populations of wine estate C was shared with wine estate B, which also explains  
315 well with the mix group seen on the network. The relation between wine estates C and B were  
316 illustrated by shared ancestral populations in 2014, and to a less extent, in 1992-1993. Except  
317 for wine estate B, where ancestral populations persisted through vintages (2012-2014), for  
318 wine estates A and C, only few ancestral populations were shared from one year to another.  
319 Moreover, in 2014, 2 new ancestral subpopulations appeared to be predominant and absent  
320 from the former vintages for both wine estates A and C. When focusing on population  
321 structure of the wine estates A and C in 2014, a temporal succession of two ancestral  
322 subpopulations was clearly related to the different batches associated to the selective picking  
323 of noble rotted grapes (figure 4).

324 The ObStruct program permits an evaluation of significance of different factors on the  
325 ancestry profile obtained from Instruct. Here we can test the effect of the wineries on the

326 population of wine estate from Sauternes or Merlot wine estates (Supplementary data, figure  
327 S1), (Supplementary data, table S4.a). The two Merlot wine estates D and F were those that  
328 had a strong influence on the global population structure. Sauternes wine estate B contributed  
329 also to this population structure shaping, but at a lower level. To focus on the Sauternes  
330 appellation, Merlot wine estates were removed from the data set. The ObStruct results on  
331 Sauterne wine estate solely (Supplementary data, figure S2, table S4.b) showed that wine  
332 estate A still had the strongest influence on the shape of Sauterne population structure in  
333 agreement as it is clearly distinguished, from the 2 other wine estates. Wine estates B and C,  
334 had a lesser influence on the population structure but with B contributing slightly more than  
335 C.

336

### 337 **Discussion**

338 Sauternes is a particular Appellation of the Bordeaux region, producing high quality sweet  
339 wines. The development of noble rot on grapes results in the production of highly  
340 concentrated grape musts and typical wines (Bely, Masneuf, and Dubourdieu 2005; Magyar  
341 2011) Fermentation conditions are highly stressful for wine yeast mainly due to high sugar-  
342 content and low level of assimilable nitrogen (Bely, Rinaldi, and Dubourdieu 2003). A total  
343 number of 653 isolates were collected over 3 consecutive years (2012, 2013 and 2014) in 3  
344 different wine estates. Moreover, 102 additional strains collected from 4 to 23 years ago in the  
345 wine estate C have been added to our population's sampling. A highly discriminating method  
346 based on 15 microsatellites markers specific to *S.cerevisiae* was used for molecular typing at  
347 strain level. In comparison to previous studies based on others methods such as PFGE,  
348 mtDNA RFLP, inter-*Delta* analysis (Schuller et al. 2005; Valero et al. 2007a; Ileana Vigentini  
349 et al. 2015), this is, to our knowledge, the first *S. cerevisiae* ecological study, combining a  
350 deep sampling and relying on the robustness of microsatellite markers method (Goddard et al.

351 2010; Hennequin et al. 2001a; Schuller et al. 2012; Francesca et al. 2012), performed with  
352 such a number of microsatellite markers (15 loci) and providing more sensitivity when  
353 compared to previous studies. Multilocus microsatellites analysis allowed us to evaluate the  
354 genetic diversity of our population. A total of 503 genotypes were revealed from an initial  
355 population of 653 *S.cerevisiae* isolates (77% of different genotypes), thus indicating high  
356 genetic diversity. By sampling 21 different white and red ferments across three different  
357 regions in New Zealand, Gayevskiy and Goddard (2012) obtained 353 *S. cerevisiae* isolates  
358 and 274 genotypes (78%) (with 10 microsatellites markers), which is in agreement with our  
359 data. However, our estimate of yeast diversity, suggests that the Sauternes region is expected  
360 to contain an extremely large diversity of *S. cerevisiae* strains, with an underling population  
361 of more than 4800 unique genotype strains, a figure far higher than the estimate of 1700  
362 inferred in the NZ vineyard (Knight and Goddard 2014). The diversity index obtained for  
363 Sauternes *S. cerevisiae* population was also similar to the diversity index obtained for Merlot  
364 red wine cellar's *S. cerevisiae* population. Because damaged grape berries state may be very  
365 rich depositaries of *S. cerevisiae*, in comparison to sound berries (Mortimer and Polsinelli  
366 1999), we might expect to obtain higher diversity index values for botrytized ferments  
367 population. However, on the contrary, the specific botrytized grape must composition with  
368 high sugars content, and the interaction with the action of *Botrytis cinerea* may constitute a  
369 highly selective medium, potentially limiting *S. cerevisiae* strains diversity.

370 The main objectives of the study were to define the population genetic structure and diversity  
371 of *S. cerevisiae* at both the Sauternes appellation and wine estate scale. The impact of  
372 commercial strains on the diversity of endogenous wine yeasts strains is still controversial  
373 since some authors showed that the use of Active Dry Yeasts reduced the variability of wine  
374 cellar strains (Beltran et al. 2002), whereas other studies did not evidence any impact (Valero  
375 et al. 2007a; Cordero-Bueso, Arroyo, Serrano, and Valero 2011; Velimir Gayevskiy and

376 Goddard 2012). In this study, only 7% of cellars strains were found related to 4 commercial  
377 strains usually used in sweet and dry white wine making of the Bordeaux region for over 25  
378 years. Moreover, no significant variation for wine estates pairwise  $F_{st}$  values were obtained  
379 before and after removing strains genetically related to commercial starters. Despite the past  
380 or present use of yeast starters to inoculate dry white wines in the wine estates studied, this  
381 practice had low impact on *S. cerevisiae* diversity and population genetic structure at the  
382 winery scale in the Sauternes region.

383 AMOVA and pairwise Fst, and ancestry profile and ObStruct analyses showed contrasting  
384 results concerning genetic differentiation between populations originated from different wine  
385 estates. While population differentiation between wine estate A and wine estates B and C  
386 were high, a much smaller differentiation was observed between wine estates B and C.  
387 Ancestry profile analysis provides evidence that wine estate B and C populations are mixed to  
388 a certain degree. Taking into account the short geographic distance between the wine estates  
389 A, B and C (distant from less than 10 km), it is not realistic to postulate that the various  
390 degree of genetic differentiation between wine estate populations is linked to their respective  
391 geographic distance. However, one of the possible explanations of the small differentiation  
392 between B and C wine estates in comparison to A is the small distance between B and C,  
393 which have juxtaposed vineyard plots. At such a close distance, insects like bees, wasps and  
394 fruit-flies, as well as birds which are known to be vectors for yeasts, could have homogenized  
395 these yeast populations (Francesca et al. 2012; Shihata and Mrak 1952; Stefanini et al. 2012).  
396 Humans can as well influence yeast population structure and promote dispersal (Goddard et  
397 al. 2010; Fay and Benavides 2005; Legras et al. 2007). The wine estates B and C shared  
398 seasonal staff and wine-growing equipment during the harvest and fermentation periods,  
399 which may also have facilitated exchanges between the *S. cerevisiae* populations of the two

400 estates. At the very small scale of Appellation, this is an illustration of possible *S. cerevisiae*  
401 dispersion.

402 During a period of 23 years, strains from the wine estate C were collected, and we could  
403 observe the systematic persistence of specific ancestral populations that were never dominant  
404 in the wine estate C. The ancestral populations observed in 1992/1993 in winery C were also  
405 detected in the sampling performed during 2012-2014 period in wine estate B but absent in  
406 wine estate A. This result demonstrates, at the small scale of two wine estates, the existence  
407 of a local and stable group of strains with shared ancestry over 20 years, as well as the  
408 occurrence of multiple yeast population exchanges between the two wine estates over time.  
409 The phenotypic traits of this local and long-term stable group of strains would be interesting  
410 to investigate, in order to better understand to what extent, those ancestral *S. cerevisiae*  
411 populations may contribute to the characteristic and typicality of the wine produced in this  
412 area.

413 Previous consecutive years follow up studies reported contrasting results concerning the  
414 possible establishment of strains as resident in a given winery (Beltran et al. 2002; Ileana  
415 Vigentini et al. 2015). The comparison of samples obtained from wine estate C over a long  
416 period by AMOVA analyses revealed that the variation between the most distant years  
417 (1993/2014), provided more differences than the comparison of different samples of the same  
418 year or from following years (1992/1993 or 2013/2014). From this preliminary analysis, we  
419 could hypothesize that time, over the long-term, may be a key factor for genetic  
420 differentiation between cellar-resident *S. cerevisiae* populations in a given winery.

421 Finally, cellar-associated *S. cerevisiae* population during the harvest period of 2014 for wine  
422 estate A and C was more closely explored. Ancestry profiles analysis revealed a clear  
423 temporal succession of two main ancestral populations for wine estate C, and to a less extent  
424 for wine estate A during the harvest campaign. The particularity of those both wine estates

425 compared to wine estate B was the use of fermented batches to inoculate the other ones. This  
426 method named “pied de cuve” was shown to better acclimatize the yeast inoculum to the  
427 high-sugar content of fermentation medium. Such stress factor provokes an up-regulation of  
428 structural genes involved in glycerol synthesis and intracellular accumulation by *S. cerevisiae*  
429 in response to external osmolarity (Blomberg and Adler 1989; Varela and Mager 1996),  
430 which results in the formation of acetic acid from acetaldehyde (Erasmus, van der Merwe, and  
431 van Vuuren 2003). The use of yeasts collected from already fermenting wine is advantageous  
432 since yeast cells already had the opportunity to acclimate to the high sugar contents of the  
433 musts and produce less acetic acid than selected starters directly inoculated (Bely, Masneuf,  
434 and Dubourdieu 2005). Our data indicated that the selection of specific ancestral *S. cerevisiae*  
435 populations through the successive fermentations may also be favored by the use of  
436 subculture in wine estate A and C. Still the factors that explain the selection of given ancestral  
437 populations remain to be elucidated. In the case of the Sauternes winemaking, the sugar  
438 contents of the musts, which is dramatically increased during the harvest with concentrations  
439 as high as 40-45 % (w/v) at the end of the campaign, is probably a key parameter. In the case  
440 of wine estate C, this selection of one ancestral population during the harvest period was  
441 highlighted but raises the underlying question: to what extent does the increase of the must’s  
442 sugar content explain this temporal succession?

