



**HAL**  
open science

# Population genetics and diversity of the species *Brettanomyces bruxellensis*: a focus on sulphite tolerance

Marta Avramova

► **To cite this version:**

Marta Avramova. Population genetics and diversity of the species *Brettanomyces bruxellensis*: a focus on sulphite tolerance. Agricultural sciences. Université de Bordeaux; University of Adelaide (Australie), 2017. English. NNT: 2017BORD0911 . tel-01808370

**HAL Id: tel-01808370**

**<https://theses.hal.science/tel-01808370>**

Submitted on 5 Jun 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THÈSE EN COTUTELLE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE  
**DOCTEUR DE**  
**L'UNIVERSITÉ DE BORDEAUX**  
**ET**  
**THE UNIVERSITY OF ADELAIDE**

ÉCOLE DOCTORALE UBX

ÉCOLE DOCTORALE UofA

Spécialité Œnologie

par **Marta AVRAMOVA**

**Génétique de populations et diversité de l'espèce**  
***Brettanomyces bruxellensis*.**

**Etude de la tolérance aux sulfites**

Sous la direction de Isabelle MASNEUF-POMARÈDE

et de Paul GRBIN

Soutenue le 19 décembre 2017

Membres du jury :

M. Philippe DARRIET  
M. Hervé ALEXANDRE  
M. Serge CASAREGOLA  
M. Vittorio CAPOZZI  
M. Paul GRBIN  
Mme Isabelle MASNEUF-POMAREDE  
Mme Warren ALBERTIN

Professeur, Université de Bordeaux  
Professeur, Université de Bourgogne  
Directeur de recherche, INRA, Jouy-en-Josas  
Docteur, Università di Foggia  
Associated professor, University of Adelaide  
Professeur, Bordeaux Sciences Agro  
Maître de conférences ENSCBP, Bordeaux INP

Président du jury  
Rapporteur  
Rapporteur  
Examineur  
Directeur de thèse  
Directrice de thèse  
Invitée



# **Titre : Génétique des populations et diversité de l'espèce *Brettanomyces bruxellensis*. Etude de la tolérance aux sulfites**

## **Résumé**

*Brettanomyces bruxellensis* est un microorganisme qui est considéré comme la cause majeure des défauts microbiologiques du vin. L'importance de cette levure à l'échelle industrielle est liée au fait qu'elle est isolée à partir de substrats différents tels que la bière, le kombucha, les molasses utilisées pour la production de bioéthanol et autres. Ce projet a pour objectif d'étudier la diversité génétique de l'espèce en se basant sur une large population d'isolats provenant de niches écologiques et géographiques variées. Pour ce faire, une méthode de génotypage robuste (analyse microsatellite) a été optimisée et appliquée sur la population, mettant en évidence la coexistence de populations diploïdes et triploïdes à l'échelle globale. Puis, la relation entre regroupement génétique et traits physiologiques a été explorée. Notamment, l'étude de la tolérance aux sulfites a été effectuée sur un sous-ensemble de souches représentatif de la population. Les résultats obtenus mettent en évidence un lien entre groupes génétiques et comportement vis-à-vis des sulfites. Des expériences de compétition en présence de dioxyde de soufre montrent un avantage sélectif des souches tolérantes aux sulfites par rapport aux souches sensibles, suggérant ainsi une adaptation spécifique au principal antiseptique utilisé en œnologie. Ce travail contribue à une meilleure connaissance de cette levure d'altération du vin en termes de diversité génétique et phénotypique et permet d'émettre des hypothèses sur les stratégies évolutives d'adaptation au milieu anthropique de cette espèce modèle non conventionnelle.

**Mots clefs :** génétique des populations, *Brettanomyces bruxellensis*, sulfites, avantage sélectif, polyploidie

Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP,  
33140 Villenave d'Ornon



# **Title: Population genetics and diversity of the species *Brettanomyces bruxellensis*. A focus on sulphite tolerance.**

## **Abstract**

*Brettanomyces bruxellensis* is a microorganism described as the first cause of microbial spoilage of wine. Its industrial relevance is highlighted by the fact that this yeast is isolated from different substrates such as beer, kombucha, bioethanol fermentation molasses and others. This project aims to explore the genetic diversity of the species by studying a large population of isolates from various geographical and ecological niches. For this purpose, a robust genotyping method (microsatellite analysis) was optimised and applied on the population, thus highlighting the coexistence of diploid and triploid populations worldwide. Further, the relation between genotypic clustering and physiological traits was studied. Namely, sulphite tolerance assay was performed on a subset of strains representative of the total population. The results reveal a link between genetic group and growth profile in the presence of sulphur dioxide. Competition experiments in presence of sulphites highlight a selective advantage of sulphite tolerant strains compared to sulphite sensitive ones, thus suggesting a specific adaptation to the main antimicrobial used in winemaking. This work contributes to a deeper understanding of this wine spoilage microorganism in means of genetic and phenotypic diversity and sheds light on putative evolutionary strategies for adaptation to human related environment of this non-conventional model yeast species.