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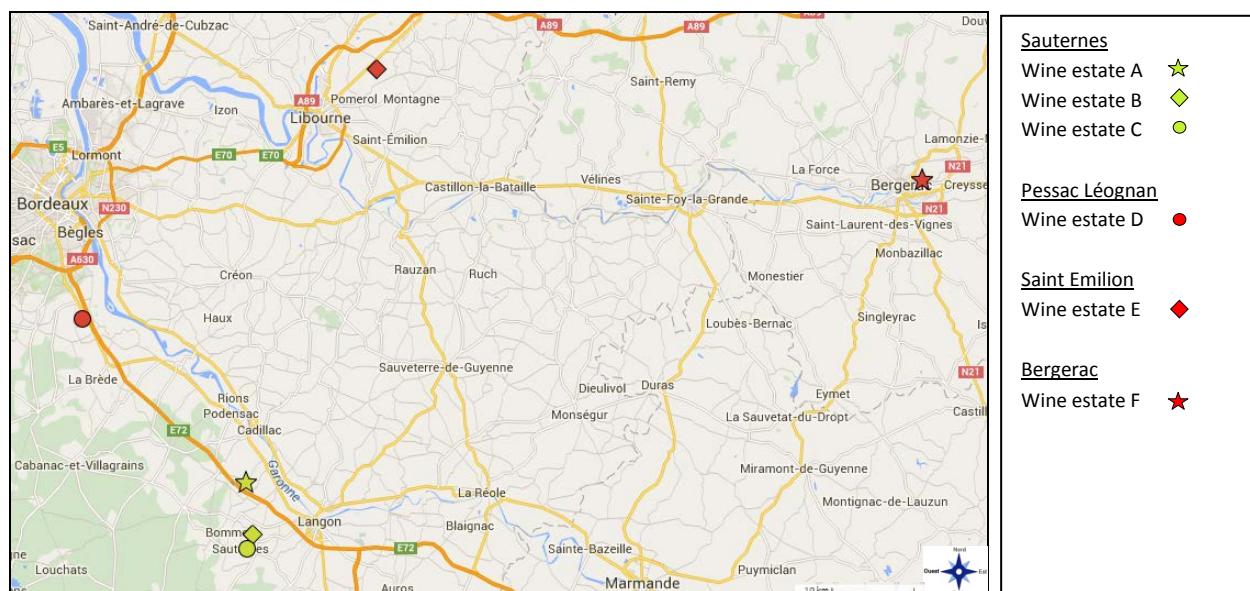
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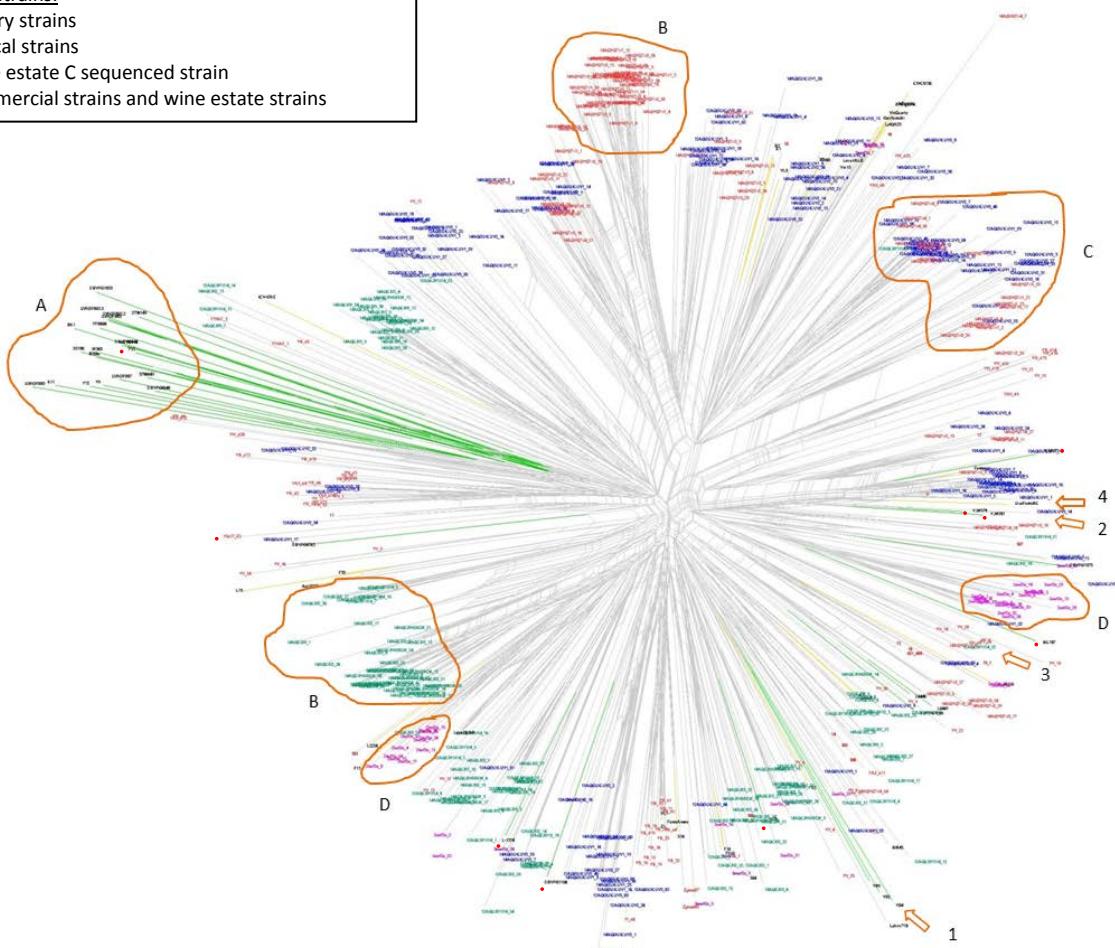
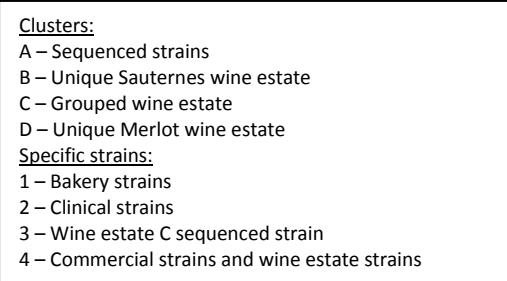
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## Figures



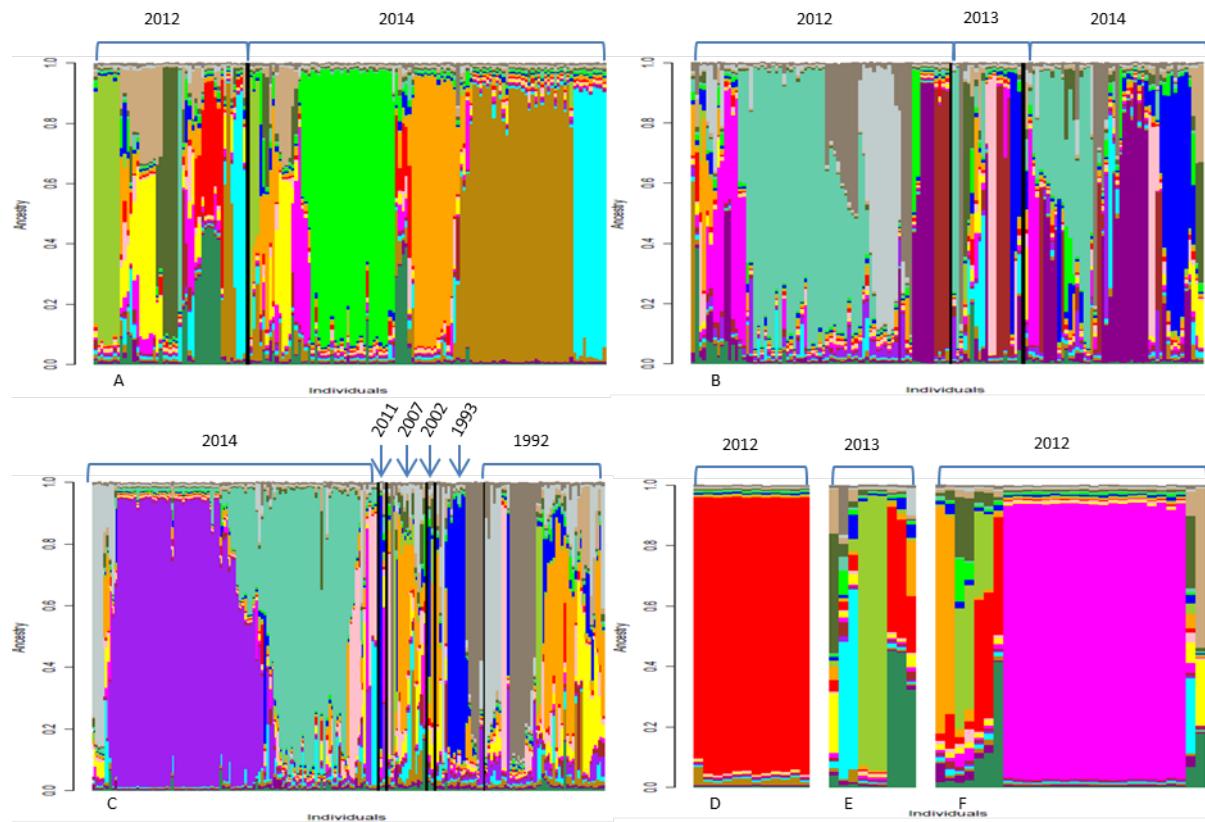
**Figure 1.**

Geographic localization of the wine estates in the appellations of Bordeaux and Bergerac regions. Yellow labels represent wines estates in white wine Sauternes appellation and red labels in red wine Pessac Léognan, Saint Emilion and Pecharmant appellations.



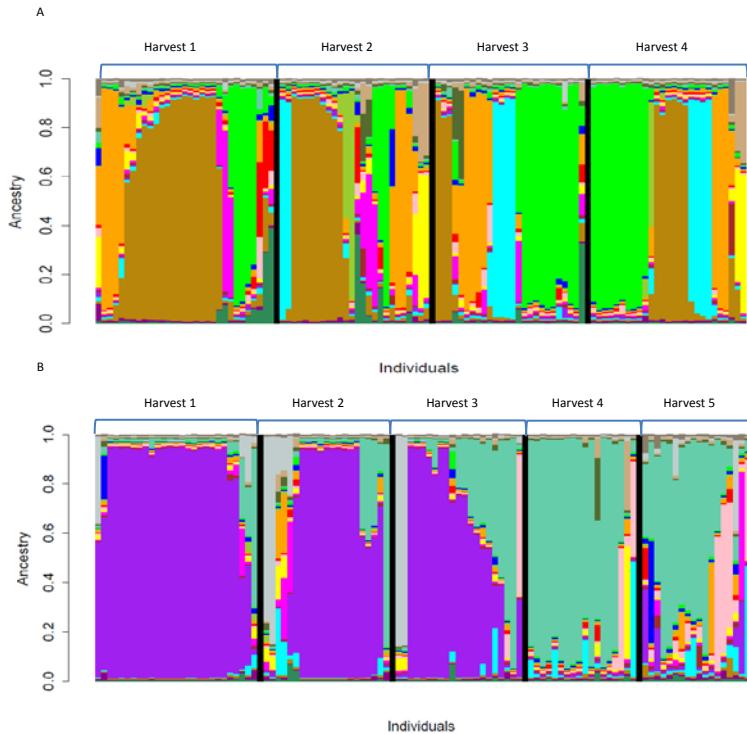
**Figure 2.**

Neighbor-net network of 636 strains from cellar of the 3 wine estates, 3 commercial strains, 33 strains from the *S.cerevisiae* sequenced data base and 49 strains from Merlot must fermentation. The network was constructed from Bruvo's distance between strains based on the polymorphism at 15 loci. Color code: wine (red dot) and non-wine sequenced strains in green, commercial strains in yellow; Domain A green labels, Domain B blue labels, Domain C red labels, wine estates D, E and F; pink labels.



**Figure 3.**

Inference of population using InStruct program on the 604 *S.cerevisiae* cellars strains with the optimal  $K = 19$  and classified depending of the years for each wine estate. Code: A. wine estate A; B. wine estate B; C. wine estate C; D. wine estate D; E. wine estate E; F. wine estate F.



**Figure 4.**

**a.** Inference of population using InStruct on the 110 *S.cerevisiae* isolates from wine estate A at K = 19. Strains isolated in 2014, classified according of the 4 different harvest batches. **b.** Inference of population using InStruct on the 105 *S.cerevisiae* isolates from wine estate C at K = 19. Strains originated in 2014 are classified depending of the 5 different harvest batches.

## TABLES

**Table 1.**

Summary of samples collected in Bordeaux and Bergerac regions with indication of wine estates, year and number of sampling, number of yeast isolates genotyped by microsatellites and number of *S.cerevisiae* profiles remaining after removing all isolates with missing values at more than 3 loci, all similar profiles, and all isolates with at least 75% of similarity with commercial strains

Wine estate	Years	Number of sampling	Number of isolates analyzed by microsatellites	Number of isolates with less than 4 missing markers*	Number of isolates after removing all similar clones **	Number of isolates after removing those with more than 75% of similarity to commercial ***
A	2012	2	55	54	52	47
	2014	4	120	114	110	110
	2012	3	120	118	72	71
B	2013	3	48	46	35	19
	2014	2	60	55	55	49
	1992	5	43	43	43	43
	1993	2	32	25	25	18
C	2002	NA	6	3	2	2
	2007	NA	2	2	2	2
	2011	NA	19	15	15	14
	2014	5	148	129	106	105
<b>Total</b>			<b>653</b>	<b>604</b>	<b>517</b>	<b>480</b>
D	2012	1				12
E	2013	1				9
F	2012	1				28
<b>Total</b>						<b>529</b>

**Table 2.**

Shannon index ( $H'$ ), equitability index ( $J'$ ) and Simpson index ( $1/D$ ) and his complement index ( $1-D$ ). Analyses of the 604 *S.cerevisiae* obtained after microsatellites analyzes depending of the 3 different wine estates of the Sauternes appellation, after combining the different years of sampling, combining all wine estates and years.

	A	B	C	Appellation
<b>Number of individuals</b>	168	219	217	604
<b><math>H'</math> (Shannon Index)</b>	5.04	4.57	5.11	5.96
<b><math>J'</math> (Equitability Index)</b>	0.81	0.73	0.81	0.98
<b><math>1/D</math> (Simpson index)</b>	0.007	0.03	0.008	0.005
<b><math>1-D</math> (Simpson complement)</b>	0.993	0.97	0.992	0.995

**Table 3.**

Pairwise  $F_{st}$  statistics values between the 6 different Sauternes and Merlot wine estates after combining the different years of sampling. All values are significant ( $P<0.001$ ). Color code: light grey, comparison between Sauternes wine estates; dark grey, comparison between Merlot wine estates.

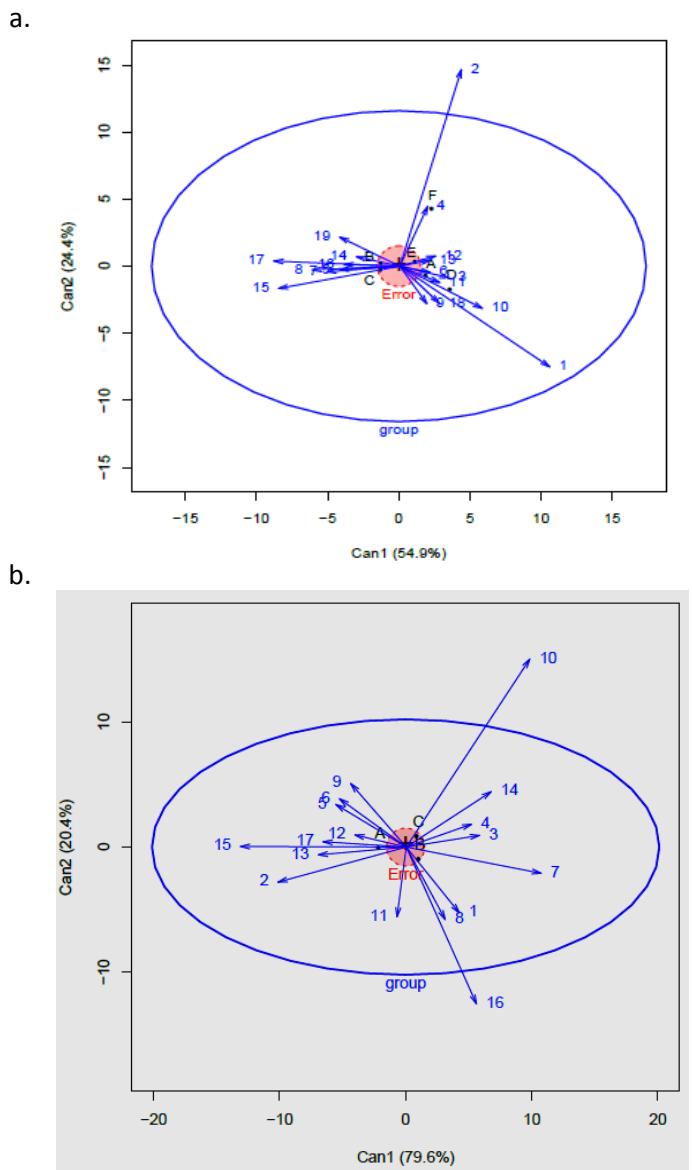
	A	B	C	D	E	F
Number of strains	162	162	193	12	9	28
A	<b>0.000</b>	0.001	0.001	0.001	0.001	0.001
B	0.109	<b>0.000</b>	0.001	0.001	0.001	0.001
C	0.145	0.038	<b>0.000</b>	0.001	0.001	0.001
D	0.222	0.299	0.325	<b>0.000</b>	0.001	0.001
E	0.103	0.105	0.139	0.327	<b>0.000</b>	0.001
F	0.147	0.120	0.165	0.399	0.138	<b>0.000</b>

**Table 4.**

AMOVA analyzes, Fst values and distribution of variance components (%) among group (AG), among population within group (APWG) and among individual within population (AIWP) based on microsatellites data of *S.cerevisiae* obtains from the indicated groups of wine estates and vintages.

Fixed parameter	Variable parameter	Percentage of variation (AG)	Percentage of variation (APWG)	Percentage of variation (AIWP)	Fst	P (r<0)
A	vintage 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
	vintage 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	-0.39	7.17	93.21	0.067	<0.000001
C	2014 - 1993 - 1992	13.14	15.02	71.83	0.281	<0.000001
	vintage 1992-1993	-3.05	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	wine estate A - B	11.69	4.19	84.11	0.158	<0.000001
2014	wine estate 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

## SUPPLEMENTAL DATA



**Figure S1.**

Canonical discriminant analysis on the *Saccharomyces cerevisiae* dataset a. from Sauternes and Merlot and b. only from Sauternes. The HE plot shows the relation of variation of the group means on two variables relative to the error variance. The arrows indicate the position of the inferred populations relative to the axes obtained by the canonical discriminant analysis and red circle reflects the pooled within-group dispersion. The black points indicate predefined populations of wine estate A, B, C, D, E and F while numbers at the arrows indicate inferred populations.

**Table S1.**

Set of 33 *S.cerevisiae* strains recently sequenced from wine and different other origins (Liti et al 2009). OS: accession number in the internal collection at the University of Nottingham. OSNNN/A: a single spore was isolated from the original diploid and OSNNN/A/A: repeated process. The absence of any /A: either the strain was haploid or monosporic culture was provided. \*Strains related to wine fermentation

OS	Strain	Geographic, Isolated by, Year and references	Source
288/A	273614X	Royal Victoria Infirmary, Newcastle UK, Galloway A	Clinical isolate (Fecal)
287/A	378604X	Royal Victoria Infirmary, Newcastle UK, Galloway A	Clinical isolate (Sputum)
181	BC187*	Napa Valley, Bisson L, USA <sup>Ref</sup>	Barrel Fermentation
150/A	DBVPG1106*	Australia, 1947, Fornachon J	Grapes
91/A	DBVPG1373	Netherlands, Capriotti A, 1952 <sup>Ref</sup>	Soil
84/A	DBVPG1788	Turku, Finland, Capriotti A, 1957 <sup>Ref</sup>	Soil
92/A	DBVPG1853	Ethiopia, Rossi, 1959 <sup>Ref</sup>	White Teff
155/A	DBVPG6040	Netherlands, 1947 <sup>Ref</sup>	Fermenting fruit juice
60/A	DBVPG6044	West Africa, Guillermond A, 1925 <sup>Ref</sup>	Bili wine, from <i>Osbeckia grandiflora</i>
3/A	DBVPG6765	Unknown <sup>Ref</sup>	Unknown
251/A/A	K11	Japan, 1981 <sup>Ref</sup>	Shochu sake strain
220/A	L-1374*	Cauquenes, Chile, Ganga A, 1999	Fermentation from must País
21/A	L-1528*	Cauquenes, Chile, Ganga A, 1999	Fermentation from must Cabernet
247/A	NCYC110	West Africa, Guillermond A, pre-1914 <sup>Ref</sup>	Ginger beer from <i>Z.officinale</i>
96	S288c	Merced, California, USA, Mrak E, 1938 <sup>Ref</sup>	Rotting fig
17/A	SK1	USA, Kane S, pre-1974 <sup>Ref</sup>	Soil
278/A	UWOPS03-461.4	Telok Senangin, Malaysia, Wiens F, 2003 <sup>Ref</sup>	Nectar, Bertram palm
280/A	UWOPS05-227.2	Telok Senangin, Malaysia, Lachance M, 2005	<i>Trigona spp</i> (Stringless bee) collected near Bertam palm flower
279/A	UWOPS05-217.3	Telok Senangin, Malaysia, Lachance M, 2005	Nectar, Bertram palm
270/A	UWOPS83-787.3	Great Inagua Island, Bahamas, 1983, Lachance M	Fruit, <i>Opuntia stricta</i>
271/A	UWOPS87-2421	Puhelu Road, Maui, Hawaii, Lachance M, 1987	Cladode, <i>Opuntia megacantha</i>
281	W303	Created by Rothstein R by multiple crossing <sup>Ref</sup>	NA
253/A/A	Y12	Ivory Cost, pre-1981 <sup>Ref</sup>	Palm wine strain
97/A	Y55*	France, Winge Ö, between 1930-60 <sup>Ref</sup>	Grape
252/A	Y9	Indonesia, pre-1962 <sup>Ref</sup>	Ragi (similar to sake wine)
174	YIIC17_E5*	Sauternes, France	Wine
308/A	YJM975*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
303/A	YJM978*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
304/A	YJM981*	Ospedali Riuniti di Bergamo, Italy, 1994-6 <sup>Ref</sup>	Isolated from vagina of patient suffering from vaginitis
182	YPS606	Pennsylvania, USA, Sniegowski P, 1999 <sup>Ref</sup>	Bark of <i>Q.rubra</i>
258/A/A	YS2	Australia <sup>Ref</sup>	Baker strain
259/A/A	YS4	Netherlands, 1975, Barnett J <sup>Ref</sup>	Baker strain
262/A/A	YS9	Singapore <sup>Ref</sup>	Baker strain

**Table S2.**

Set of 35 *S.cerevisiae* strains commonly used in Aquitaine region, as industrial yeast starters in organic and conventional farming.

Commercial name	Strain	Distributor
Actiflore cerevisiae	522D	Laffort
Excellence B2		Lamothe Abiet
Lalvin BM 45		Lallemand
Levuline CHP	CIVC8130	Oenofrance
Lalvin CY 3079	BourgoBlanc	Lallemand
Excellence C1 Val oeno		Lamothe Abiet
Zymaflore F10	FZ 182	Laffort
Zymaflore F15		Laffort
Actiflore F33	F33	Laffort
Fermol Arome plus	PB2010	Spindal
Fermivin	7013	Littorale
Zymaflore FX10		Laffort
ICV-GRE	138 grenache	ICV
K1	Killer non marquée	Lallemand
L.A. L13	L13	Lamothe Abiet
Rhône L2226		Lallemand
Lallferm bio		IOC / Lallemand
Lalvin QA23	QA23	Lallemand
Lalvin 71B	71B	Lallemand
Levuline ALS	EG8	Oenofrance
Levuline BRG	UP 30Y5	Oenofrance
Oenoferm Bio		Littorale
Vitilevure KD	R2	Martin Vialatte
Zymaflore RX60	rx60	Laffort
SP organic		Martin Vialatte
Uvaferm CEG	CEG - Epernay 2	Lallemand
Achor vin 13	vin 13	Littorale
Vitilevure quartz		Martin Vialatte
Zymaflore VL1	vl1	Laffort
Zymaflore VL3	lv3	Laffort
Zymaflore X16	x16	Laffort
Zymaflore X5	x5	Laffort
Zymaflore ST		Laffort
Zymaflore 011 organiq		Laffort
UvaFerm BC		Lallemand

**Table S3.**

Characteristics of the microsatellite loci used for *Saccharomyces cerevisiae* genotyping: repeated motif, closest ORF on *S. cerevisiae* genome, primer sequence, fluorescence dye, mix number and concentration used for 8 samples PCR mix preparation for each marker.