**Keywords:** population genetics, *Brettanomyces bruxellensis*, sulphites, selective advantage, polyploidy

Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP,  
33140 Villenave d'Ornon



## Résumé détaillé

*Brettanomyces bruxellensis* est un microorganisme qui est considéré comme la cause majeure des défauts microbiologiques du vin. L'importance de cette levure à l'échelle industrielle est liée au fait qu'elle est isolée à partir de substrats différents tels que la bière, le kombucha (thé fermenté), les molasses utilisées pour la production de bioéthanol, etc. où cette levure pourrait être considérée comme étant bénéfique (voire nécessaire) ou néfaste pour l'élaboration et la qualité du produit. Au cours des dernières décennies, l'étude scientifique de cette levure ubiquiste ont révélé une variabilité phénotypique importante pour l'espèce en terme de croissance, métabolisme, tolérance à des facteurs biotiques et abiotiques variées, et autres. Particulièrement, dans le domaine de l'œnologie, cette variabilité rend difficile la prévention et l'élimination de ce microorganisme d'altération. Pour chercher à comprendre ces variations phénotypiques intraspécifiques, la diversité génétique de *B. bruxellensis* a été explorée, mettant en évidence une variabilité génétique importante au sein de l'espèce. Notamment, une étude des souches isolées en Australie a mis en évidence différents groupes génétiques, avec un génotype prépondérant parmi les vins australiens. De plus, les souches de ce génotype se sont avérées très tolérantes aux sulfites. Plus tard, une étude de génomique comparative de 4 souches de cette espèce a mis en évidence des souches présentant niveaux de ploïdie différents, certaines souches étant diploïdes et autres – triploïdes, avec la souche appartenant au génotype le plus répandu en Australie étant triploïde. Ces études ont souligné un possible lien entre génotype et tolérance aux sulfites pour cette population d'isolats australiens. Les sulfites étant le moyen le plus utilisé pour prévenir et éliminer le développement de *B. bruxellensis* dans le domaine de l'œnologie, ce lien méritait d'être exploré à plus grande échelle. De plus, à ce stade de la connaissance de cette levure d'intérêt industriel, il était intéressant d'explorer la génétique des populations de l'espèce et les facteurs qui la déterminent.

Dans ce contexte, ce projet a pour objectif d'étudier la diversité génétique de l'espèce *B. bruxellensis* en se basant sur une large population d'isolats provenant de niches écologiques et géographiques variées. Pour ce faire une collection riche composée de 1488 isolats de substrats et origines géographiques a été utilisée. Ensuite, une méthode de génotypage robuste (analyse microsatellite) a été optimisée et appliquée sur la population, confirmant la diversité génotypique de l'espèce et mettant en



évidence la coexistence de populations diploïdes et triploïdes à l'échelle globale. Par des analyses statistiques, il est démontré que la population est structurée en fonction du niveau de ploïdie, le type de substrat et l'origine géographique des isolats, suggérant une influence anthropique sur la biodiversité spatiale de *B. bruxellensis*. Dans un deuxième temps, la relation entre regroupement génétique et traits physiologiques a été explorée. Notamment, l'étude de la tolérance aux sulfites a été effectuée sur un sous-ensemble de souches représentatif de la population (39 souches de substrats et origines géographiques différents). Des fermentations en petite échelle (fermenteurs de 3 mL) ont été effectuées dans un milieu modèle et à des différentes concentrations en sulfites variant de 0 à 0.6 mg/L de SO<sub>2</sub> moléculaire. Les paramètres de croissance phase de latence, vitesse de croissance et population maximale ont été suivis et la base de données ainsi obtenue a été traitée par des analyses statistiques. Les résultats confirment le lien entre regroupement génétique et comportement vis-à-vis des sulfites pour ce sous-ensemble d'isolats représentatifs de l'espèce.