Site name	Motif and type	ORF or coordinates	Primers	fluorescent dye	Multiplex	Quantity (nM)	Authors
ScAAT2	TAA	YBL084c	FW: CAGCTTATTGCCTGAACGA RV: GTCTCCATCCTCAAACAGCC	PET	1	100	4
ScAAT3	TAA	YDR160w	FW: TGGGAGGAGGGAAATGGACAG RV: TTCAGTTACCGCACAATCTA	NED	1	200	1, 3
C5	GT	VI-210250/210414	FW: TGACACAATAGCAATGGCCTCA RV: GCAAGCGACTAGAACAAACATACA	VIC	1	50	5
C3	CAA	YGL139w	FW: CTTTTATTACGAGCGGGCAT RV: AAATCTCATGCCTGTGAGGGTAT	NED	1	100	5
C8	TAA	YGL014w	FW: CAGGTCGTTCTAACGTTGGTAAATG RV: GCTGTTGCTGTTGGTAGCATTACTGT	6FAM	1	25	5
C11	GT	X-518870/519072	FW: TTCCATCATAACCGTCTGGGATT RV: TGCCCTTTCTTAGATGGGCTTC	6FAM	1	50	5
YKR072c	GAC	YKR072c	FW: AGATACAGAAAGATAAGAACGAAAA RV: TTATTGATGCTTATCTATTATACC	PET	1	50	1, 2
SCYOR267c	TGT	YOR267c	FW: TACTAACGTCAACACTGCTGCCAA RV: GGATCTACTTCAGTATAACGGG	VIC	1	100	1, 4
YKL172w	GAA	YKL172w	FW: CAGGACGCTACCGAAGCTAAAG RV: ACTTTGGCCAATTCTCAAGAT	6FAM	2	25	2
ScAAT1	TTA	XIII-86902/87140	FW: AAAGCGTAAGCAATGGTGTAGACTT RV: CAAGCCTCTCAAGCATGACCTTT	VIC	2	100	1, 3, 4
C4	TAA+TAG	XV-110701/110935	FW: AGGAGAAAATGCTGTTATTCTGACC RV: TTTCTCTGGGGACGTGAAATA	NED	2	200	5
C9	TAA	YOR156c	FW: AAGGGTTCGTAAACATATACTGGCA RV: TATAAGGGAAAAGAGCACGATGGC	NED	2	100	5
ScAAT5	TAA	XVI-897051/8970210	FW: AGCATAATTGGAGGCAGTAAAGCA RV: TCTCCGTCTTTTGACTGCGTG	NED	2	100	5
C6	CA	XVI-485898/485996	FW: GTGGCATCATCTGTCAATTATCAC RV: CAATCAAGCAAAGATCGGCCT	VIC	2	50	5
YPL009c	CTT	YPL009c	FW: AACCCATTGACCTCGTTACTATCGT RV: TTGATGGCTCTGATAACTCCATT	6FAM	2	50	1, 4

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**Table S4.**

Pairwise matrix of R2 values between **a.** the 3 Sauternes and 3 Merlot wine estates and **b.** the only 3 Sauternes wines estates (with P.value).

**a**

	C	A	B	D	E	F
C	<b>NA</b>	0.02 (0.06)	0.11 (<0.001)	0.06 (<0.001)	0.09 (<0.001)	0.09 (<0.001)
A	0.02 (0.06)	<b>NA</b>	0.02 (<0.001)	0.58 (<0.001)	0.03 (<0.001)	0.18 (<0.001)
B	0.11 (<0.001)	0.02 (<0.001)	<b>NA</b>	0.09 (<0.001)	0.04 (<0.001)	0.10 (<0.001)
D	0.06 (<0.001)	0.58 (<0.001)	0.09 (<0.001)	<b>NA</b>	0.10 (<0.001)	0.50 (<0.001)
E	0.09 (<0.001)	0.03 (<0.001)	0.04 (<0.001)	0.10 (<0.001)	<b>NA</b>	0.09 (<0.001)
F	0.09 (<0.001)	0.18 (<0.001)	0.10 (<0.001)	0.50 (<0.001)	0.09 (<0.001)	<b>NA</b>

**b**

	A	C	B
A	<b>NA</b>	0.11 (<0.001)	0.10 (<0.001)
C	0.11 (<0.001)	<b>NA</b>	0.04 (<0.001)
B	0.10 (<0.001)	0.04 (<0.001)	<b>NA</b>

## **Chapitre 3**

# **The “Pied de Cuve” as an alternative way to manage alcoholic fermentation: influence on fermentative process and the *Saccharomyces cerevisiae* diversity**

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## **Abstract**

Winemakers, especially in accordance with organic farming guidelines, are more and more keen in avoiding the use of commercial yeasts in order to improve wine complexity and to reduce the enological inputs. The preparation of winery-made fermentation starter from grapes called ‘Pied de Cuve’ (PdC) is getting popular. However, the implementation of the PdC method is still empirical and there is a lack of knowledge concerning the impact of PdC on the *S. cerevisiae* diversity during the alcoholic fermentation. In this study, the impact of different PdC modalities on the microbial, chemical and sensory composition of the wine was evaluated at the industrial scale. Despite very low population level of *S. cerevisiae* before inoculation, the use of PdC was as efficient as ADY in terms of chemical and sensory analysis of the resulting wines, except for one modality. The *S.cerevisiae* diversity from PdC fermentations were not as clonal as from Active Dry Yeast modality, but the strains isolated from PdC were different from one modality to another and from the spontaneous fermentation ones, guessing for specific strains selection. Our results provided evidence that the use of PdC could contribute to secure the fermentation process of white wines by selecting native indigenous yeast.

**Keywords:** Pied de Cuve, diversity, *Saccharomyces cerevisiae*, microsatellites, wine

## **Introduction**

Winemaking implies the presence of various and successive population of microorganisms, like fermentative yeasts to proceed the alcoholic fermentation. The use of Active Dry Yeast (ADY) as fermentation starters is wildly recommended to secure the process and avoid fermentation difficulties. In an opposite direction, there is an increasing demand especially from organic wine producers to enhance this idea of “terroir” microorganisms, by using indigenous *S. cerevisiae* strains from their own vineyards but also to limit the oenological inputs during the process. However, in some case, spontaneous fermentation could lead to irregular wine quality due to lack of alcoholic fermentation control, thus resulting to potential aromatic deviations or alterations of the final products. Thus there is a need for new developments to improve technological process exploiting the indigenous microorganisms.

Besides the classical method of direct use of ADY, some wineries traditionally elaborate wines with “Pied de Cuve” (PdC). This method is used to trigger alcoholic fermentation and control its progression, especially for the first vats filled at the beginning of the harvest period. The PdC is based on the use of a small volume of fermented musts prepared few days before harvest, to inoculate the entered grape juice batches. In details, the technique consist in collecting 10-20 kg of grapes 8 to 10 days before harvest and to obtain after grape crushing, a pre-culture of fresh juice. To avoid possible aromatic deviations, several grapes harvests are performed, in order to select the lot with the best fermentation without olfactory off-flavors such as acetic acid or ethyl acetate. The implementation of PdC can then continue with the use of must from fermenting tanks to inoculate the following. This process permits the entrance of already must-adapted yeast population to the fresh must that is usually a step of selection for fermentative yeasts. In a recent study, fortified PdC (with the addition of ethanol) was successfully used to accelerate spontaneous alcoholic fermentation and to influence de sensory profile of red wines (Moschetti et al. 2015). In the case of high sugar concentration

fermentation, different inoculum protocols using yeast collected from a fermented must or pre-culture yeast were shown to limit the amount of volatile acidity of the resulting wines (Bely, Masneuf, and Dubourdieu 2005). However, the implementation of the PdC method is still empirical in wineries and there is a lack of knowledge concerning the impact of PdC on the *S. cerevisiae* diversity during the alcoholic fermentation. The main objective of this study was to evaluate the impact of PdC on the microbial, chemical and sensory composition of the wine.

## **Materials and Methods**

### PdC protocol preparation

In 2012, 4 modalities of PdC were managed from Ugni Blanc and Sauvignon Blanc grape varieties in the wine estate Du. Undamaged grapes (15kg) were manually harvested for each variety 4 days before harvest time. The grapes were crushed in clean containers giving four lots of around 12 liters of must. For the 2 grape varieties, 1 lot was treated with sulfur dioxide ( $\text{SO}_2$ ) at 3g/hL whereas the other received no treatment. The lots were kept in the white-wine cellar at temperature between 18 and 20°C. After 2 days of alcoholic fermentation, ammonium phosphate was added to reach the level of 200mg/L and 60mg/hL of thiamine was added. The PdC were then kept until reaching around 30-50% of the alcoholic fermentation. The density and temperature of each PdC were daily monitored. Finally, the different PdC modalities were subjected to sensory and chemical analysis to evaluate possible off-flavors deviations and acetic acid content. If one of the PdC had failed to one of these analyses, it would not have been retained for this project.

### Barrels inoculation

The PdC were transferred into four 225 liters barrels which were filled with Sauvignon Gris “cold settled” must, with a PdC inoculum ratio of 1/20 of Sauvignon Gris must. Two

additional barrels were prepared as controls, one was inoculated with the commercial strains ZymafloreX5 at 20g/hL following the industrial recommendations and one was spontaneously fermented. The density and temperature of each barrel were monitored every day. At the end of the alcoholic fermentation, samples were taken for chemical analyses and each barrel was judged for tasting criteria.

#### Analytical and population counting analyzes

Total acidity (TA), reducing sugars (g/L), turbidity (NTU - Nephelometric Turbidity Unit) and assimilable nitrogen were measured for each PdC before fermentation and for Sauvignon Gris must. Sugar (g/L), acetic acid (g/L) and ethanol concentration (% vol.) were measured by infrared reflectance (Infra Analyser 450; Technicon, Plaisir, France) at 30-50% of fermentation of the PdC modalities and at the end of alcoholic fermentation in barrels. The volatile acidity (expressed in g/L acetic acid) was determined chemically by colorimetry (A460 nm) in continuous flux (Sanimat, Montauban, France). The analyses were performed in accordance to the official methods described by the European Commission (1990). The number of indigenous yeast population (CFU/mL - Colony-Forming Unit), was calculated by YPD-plate counting after SO<sub>2</sub> addition in the PdC before fermentation and at 30-50% of fermentation.

#### Microbial sampling

Samples were taken from the PdC must at around 30-50% of the alcoholic fermentation, just before the barrel's inoculation, directly plated onto Total Yeast (TY) and Non-*Saccharomyces* (NS) medium at different serial dilutions (10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup>) and kept 2 days at 26°C. TY and NS medium were based on YPD (yeast extract, 1% w/v, peptone, 1% w/v, glucose, 2% w/v, agar 2% w/v) with 100µg.ml<sup>-1</sup> of chloramphenicol and 150µg.ml<sup>-1</sup> of biphenyl to delay bacterial growth for the (TY) medium and cycloheximide at 500µg/L in addition for (NS)

medium. Microbial samplings were done on the Sauvignon Gris must before alcoholic fermentation and on the 6 fermented barrels when they reached the 75% of the fermentation. For PdC just before inoculation, 30 randomly chosen colonies were collected. For barrels at 75%, 10 colonies were collected. After two sub-cloning on YPD plates, each colony was stored in (30%, v/v) glycerol at -80°C. In addition to those fermentation samples and as a referenced out-group, 33 commercial wine strains (Supplementary data, table S1) widely used as yeast starters were added to the data set. Another group of 29 cellar *S.cerevisiae* strains from the same wine estate isolated from spontaneously fermenting Semillon grape must (at 75% of the fermentation) was added to the data set.