En effet, le lien entre configuration génétique et tolérance aux sulfites, combiné à la dissémination des souches triploïdes tolérantes au SO<sub>2</sub> mènent à l'hypothèse que cette configuration génétique pourrait apporter un avantage sélectif dans les conditions œnologiques, notamment en présence de SO<sub>2</sub>. Pour vérifier cette hypothèse, des expériences de compétition entre souches ont été menées en présence de dioxyde de soufre. Les isolats représentatifs des trois génotypes majeurs rencontrés en milieu œnologique ont été marqués avec des gènes de résistance aux antibiotiques en utilisant un protocole de transformation basé sur le mécanisme de jonction des extrémités non homologues. Les résultats montrent un avantage sélectif des souches tolérantes aux sulfites par rapport aux souches sensibles, suggérant ainsi une adaptation spécifique au principal antiseptique utilisé en œnologie.

Dans un dernier temps, l'étude du lien groupe génétique/tolérance aux sulfites a été approfondi en élargissant le panel de souches phénotypes à 145 souches représentatives de la population *B. bruxellensis* étudiée. Cette démarche a permis de confirmer ce lien ainsi que d'explorer la possibilité d'utiliser des marqueurs moléculaires pour discriminer les souches sensibles des tolérantes. De plus, des différents types de comportements en terme de croissance ont été observés parmi les

souches de *B. bruxellensis* qui survivent à des fortes de doses de SO<sub>2</sub> soulignant des phénotypes résistants et tolérants.

Globalement, ce travail contribue à une meilleure connaissance de *B. bruxellensis*, levure d'altération du vin, en termes de diversité génétique et phénotypique et permet d'émettre des hypothèses sur les stratégies évolutives d'adaptation au milieu anthropique de cette espèce modèle non conventionnelle.

**Mots clefs :** génétique des populations, *Brettanomyces bruxellensis*, sulfites, avantage sélectif, polyploïdie



## **Acknowledgements**

First, I would like to thank Hervé Alexandre and Serge Casaregola for accepting to revise this manuscript. Thanks to Philippe Darriet, Vittorio Capozzi, and Warren Albertin for participating in the jury of the thesis defence.

To Isabelle Masneuf-Pomarède and Warren Albertin I am thankful for their support, their ability to transmit their passion for science and wine, their constant interest and implication in the project, their receptivity and constant advices throughout the work. Thank you for being my advisors and for being the main leaders of this project, I feel lucky that I had the opportunity to work with you.

Thanks to Chris Curtin and Paul Grbin for co-supervising the work. Chris, thank you first for your work which was in the basis of my thesis project, for participating in the construction of the project and for yours ideas, excitement and guidance throughout the project. Paul, thank you for your friendly support during my stay in Adelaide, it really means a lot to me and I was so happy to have you as a supervisor.

I thank also Cristian Varela and Anthony Borneman for their advices, for their work, their constant help during my work in AWRI and the University of Adelaide. Cristian, thank you for your energy and your serious attitude towards my work, thank you for your practical contribution to the work. Anthony, thank you for the weekly advices and the conversations on Brett and wine yeast science in general.

Thanks to my colleagues from Bordeaux. Thanks to Alice Cibrario for being my professional twin and for her friendship, for the conversations and valuable discussions, the shared knowledge (and the shared beers). Alice, you were one of the people who showed me how work and friendship can (and probably should) go hand in hand. Thanks to Margaux, Cécile, Marion for creating the family of the 2018 PhD girls – a true family in the ISVV, it was a true

happiness and honour for me to be a part of this family. Thanks to F  ty, Mariette, Hugo, Lucie, Geoffrey, Marie, Marion, Maroula, Julie, Am  lie, Emilien, Philippe for their professional advices and friendly presence in the lab. Thanks to Marina Bely for motivating me to undertake this PhD and for her support. Thanks to all of the scientific team of the ISVV for their work - I feel lucky that I was a part of your team. Also, thanks to all the people who contributed to our comfort at work, everyone looking after the administrative and technical work in the building. Thank you!

Thanks to my colleagues in Adelaide. Thank you, Marlize for being my desk neighbour, my friend and a great example to follow as a person and as a scientist. Thanks to Paul Chambers, Paul Henschke, Simon Schmidt for the great conversations on wine yeast and science. Thanks Radka, Amy, Caroline, Kate, Jane, Jenny, Simon Dillon for your indirect (and sometimes direct) participation in my work. Thanks to Angus for taking such good care of us and the lab. Thank you Jelena and Eve for your work and for adopting me during the last weeks of my stay in Adelaide (you know what I mean). Thank you to all of the great team of AWRI for being such great specialists and for making me a part of your family during my stay – both professionally and personally.