#### Molecular methods

All clones were put on Sigma-Aldrich Whatman® FTA® card, a small amount of fresh colony was suspended in 50µl of MilliQ water and 7µl of this suspension was dropped on the card for DNA preservation.

The clones were first analyzed with PCR amplification of the ITS region with primers ITS1 (<sup>5'</sup>-TCCGTAGGTGAAACCTGCGG-<sup>3'</sup>) and ITS4 (<sup>5'</sup>-TCCTCCGCTTATTGATATGC-<sup>3'</sup>) (White et al. 1990) to differentiate *S.cerevisiae* strains from others non-*Saccharomyces*. The PCR reaction was made for 25µL of final mix with 5µL of Taq-&GO™ Ready-to-use PCR Mix from MP, 0.5µL of each primer, 19µL of MilliQ water and the DNA disc. The following PCR program was used in routine: initial denaturation at 95 ° C for 5 minutes followed by 35 cycles of 94° C for 1 minute, 55.5° C for 2 minutes, 72° C for 2 minutes and finally a final extension at 72° C for 10 minutes. PCR products were sized on the Shimadzu Microchip Electrophoresis System for DNA/RNA Analysis MCE®-202 MultiNA using the Shimadzu DNA-1000 reagent kit.

Then the selected *S.cerevisiae* isolates were analyzed by microsatellites. Isolates were genotyped using 2 multiplex PCR reaction of 8 and 7 microsatellites loci, respectively for mix 1 and 2, (Supplementary data, table S2) (Pérez et al. 2001b; Legras et al. 2005a; Hennequin et al. 2001a; González Techera et al. 2001b; D Field and Wills 1998; J.E. Bradbury et al. 2005). Mixes were prepared for at least 8 samples for a volume of 100 $\mu$ l with in both 50 $\mu$ l of 2X QIAGEN Multiplex PCR Master Mix. Mix 1 had 8 multiplexed primers and mix 2 the 7 others, each them had specific concentration specified in Table S2. The PCRs were run in a final volume of 12 $\mu$ l containing 2 $\mu$ l of cell suspension. The following PCR program was used in routine: initial denaturation at 95 ° C for 15 minutes followed by 35 cycles of 95 ° C for 30 seconds, 57 ° C for 2 minutes, 72 ° C for 1 minute and finally a final extension at 60 ° C for 30 minutes. PCR products were sized on a capillary electrophoresis ABI3730 (Applied Biosystems) using size standard 600LIZ® (GeneScanTM).

#### Data analyses

ABI3730 genotyping results were read using GeneMarker (V2.4.0, Demo). The presence of missing value was allowed to a maximum of 3 markers per samples. GenClone (V2.0) software was used to remove from our dataset strains with exact similar profiles resulting from potential clonal expansion. Dendrogram was constructed using Bruvo's distance (R Bruvo et al. 2004) and neighbor-joining clustering by means of the R program (R Development Core Team 2013b) with the following packages: ape (Paradis, Claude, and Strimmer 2004b) and poppr (Kamvar, Tabima, and Grünwald 2014b). In order to assess the robustness of trees nodes, bootstrap resampling was performed by means of R and the pvclust package (Suzuki and Shimodaira 2014b) and trees were drawn with MEGA6. All bootstraps lower than 25 were not showed in the trees.

## Results

### PdC analyses

Sauvignon Blanc (SB) and Ugni Blanc (UB), late and early ripening grape varieties, respectively, were collected four days before the harvest time to produce two PdC, with the aim to obtain PdC from two grape samples with typically different maturities. As expected, chemical analyses of the juice just after grape crushing showed different maturity level in terms of sugar content (182 g/L and 135 g/L for SB and UB, respectively) and total acidity (5.9 g/L and 8.6 g/L for SB and UB, respectively) (Table 1). Assimilable nitrogen content of UB was below the deficiency level of 140 mg/L (Bely, Sablayrolles, and Barre 1990). Turbidity level was high (1958 NTU and 1348 NTU for SB and UB, respectively) comparing with the recommended values between 50 and 200 NTU for white winemaking (Ollivier 1987).

Chemical analyses were then performed just before the inoculation of the barrels to confirm the state of fermentation and to evaluate if the PdC were good potential starters (Table 2). As the fermentation has proceeded, lower sugar concentrations were found compared to the fresh musts. Just before inoculation, the sugar consumption of the Sauvignon Blanc PdC was between 34-38% whereas it was between 42-50 % for the Ugni Blanc PdC. The acetic acid content was similar whatever the modality considered, ranging from 0.35 to 0.40. The possibility to use the PdC to inoculate the barrels was further confirmed by sensory analysis, since no defect was detected.

### Yeast population and *S. cerevisiae* diversity analysis

For the initial PdC fresh must, the colonies counts on both medium were higher in Sauvignon Blanc juice than in Ugni Blanc,  $1.10^5$  and  $1.10^4$  UFC/mL respectively for total yeast, and  $8.10^4$  and  $7.10^3$  UFC/mL for non-*Saccharomyces* yeast (Table 3). The higher yeast population

level in the SB juice comparing with UB is probably due to the differences in the composition of the two musts, such as pH (Fleet 1993), that can result as well from the differences in ripening (Martins et al. 2014). Just before the inoculation of the barrels, the different PdC modalities had similar population levels, reaching from  $1.10^7$  to  $6.10^7$  UFC/mL for TY and  $2.10^7$  to  $3.10^7$  for NS (Table 3), thus suggesting that the non-*Saccharomyces* yeast population seemed to be the dominant one.

From the TY medium, 30 yeasts isolates obtained from the 4 fermented PdC before inoculation and 10 isolates at 75% of alcoholic fermentations of the 6 barrels were analyzed by ITS-PCR-sequencing (Table 4). From the different PdC, only five isolates out of 120 were identified as *S.cerevisiae* whereas the resulting 115 isolates were identified as *Hanseniaspora uvarum*. *S.cerevisiae* was isolated from the SB PdC, two from the experiment without SO<sub>2</sub> and three from the experiment with SO<sub>2</sub> (Table 4). Isolates collected from the six barrels were all identified as *S.cerevisiae*, thus resulting in a total number of 65 *Saccharomyces* isolates that were analyzed through microsatellites. Only strains with less than 4 missing values were retained in the data set, which resulted into 59 *S.cerevisiae* isolates for further analysis (Table 4).

The genetic diversity of the 59 *S.cerevisiae* isolates was analyzed through a neighbor-joining tree, included 33 commercial starters and the 29 isolates collected from Semillon spontaneous fermentation (Figure 1). It was possible to observe that the four *S.cerevisiae* isolates originated from the PdC were spread over the tree and only one (2duSBp-2) was close to an isolate collected from the barrel that was inoculated with it. Thus, the strains isolated from the different PdC seemed not implanted in the barrels. A cluster of almost identical isolates resulting from clonal expansion was observed (groups A), especially for the Ugni Blanc without SO<sub>2</sub> PdC, grouping 9 out of the 10 strains analyzed. To a less extend, similar clusters of almost identical isolates resulting from clonal expansion can be noticed for strains isolated

from PDC Sauvignon Blanc with and without SO<sub>2</sub>, grouping 4 out of 8 and 3 out of 10 strains analyzed, and for the Ugni Blanc PDC with SO<sub>2</sub>, a cluster grouping 4 out of 10 strains analyzed. As expected, 8 isolates collected in the inoculated barrel with the industrial starter X5 clustered with the X5 strain. Surprisingly, one *S.cerevisiae* isolates originated from the PdC (2duSBp-16) grouped in the same cluster. No clonal cluster was observed for the 8 isolates of the spontaneous fermentation barrel which however clustered with strains isolated from the Semillon spontaneous fermentation isolates.

#### Wine analyses and fermentation time

At the beginning of the harvest period, a Sauvignon Gris grape lot was used for the PdC experiment. Chemical analysis of the must is given (Table 5). Assimilable nitrogen level was adjusted at 200 mg/L. Colonies counts on both medium were 4.10<sup>6</sup> and 9.10<sup>5</sup> UFC/mL respectively for total yeast and non-*Saccharomyces* yeast, respectively. Chemical analysis and fermentation time of the samples collected at the end of the alcoholic fermentation of the six modalities (four barrels fermented with the PdC plus two controls, one with spontaneous fermentation and one directly inoculated with X5 commercial strains fermentation) are given in Table 6. The fermentation time was similar for the inoculated modality (11 days) and for the Sauvignon Blanc PdC with and without SO<sub>2</sub> and the Ugni Blanc PdC with SO<sub>2</sub> (12 and 13 days). However, fermentation time of the PdC ‘Ugni Blanc without SO<sub>2</sub>’ modality was longer as for the spontaneous fermentation modality with 18 and 17 days, respectively. Volatile acidity level (0.44 to 0.57 g/L acetic acid) was similar for all the modalities, with the exception of the PdC ‘Ugni Blanc without SO<sub>2</sub>’ modality for which the level was higher (0.61 g/L acetic acid). Finally, sensory analysis was performed with a panel of 15 professional tasters using black ISO glasses (NF V09-110, 1971), based on a descriptive analysis of the wines. No significant differences were showed between all the modalities (results not shown).

## **Discussion**

There is an increased demand, especially for organic wine producers, to limit the inputs and to better use the microbiological diversity present in their vineyards. Two methods are possible to achieve this goal: i) the selection of high fermentative *S.cerevisiae* yeasts from the endogenous population followed by a small scale industrial production, according to organic production rules in the case of organic producers (Settanni et al. 2012; Liu et al. 2015), and ii) to constitute a home-made yeast inoculum from selected grape juice few days before harvest or from the first spontaneous fermented vat. This latter methods was reported to have some positives impacts on the fermentation and final product (Bely, Masneuf, and Dubourdieu 2005; Santamaría et al. 2005).

In 2012, 4 different PdC modalities were prepared to test the impact of the PdC (grape varieties and the addition of SO<sub>2</sub>) in comparison with a more traditional way to manage alcoholic fermentation, by using ADY or to ferment spontaneously.

With the exception of one modality, the PdC inoculated barrels had fermentation time similar to those observed for the control fermentation inoculated with X5, showing that the use of the PdC method can be in those cases consistent comparing to a classic use of commercial strains in terms of alcoholic fermentation control. Only the barrels inoculated with the PdC ‘Ugni Blanc without SO<sub>2</sub>’ and the spontaneous fermentation modality had longer fermentation duration and higher volatile acidity. The fermentation kinetic of this PdC modality was similar to the Ugni Blanc with SO<sub>2</sub> PdC one, except for the end of the process resulting in a sluggish fermentation (Figure S1). In that case, the selected population of *S. cerevisiae* may not be considered as strong fermenter. Except for the latter modality, the use of the fresh PdC must was as efficient as ADY in terms chemical and sensory analysis of the resulting wines. It

was not possible to conclude concerning the impact of the different grape varieties and the SO<sub>2</sub> addition on the efficiency of the PdC since no repetitions was available.

The microbial and *S.cerevisiae* diversity analysis of the PdC revealed surprising results. First of all, the alcoholic fermentation of the PdC occurred through mainly non-*Saccharomyces* yeast, especially *H.uvarum* and *S.cerevisiae* was rare, even more, by using culture method, it was not possible to isolate *S.cerevisiae* strains in the 2 Ugni Blanc PdC, with or without SO<sub>2</sub>, probably due to the lack of ripening for the grapes when they were collected. In our experimental conditions, due to the low *S.cerevisiae* population level in the PdC, a higher number of colonies analyses would have been required to provide a better estimation of the *S.cerevisiae* diversity. Regarding the barrels *S.cerevisiae* diversity, the implantation of the ADY was confirmed and revealed a group of sub-clonal isolates around the commercial yeast X5. This result is in accordance with clonal variation highlighted for industrial batch of this strain (Chapter 1). In opposition, the other control barrel from spontaneous fermentation showed high *S.cerevisiae* diversity, indicating that during spontaneous fermentations, different strains were selected giving polyclonal population compared to ADY inoculation. For the PdC inoculated barrels, clonal expansion of *S.cerevisiae* was highlighted during fermentation in barrels, especially for the ‘Ugni Blanc without SO<sub>2</sub>’ modality, and to a less extends for the other modalities giving less than 50% of the strains that were clustered together. Then by using PdC, the winemakers seemed to select endogenous *S.cerevisiae* strains, different from one modality to another and to the *S. cerevisiae* population that occurred during spontaneous fermentation in the cellar. A dominant population, in line with what could be obtained by using ADY, may process the alcoholic fermentation. However, the results could be uncertain since one modality failed in terms of fermentation kinetic and high acetic acid level in the resulting wine. Then, the preparation of different PdC modalities could be of importance to secure the process.