Thanks to the team of the University of Adelaide. Thank you Paul, for your contribution to the work as a scientist but also for the cycling conversations! Thank you, Nick for your help and for being there for keeping up the good humour in the lab. Thank you, Federico and Ana for being such great colleagues and friends. Thank you all for the meetings, the exchange, the conversations. Thank you for making me part of your team. I have learnt a lot from you and I hope that I will be able to make it up to you one day.

Thanks to my family! Thank you to my parents and my grandmother baba Nina who were always motivating me to undertake new and interesting projects. Thank you for being a support

but also an example to follow professionally with your innate scientific robustness and rigorous attitude towards your work. Thank you for being my personal and professional advisors and most of all for your love and true friendship. Big thank you to Nona, who was always there for me as my dearest friend and also advisor, and for supporting all of my ups and downs. Thank you for your friendship and for being who you are. Thanks to Carles, who motivated me and always believed in me as a scientist and as a person. Thank you for your love and your advices. Thank you to all of my friends who believed in me more than I did – Nelly, Mitty, Radina, Lyubo, Charly, Bill, Ben, Lizzy, Marcell, Federico, Joël, Perrine, Agathe. Thank you – your friendship is priceless and has definitely contributed to this work.

Thank you all, a scientific work is the work of a team.



## Outputs related to this work

### Scientific communications

#### Written communications

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Joseph Schacherer, Giuseppe Spano, Vittorio Capozzi, Giuseppe Blaiotta, Franck Salin, Marguerite Dols-Lafargue, Paul Grbin, Chris Curtin, Warren Albertin, Isabelle Masneuf-Pomarede; “*Brettanomyces bruxellensis* population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution”, submitted in *Scientific Reports*

#### Poster presentations

**Marta Avramova**, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Intraspecific diversity of *Brettanomyces bruxellensis* (*B. bruxellensis*) established with microsatellite markers” **ISSY32, International Specialized Symposium on Yeasts**, September 2015, Italy

Alice Cibrario, **Marta Avramova**, Isabelle Masneuf-Pomarede, Warren Albertin, Patricia Ballestra, Gilles de Revel, Marguerite Dols-Lafargue. *Brettanomyces bruxellensis* intra-species genotypic and phenotypic diversity. Consequences on adaptation to wine. **LMO12, 12<sup>ème</sup> conférence Levures: Modèles et Outils**, April 2016, Belgium

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Coexistence of diploid and triploid populations in the wine spoilage yeast species *Brettanomyces bruxellensis*”, **ICPHB2016, International Conference on Polyploidy, Hybridization and Biodiversity**, May 2016, Croatia

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Diversity of the species *Brettanomyces bruxellensis*: genotypic and phenotypic study” **AWITC2016, Australian Wine Industry Technical Conference**, July 2016, Australia

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Marguerite Dols-Lafargue, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “The wine spoilage yeast species *Brettanomyces bruxellensis*: a diploid-triploid complex associated with contrasted sensibility to sulphites” **ISSY33, 33rd International Specialised Symposium on Yeast**, June 2017, Ireland

Alice Cibrario, **Marta Avramova**, Hany Abdo, Emilien Peltier, Margaux Paulin, Isabelle Masneuf-Pomarede, Patricia Ballestra, Gilles de Revel, Marguerite Dols-Lafargue. “*Brettanomyces bruxellensis* intra-species phenotypic diversity and carbohydrate



preferences. Consequences on adaptation to wine and spoilage ability.” **ISSY33, 33rd International Specialised Symposium on Yeast** , June 2017, Ireland

## **Technical communications**

### Written communications

Warren Albertin, **Marta Avramova**, Alice Cibrario, Patricia Ballestra, Marguerite Dols-Lafargue, Chris Curtin, Isabelle Masneuf-Pomarède, “*Brettanomyces bruxellensis* : diversité génétique et sensibilité aux sulphites”, paru dans *La Revue des Oenologues*

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Why is genetic diversity of *Brettanomyces bruxellensis* important for winemakers and is it related to sulphur dioxide tolerance?”, **AWITC2016 Proceedings, Australian Wine Industry Technical Conference**. Attached in Appendix.