A question still remained to be answered. It was showed here that the different PdC, besides the ‘Ugni Blanc without SO<sub>2</sub>’ modality, showed very good monitored fermentations with proper final product characteristics similar to the direct ADY inoculation ones. Despite the very low frequency of *S.cerevisiae* yeast identified over the 30 *S.cerevisiae* analyzed for each PdC, the fermentations proceeded till the final product. It has been shown that *S.cerevisiae* was capable to survive in must conditions, permitting it to grow from 0.1% to 99.9% of the global yeast population in only few days (Goddard 2008). *S.cerevisiae* colonizes the medium by producing high amount of alcohol and heat through the Crabtree effect, and this latter trait provides a 7% fitness advantage over the other members of the community (Goddard 2008). The fitness advantage of *S.cerevisiae* over non-*Saccharomyces* yeast could explain the short lag-phase and complete fermentation of 3 out of 4 PdC modalities despite very low population level in the PdC before barrels inoculation.

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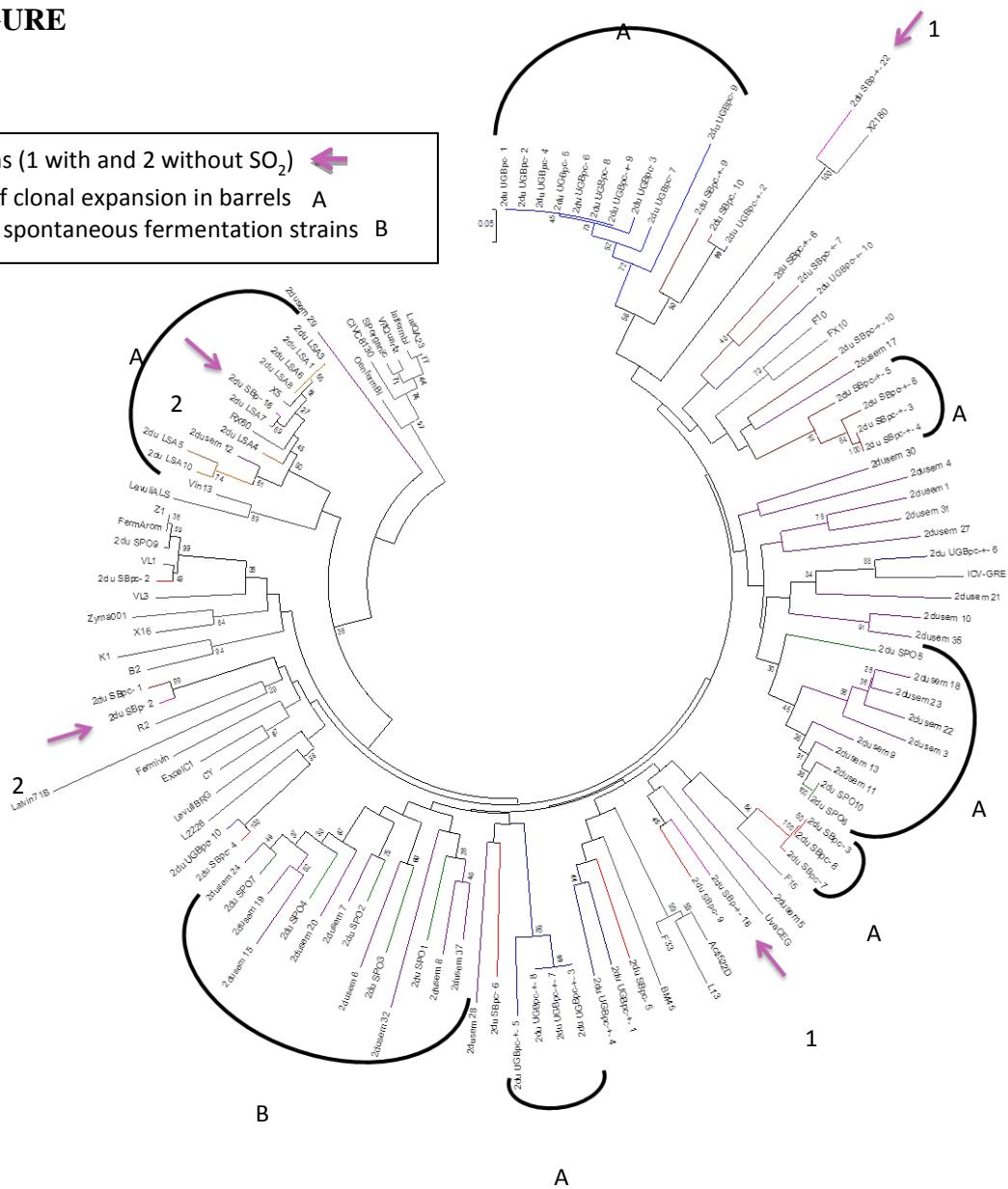
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## FIGURE

PdC strains (1 with and 2 without SO<sub>2</sub>) ←  
 Clusters of clonal expansion in barrels A  
 Cluster of spontaneous fermentation strains B



**Figure 1.**

Neighbor joining tree clustering 59 strains from PdC and barrels fermentations, 33 commercial strains and 29 cellar strains. The tree was constructed from Bruvo's distance between strains based on the polymorphism at 16 loci. Color code: Grey; commercial strains, Pink; Sauvignon Blanc PdC with and without SO<sub>2</sub>, Dark and light red; respectively Sauvignon Blanc barrels with and without SO<sub>2</sub>, Dark and light blue; respectively Ugni Blanc barrels with and without SO<sub>2</sub>, Orange; Sauvignon Gris barrels inoculated with commercial strain X5, Green and Purple; respectively Sauvignon Gris and Semillon barrels from spontaneous

## TABLES

**Table 1.**

Chemical analyses of PdC fresh musts just after crushing grape step. SB and UB meant respectively Sauvignon Blanc and Ugni Blanc.

	AQ/Du SB P	AQ/Du UB P
Sugar - g/L	182	135
pH	3.15	2.9
Total acidity - g/L (of tartaric acid)	9.0	13.6
Assimilable nitrogen - mg/L	175	101
Turbidity - NTU	1958	1348

**Table 2.**

Chemical analyses of PdC musts before barrels inoculation. SB and UB meant respectively Sauvignon Blanc and Ugni Blanc. Pc+ and Pc- represented the 2 modalities, with and without SO<sub>2</sub> addition at 30mg/L, respectively.

	AQ/Du SB Pc+	AQ/Du SB Pc-	AQ/Du UB Pc+	AQ/Du UB Pc-
Sugar - g/L	120	113	78	67
pH	3.14	3.12	2.95	2.94
Volatile acidity - g/L (of acetic acid)	0.48	0.43	0.43	0.49

**Table 3.**

Population numeration of the fresh must used for PDC immediately after grape crushing, and of the 4 PdC before inoculation. SB and UB meant respectively Sauvignon Blanc and Ugni Blanc. Pc+ and Pc- represented the 2 modalities respectively with and without SO<sub>2</sub> addition at 30mg/L. Code: TY and NS meant respectively Total Yeast and Non-*Saccharomyces* culture mediums. Values in brackets represent standard deviation (n=3)

	Total yeast count (ufc.mL <sup>-1</sup> )	Non- <i>Saccharomyces</i> count (ufc.mL <sup>-1</sup> )
<b>PDC fresh must</b>		
AQ/Du SB P	1.10 <sup>5</sup> (±7.10 <sup>3</sup> )	8.10 <sup>4</sup> (± 3.10 <sup>3</sup> )
AQ/Du UB P	1.10 <sup>4</sup> (± 1.10 <sup>3</sup> )	7.10 <sup>3</sup> (± 5.10 <sup>3</sup> )
<b>PDC at 30-50% fermentation</b>		
AQ/Du SB Pc+	1.10 <sup>7</sup> (± 1.10 <sup>3</sup> )	2.10 <sup>7</sup> (± 2.10 <sup>6</sup> )
AQ/Du SB Pc-	6.10 <sup>7</sup> (± 2.10 <sup>6</sup> )	3.10 <sup>7</sup> (± 1.10 <sup>7</sup> )
AQ/Du UB Pc+	4.10 <sup>7</sup> (±0)	2.10 <sup>7</sup> (± 3.10 <sup>6</sup> )
AQ/Du UB Pc-	3.10 <sup>7</sup> (±4.10 <sup>6</sup> )	2.10 <sup>7</sup> (± 8.10 <sup>6</sup> )

**Table 4.**

Summary of samples collected with PdC or barrel origin, indication of the samples code, number of yeast isolates analyzed by ITS PCR, microsatellites and final number of *S.cerevisiae* profiles after removing all strains with more than 3 missing markers values.

Origin	Samples	Number of isolates analyzed by ITS	Number of isolates analyzed by microsatellites	Number of strains with less than 4 missing markers
PdC	AQ/Du SB P+	30	3	2
	AQ/Du SB P-	30	2	2
	AQ/Du UB P+	30	0	-
	AQ/Du UB P-	30	0	-
Barrels	AQ/Du SG/SB Pc+	10	10	8
	AQ/Du SG/SB Pc-	10	10	10
	AQ/Du SG/UB Pc+	10	10	10
	AQ/Du SG/UB Pc-	10	10	10
	AQ/Du SG Spo	10	10	<b>9</b>
	AQ/Du SG LSA	10	10	8
Total		180	65	59

**Table 5.**

Chemical analyses of the first entered barrel just after crushing grape step. SG meant the grape variety Sauvignon Gris.

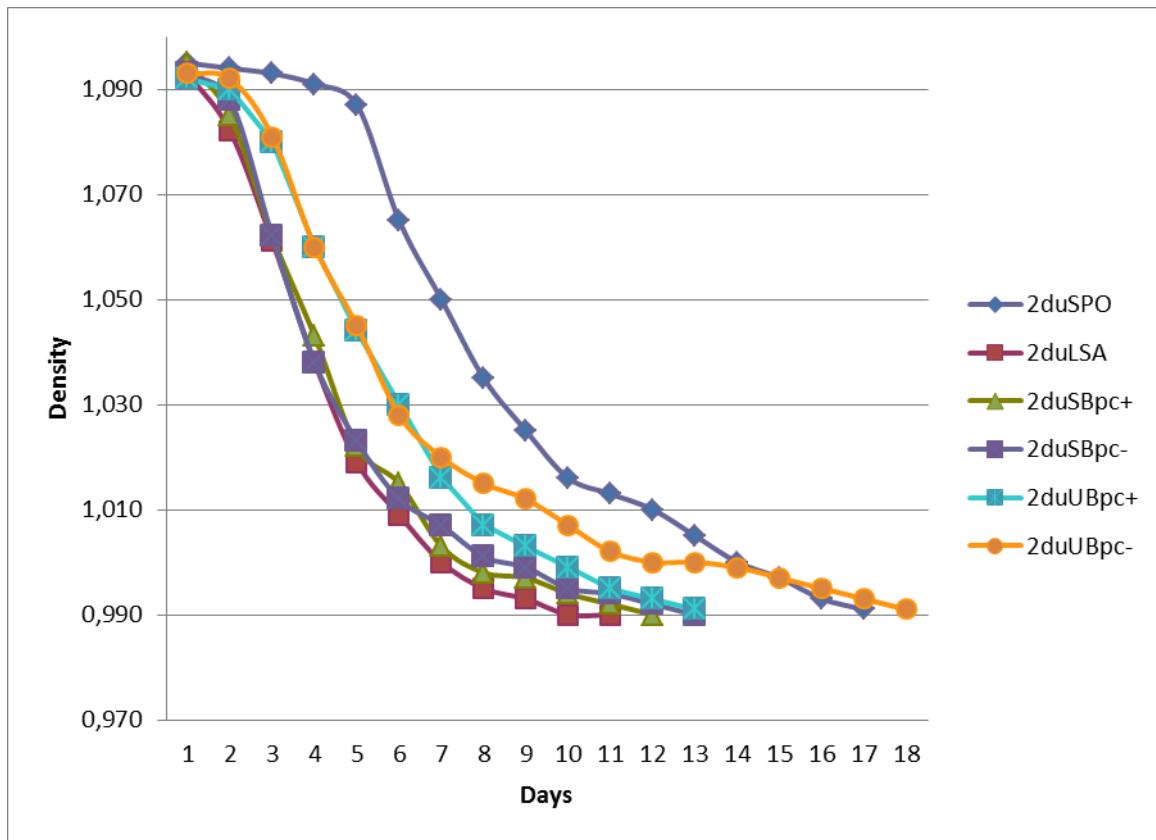
	AQ/Du SG Bq
Sugar - g/L	231
pH	3.35
Total acidity - g/L (of tartaric acid)	6.1
Assimilable nitrogen - mg/L	133
Turbidity - NTU	51
Total yeast count (ufc.mL <sup>-1</sup> )	4.10 <sup>6</sup> (± 1.10 <sup>6</sup> )
Non- <i>Saccharomyces</i> count (ufc.mL <sup>-1</sup> )	9.10 <sup>5</sup> (± 8.10 <sup>4</sup> )

**Table 6.**

Chemical analyses of the barrels musts at the end of the alcoholic fermentation (or total sugar consumption). SB, UB and SG meant respectively grape varieties Sauvignon Blanc, Ugni Blanc and Sauvignon Gris. Pc+, Pc-, Spo and LSA represented the 4 modalities respectively with and without SO<sub>2</sub> addition at 30mg/L, spontaneous fermentation and direct inoculated fermentation with X5 commercial strain.

	AQ/Du SG/SB Pc+	AQ/Du SG/SB Pc-	AQ/Du SG/UB Pc+	AQ/Du SG/UB Pc-	AQ/Du SG Spo	AQ/Du SG LSA
<b>Fermentation time (days)</b>	12	13	13	18	17	11
<b>Final alcohol - % vol.</b>	14.20	14.35	14.20	13.98	14.20	14.00
<b>pH</b>	3.28	3.31	3.30	3.29	3.24	3.23
<b>Volatile acidity - g/L (of acetic acid)</b>	0.50	0.44	0.57	0.61	0.56	0.54

## SUPPLEMENTAL DATA



**Figure S1.**

Fermentation curves of the 4 inoculated barrels with PdC (2duSBpc+, 2duSBpc, 2duUBpc+, 2duUBpc-), spontaneous fermentation (2duSPO) and X5 inoculated barrel (2duLSA).

**Table S1.**

33 *S.cerevisiae* strains from industrial yeast production commonly used in Aquitaine region, in organic and conventional farming system.

Commercial name	Strain	Distributor
Actiflore cerevisiae	522D	Laffort
Excellence B2		Lamothe Abiet
Lalvin BM 45		Lallemand
Levuline CHP	CIVC8130	Oenofrance
Lalvin CY 3079	BourgoBlanc	Lallemand
Excellence C1 Val oeno		Lamothe Abiet
Zymaflore F10	FZ 182	Laffort
Zymaflore F15		Laffort
Actiflore F33	F33	Laffort
Fermol Arome plus	PB2010	Spindal
Fermivin	7013	Littorale
Zymaflore FX10		Laffort
ICV-GRE	138 grenache	ICV
K1	Killer non marquée	Lallemand
L.A. L13	L13	Lamothe Abiet
Rhône L2226		Lallemand
Lallferm bio		IOC / Lallemand
Lalvin QA23	QA23	Lallemand
Lalvin 71B	71B	Lallemand
Levuline ALS	EG8	Oenofrance
Levuline BRG	UP 30Y5	Oenofrance
Oenoferm Bio		Littorale
Vitilevure KD	R2	Martin Vialatte
Zymaflore RX60	rx60	Laffort
SP organic		Martin Vialatte
Uvaferm CEG	CEG - Epernay 2	Lallemand
Achor vin 13	vin 13	Littorale
Vitilevure quartz		Martin Vialatte
Zymaflore VL1	vl1	Laffort
Zymaflore VL3	lv3	Laffort
Zymaflore X16	x16	Laffort
Zymaflore X5	x5	Laffort
Zymaflore 011 organiq		Laffort

**Table S2.**

Microsatellite loci for *Saccharomyces cerevisiae* genotyping with, repeated motif, ORF, primer sequence, fluorescence dye, mix number and concentration used for 8 samples PCR mix preparation for each marker.

Site name	Motif and type	ORF or coordinates	Primers	Fluorescent dye	Multiplex	Quantity (nM)	Authors
ScaAT2	TAA	YBL084c	FW: CAGTCTTATTGCCCTGAACGGA RV: GTCTCCATCCTCCAAACAGCC	PET	1	100	4
ScaAT3	TAA	YDR160w	FW: TGGGAGGAGGGAAATGGACAG RV: TTCAGTTACCGCACAATCTA	NED	1	200	1, 3
C5	GT	VI- 210250/210414	FW: TGACACAATAGCAATGGCTCA RV: GCAAGCGACTAGAACACAATCACA	VIC	1	50	5
C3	CAA	YGL139w	FW: CTTTTTATTACAGAGCGGGCAT RV: AAATCTCATGCCTGTGAGGGGTAT	NED	1	100	5
C8	TAA	YGL014w	FW: CAGGTCGTTCTAACGTTGGAAAATG RV: GCTGTTGCTGTTGGTAGCATTACTGT	6FAM	1	25	5
C11	GT	X- 518870/519072	FW: TTCCATCATAACCGTCTGGGATT RV: TGCCCTTTCTTAGATGGGTTTC	6FAM	1	50	5
YKR072c	GAC	YKR072c	FW: AGATACAGAAGATAAGAACGAAAA RV: TTATTGATGCTTATCTATTATACC	PET	1	50	1, 2
ScaAT6	TAA	IX- 105711/105883	FW: TTACCCCTCTGAATGAAAACG RV: AGGTAGTTAGGAAGTGAGGC	PET	1	100	1, 3
SCYOR26 7c	TGT	YOR267c	FW: TACTAACGTCAACACTGCTGCCAA RV: GGATCTACTTGCACTATACGGG	VIC	1	100	1, 4
YKL172w	GAA	YKL172w	FW: CAGGACGCTACCGAAGCTAAAG RV: ACTTTGGCCAATTCTCAAGAT	6FAM	2	25	2
ScaAT1	TTA	XIII-86902/87140	FW: AAAGCGTAAGCAATGGTAGATACTT RV: CAAGCCTCTCAAGCATGACCTTT	VIC	2	100	1, 3, 4
C4	TAA+TAG	XV- 110701/110935	FW: AGGAGAAAATGCTTTATTCTGACC RV: TTTTCCCTCGGGACGTGAAATA	NED	2	200	5
C9	TAA	YOR156c	FW: AAGGGTTCGTAACATATACTGGCA RV: TATAAGGGAAAAGAGCACGATGGC	NED	2	100	5
ScAAT5	TAA	XVI- 897051/8970210	FW: AGCATAATTGGAGGGCAGTAAAGCA RV: TCTCCGTCTTTGTACTGCGTG	NED	2	100	5
C6	CA	XVI- 485898/485996	FW: GTGGCATCATATCTGCAATTATCAC RV: CAATCAAGCAAAGATCGCCT	VIC	2	50	5
YPL009c	CTT	YPL009c	FW: AACCCATTGACCTCGTTACTATCGT RV: TTCGATGGCTCTGATAACTCCATT	6FAM	2	50	1, 4

## Authors:

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## Conclusion – Perspectives

### CONCLUSION

Cette thèse avait pour objet principal d'étude la levure *S.cerevisiae* dans la région viticole du Bordelais et du Bergeracois. Ces deux régions sont connues pour leur grande variabilité de types de sols, de climats (Bois 2007) mais également pour la diversité des cépages cultivés ou encore des pratiques culturales. Les microclimats en relation avec différentes natures de sols ont permis de définir des milieux environnants d'étendue limitée ou terroir, à la base de la notion d'Appellations d'Origines Contrôlées. Notre étude s'est située naturellement à l'échelle de l'Appellation mais également à l'échelle de l'exploitation viticole ou Cru. Une première approche de la diversité de *S.cerevisiae* présente dans ces différents vignobles avait été menée il y a vingt ans, à partir de l'analyse des caryotypes par électrophorèse en champs pulsés (Frezier and Dubourdieu 1992b). Notre travail a permis de revisiter ces résultats, par l'utilisation d'une méthode plus performante de typage moléculaire basées sur l'utilisation de marqueurs microsatellites qui autorise les approches de génétique des populations afin de structurer la diversité des levures de l'espèce *S.cerevisiae*, mais également de mesurer l'effet de différents facteurs sur cette diversité.

*S.cerevisiae* a été étudiés au cours de cette thèse dans deux compartiments : la grappe de raisin au vignoble et le chai, à partir de prélèvements réalisés dans des cuves en fin de fermentation alcoolique. Au total, 1374 isolats de *S.cerevisiae* provenant de prélèvements de raisins et 1048 isolats provenant de prélèvements de cuves ont été collectés durant deux années consécutives. La diversité génétique a été établie après analyse de marqueurs microsatellites spécifiques à l'espèce *S.cerevisiae*, et en utilisant pour la première fois un nombre important de marqueurs différents (de 15 à 17). Nos travaux ont permis de mettre en

évidence une très grande diversité génétique pour l'ensemble des prélèvements (plus de 75% de génotypes différents). Cependant, malgré un large dispositif d'échantillonnage, seule une fraction de cette diversité a été appréhendée, et l'utilisation des courbes de raréfaction a permis d'estimer une diversité maximale de 4895 génotypes différents pour la région du Sauternais et de 6770 pour la population du raisin à l'échelle de la région Bordelaise.

La géographie est l'un des premiers facteurs qui peut expliquer les structures de populations, comme montré pour les végétaux (Zanetto and Kremer 1995). Aussi, nous avons recherché l'impact de ce facteur sur la structuration de la population de *S.cerevisiae* présente sur la baie de raisin, à l'échelle des vignobles de la région bordelaise, ce qui se traduit par des distances entre chaque appellation variant de 20 à 80 km. L'analyse de différentiation par Fst a montré une différentiation génétique significative des populations selon les appellations. Cette différentiation ne semble pas reliée à la distance entre les sites, ni clairement aux barrières naturelles que sont la Garonne et la Gironde. En revanche, les échanges entre appellations pointent une position centrale de l'appellation Pessac-Léognan puisque tous les flux estimés semblent plus élevés dans le sens de Pessac-Léognan vers les autres régions viticoles, comme si cette région pouvait jouer le rôle de réservoir primaire. Il est intéressant de souligner ici que, d'un point de vue historique, la région des Graves est considérée comme la première région productrice de raisins en Gironde dès le Moyen Age. Ce point devrait être évalué de manière plus approfondie afin de mieux expliciter les liens entre populations et faire éventuellement apparaître les liens historiques. Il a également été observé, au sein de chaque appellation, des différenciations des populations de levures *S. cerevisiae* selon le domaine.

A côté de ces paramètres environnementaux naturels, l'homme par son action peut intervenir sur la diversité des communautés levures en modifiant leur environnement.

Le choix du mode de conduite de la vigne, qui dépend notamment de l'usage de produits phytosanitaires, peut potentiellement affecter la communauté microbienne (Cordero-Bueso, Arroyo, and Valero 2014; Milanović, Comitini, and Ciani 2013) et en particulier la diversité des levures *S. cerevisiae*. Afin, d'aborder cette question, le dispositif d'échantillonnage avait été construit de manière à inclure pour chaque appellation, hors Bergerac, deux domaines proches, l'un en agriculture biologique et l'autre en agriculture conventionnelle, ceci afin d'obtenir pour chaque microclimat un échantillonnage équilibré. A partir de notre jeu de données, la différentiation associée à ces deux modes de conduite mesurée à l'aide de statistique était faible (0.03) indiquant que celui-ci impacte peu la diversité des levures *S. cerevisiae*. Néanmoins, les résultats obtenus au cours de deux années d'études montrent une diversité des souches de *S. cerevisiae* plus élevée pour les raisins issus de parcelles conduites en agriculture conventionnelle par rapport à celles conduites en agriculture biologique.

L'impact du développement important de la pratique du levurage depuis une quarantaine d'années sur la diversité des levures est également une question qui préoccupe un grand nombre de vinificateurs. Aussi, nous avons évalué systématiquement la présence des levures commerciales les plus couramment utilisées dans le bordelais dans les prélèvements de raisins et de cuves. Les études antérieures avaient abordé ce sujet de deux manières distinctes. Dans un premier cas, la présence de souches commerciales dans des fermentations spontanées ou sur des prélèvements de baie de raisin était recherchée (Valero et al. 2005; Schuller et al. 2007; Schuller and Casal 2006) et dans le second cas la persistance dans un vignoble après dissémination volontaire des souches commerciales a été mesurée durant 3 ans (Cordero-Bueso, Arroyo, Serrano, and Valero 2011). La première originalité de cette étude provient de l'échelle régionale à laquelle elle a été menée, sur plus de 23 domaines, avec la prise en compte non seulement des génotypes de levures commerciales mais également les génotypes apparentés (75% des allèles en commun). La présence de souches apparentées aux levures

commerciales dans les chais ne pratiquant pas le levurage est relativement faible (7%), mais ce pourcentage n'est pas représentatif du bordelais dans son ensemble, puisque ce constat a été principalement obtenu à partir des domaines conduits en agriculture biologique (6/7 domaines). En revanche, nous avons pu constater une présence importante de souches apparentées à des souches commerciales dans les prélèvements de baies de raisins (25%) situés à une distance maximale de 400 m d'un chai de vinification, sans qu'un gradient de fréquence n'apparaisse clairement. Ceci indique ainsi l'importance du retour jusqu'alors sous-estimé des levures du chai vers le vignoble. Ceci est confirmé par l'estimation des flux de souches entre raisin et chai qui suggère des flux similaires et importants entre ces deux compartiments, dans le sens chai-raisin et également dans le sens raisin-chai. La différentiation faible à moyenne mais significative des meta-populations issues de raisin et de chai suggère l'existence de deux compartiments distincts potentiellement associés aux modes de vie divergents entre le chai et le vignoble avec éventuellement des processus de sélection distincts dans chaque compartiment. Ces résultats closent enfin les désaccords entre les théories présentant *S. cerevisiae* comme une levure exclusivement de chai proposée par (Vaughan-Martini and Martini 1995), ou au contraire comme une levure de raisins ainsi indiqué par Mortimer et Polsinelli en 1999 (Mortimer and Polsinelli 1999), au profit d'une vision dynamique entre ces deux compartiments.

Cette exploration de la structure de population des levures *S.cerevisiae* du vignoble bordelais a été complétée par l'analyse des fermentations spontanées à l'échelle de trois domaines d'une même appellation. La disponibilité de souches de levures collectées il y a plus de 20 ans pour l'un des domaines, nous a permis pour la première fois, d'ancrer cette dynamique des populations de *S. cerevisiae* sur une longue période. Le concept de l'existence de levures de zone (et non pas de levures de Cru) sur une période de 3 années consécutives, indépendamment du cépage et du mode de vinification avait été avancée pour le bordelais par

les travaux précurseurs de Frézier en 1992. Nos résultats montrent que le « domaine » contribue à la structuration des populations du chai. L'analyse des populations de levures nous a permis d'observer des différenciations génétiques faibles entre populations issues d'un même chai pour 2 à 3 années consécutives, et plus importante sur une période plus longue d'observation (ici > 20 ans), montrant ainsi que le temps pourrait être un élément permettant d'expliquer partiellement la différenciation des populations au sein d'un même Cru. De plus, la forte proximité entre deux domaines, présentant des parcelles jointives et partageant du matériel et du personnel en commun, favorise vraisemblablement les échanges de populations de *S. cerevisiae* au vignoble et dans le chai. Mais un des points marquant de ce travail était la présence de populations ancestrales communes dans des prélèvements anciens et récents pour ces deux domaines proches qui suggère la stabilité globale des populations sur le long-terme dans une région et incite à poser la question de la contribution éventuelle de ces populations aux caractéristiques et typicité des vins produits.

Enfin, la diversité et la structure de population de *S. cerevisiae* ont été étudiées dans les fermentations d'un même chai au cours de la campagne de vendange. Les chais échantillonnés ont utilisé des modes d'inoculation originaux, basés sur la mise en œuvre de pieds de cuve, permettant ainsi l'utilisation de la communauté levure indigène, soit à partir d'une quantité de raisins ramassée 8 à 10 jours avant les vendanges, soit en utilisant la première cuve remplie comme apport de biomasse pour ensemencer les cuves suivantes. Dans le cas de la vinification classique, la préparation d'un pied de cuve à l'aide de raisins ramassés avant vendange s'est révélée être une méthode efficace pour contrôler le démarrage et la conduite des fermentations de façon comparable à l'utilisation d'une levure commerciale. Dans ce cas, le viticulteur sélectionne une population de levures spécifiques, différente d'un pied de cuve à l'autre et de celles des fermentations spontanées. Dans nos conditions

expérimentales, ces pieds de cuve présentaient en fait une population de *S. cerevisiae* sous dominante, capable néanmoins d'assurer le déclenchement et l'achèvement des fermentations.

D'un point de vue plus appliqué, un volet de ce travail mené en collaboration avec des viticulteurs en agriculture biologique, avait comme objectif de proposer des souches candidates, issues de chaque propriété, afin de réaliser une micro-sélection dans le but de développer des levains « à façon » disponibles à la demande, pour les utiliser en fonction des caractéristiques du millésime. Nous avons ainsi pu proposer des individus répartis dans les dendrogrammes, en ciblant des génotypes rémanents d'une année à l'autre pour un chai donné. Des tests phénotypiques ont ensuite été réalisés au laboratoire afin de valider les potentialités technologiques de ces individus. Néanmoins, sur 27 souches testées (12 en vinification en blanc et 15 en vinification en rouge), seulement 2 ont été retenues et proposées pour un développement éventuel de levains à façon.

## PERSPECTIVES

La collection de souches de *S.cerevisiae* ainsi collectée dans ce travail comporte un grand nombre des souches obtenues à partir de prélèvements de raisins dont un groupe de souches ayant une parenté forte avec des souches commerciales. Par ailleurs, nous avons pu observer que vigne et chai représentent deux compartiments apparemment disjoints, en suggérant que leurs populations associées subissent des contraintes différentes. Il est à noter que les souches sont apparentées aux LSA mais présentant de faibles variations. Aussi, la question de l'évolution de ces souches de levures se pose lors des retours dans l'environnement avec la transition du milieu chai/moût « riche » générant des populations très élevées pendant un temps très court, suivi à priori d'une phase de survie, opposé au milieu raisin plutôt pauvre, où les populations de levures sont faibles et diffuses, et se maintenant tout au long de l'année.

Les analyses de génomique comparative par hybridation et de séquençage d'ADN de 4 variant clonaux de VL1 obtenus à partir de raisin (Franco-Duarte et al. 2015) suggèrent en effet l'amplification de gènes associés au retour dans le vignoble. Appliquer des approches de génomique des populations à notre collection d'individus comportant différents fonds génétiques, complétée par des évaluations phénotypiques pourrait permettre de mettre en évidence des gènes amplifiés partagés par plusieurs souches. Dans cette perspective, un travail a débuté visant à étudier la fréquence d'apparition des translocations impliquées dans la résistance au SO<sub>2</sub> dans les populations issues du raisin ou du chai constituées lors de cette étude (Zimmer et al. 2014) . De la même manière, la comparaison des populations des levures de chai et de raisin à l'aide d'un scan génomique pourrait permettre de faire apparaître des polymorphismes en lien avec les contraintes différentielles entre les deux compartiments susceptibles d'être exercées sur les génomes des levures. Cette comparaison serait également à envisager pour des comparaisons de populations de génomes de souches isolées de fermentations de raisins botrytisés avec ceux de génomes de levures de vinification classique, ou tout simplement de populations isolées de régions éloignées comme par exemple de vignobles septentrionaux et méditerranéens ou d'autres. Ces approches peuvent nous fournir des éléments de compréhension de l'adaptation des levures *S. cerevisiae* à la vinification en général, mais également à des vinifications particulières, tout en apportant des informations nouvelles sur l'écologie de *S.cerevisiae*.

Enfin, durant cette étude, de nombreuses autres espèces de levures non-*Saccharomyces* ont également été mises en collection. Cette collection regroupe 1995 isolats obtenus à partir de prélèvements de raisin issus de parcelles conduites en agriculture biologique et conventionnelle. Cette collection pourrait faire l'objet d'une étude similaire à ce que nous avons pu mener chez *S. cerevisiae* afin d'évaluer la diversité et de déterminer l'existence d'éventuelle structure de population au vignoble pour ces espèces. Cela ne devrait pas être le

cas pour *Starmerella bacillaris* (synonyme *Candida zemplinina*) qui ne montrent pas de structure de population claire (Masneuf-Pomareda et al. 2015), mais davantage pour *Torulaspora delbrueckii* qui a été souvent isolée sur les raisins (Saffran et al. 2014). Pour les autres espèces, peu de données existent concernant la structure de population. Le décryptage de la diversité de ces espèces permettrait de faire apparaître des spécificités liées à l'environnement vin, et nous aider à mieux comprendre comment l'histoire de la vinification a façonné la diversité de ces microorganismes.

## Liste des publications et communications

### Article

Microsatellite analysis of *Saccharomyces uvarum* diversity reveals a different “life style” than *S. cerevisiae* one. Masneuf-Pomarede Isabelle; Salin Franck; Börlin Marine; Coton Emmanuel; Coton Monika; Le Jeune Christine; Legras Jean-Luc.  
Soumis dans FEMS Yeast Research. En révision majeure.

### Oral

Grape berries *saccharomyces cerevisiae*'s population structure in the Bordeaux and Bergerac region revealed by multilocus microsatellite analyses. M. Börlin, F. Salin, J-L. Legras, I. Masneuf-Pomarede. 10th International Symposium of Oenology, Oeno 2015, Bordeaux  
**Posters**

Bordeaux region *S. cerevisiae* strains diversity isolated from grapes and wineries revealed by multilocus microsatellite analysis. M. Börlin, F. Salin, J-L. Legras, I. Masneuf-Pomarede1. Levures, Modèles & Outils. 11ème rencontre internationale des levuristes francophones, LMO2014, Bordeaux

Wine grape *S.cerevisiae* diversity and population structure reveal possible genetic admixture of vineyard and commercial wine strains. M. Börlin, F. Salin, J-L. Legras, I. Masneuf-Pomarede. International Specialized Symposium on Yeasts, ISSY32, Perugia

## Références bibliographiques (Introduction et Conclusion – Perspectives)

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