### Oral communications

**Marta Avramova**, Warren Albertin, Isabelle Masneuf-Pomarède, Chris Curtin “*Brettanomyces bruxellensis*: diversité de l’espèce” **Journée technique Vins Bio 2016**, February, 2016, France

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Monika Coton, Emmanuel Coton, Franck Salin, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Why is genetic diversity of *Brettanomyces bruxellensis* important for winemakers and is it related to sulphur tolerance?”, **AWITC2016, Australian Wine Industry Technical Conference**, July 2016, Australia; Price for best wine presentation in Fresh Science Session

### Poster communications

**Marta Avramova**, Alice Cibrario, Franck Salin, Patricia Ballestra, Marguerite Dols-Lafargue, Warren Albertin, Chris Curtin, Isabelle Masneuf-Pomarede, “Tolérance aux sulphites de *Brettanomyces bruxellensis* : lien avec sa diversité génétique et diagnostic”, **Journées Techniques CIVB 2017, Conseil Interprofessionnel des Vins de Bordeaux**, February 2017, France

**Marta Avramova**, Alice Cibrario, Emilien Peltier, Amélie Vallet-Courbin, Julie Maupeu, Franck Salin, Marguerite Dols-Lafargue, Isabelle Masneuf-Pomarede , Warren Albertin , “The wine spoilage yeast *Brettanomyces bruxellensis*: predicting its tolerance to sulphite treatments using TYPEBRETT test” **Spoilers2017, Microbial spoilers in food** June, 2017, France

## **Other**

**Marta Avramova**, “Génomique fonctionnelle et comparative de l’espèce *Brettanomyces bruxellensis*” **MT180s, Ma Thèse en 180s**, March 2016, France

# Contents

<b>Chapter 1. Bibliographical research .....</b>	<b>1</b>
<b>1.1. Of yeast and men.....</b>	<b>1</b>
1.1.1. Yeast and fermentation .....	1
1.1.2. Focus on wine yeast .....	2
1.1.3. Yeast as human commensals and pathogens .....	4
1.1.4. Yeast as model organisms.....	4
1.1.5. Yeast as “cell factories” .....	4
<b>1.2. Yeast population genetics – an approach to study yeast evolution .....</b>	<b>5</b>
<b>1.3. Ploidy level among wine (and other) yeast of interest .....</b>	<b>10</b>
1.3.1. What is polyploidy? .....	12
1.3.2. How does polyploidy occur? .....	12
1.3.3. Polyploidy in yeast.....	16
<b>1.4. <i>Brettanomyces bruxellensis</i> .....</b>	<b>21</b>
1.4.1. History of the species <i>Brettanomyces bruxellensis</i> .....	21
1.4.2. Genetics of <i>Brettanomyces bruxellensis</i> .....	24
1.4.3. Occurrence of <i>Brettanomyces bruxellensis</i> in wine.....	32
1.4.4. Impact of <i>Brettanomyces bruxellensis</i> on organoleptic wine qualities.....	33
1.4.5. <i>Brettanomyces bruxellensis</i> spoilage prevention and elimination .....	34
1.4.5.1 Prediction and prevention methods in wine .....	34
1.4.5.2 Detection of <i>Brettanomyces bruxellensis</i> in wine .....	35

1.4.5.3	Elimination methods for <i>Brettanomyces bruxellensis</i> .....	36
1.4.5.4	Post-spoilage curative methods .....	38
1.4.5.5	Focus on sulfur dioxide use in wine .....	39
1.4.5.5.1	Use of sulfur dioxide in wine .....	39
1.4.5.5.2	Sulfur dioxide tolerance in <i>Saccharomyces cerevisiae</i> .....	42
1.4.5.5.3	Sulfur dioxide tolerance in <i>Brettanomyces bruxellensis</i> .....	46

**Chapter 2. *Brettanomyces bruxellensis* population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution.....51**

<b>2.1.</b>	<b>Abstract.....</b>	<b>53</b>
<b>2.2.</b>	<b>Introduction.....</b>	<b>55</b>
<b>2.3.</b>	<b>Materials and methods .....</b>	<b>58</b>
2.3.1.	Yeast strains .....	58
2.3.2.	Genotyping by microsatellite analysis .....	59
2.3.2.1	DNA extraction.....	59
2.3.2.2	Microsatellite loci identification and primers design .....	59
2.3.2.3	Microsatellites amplification .....	59
2.3.2.4	Microsatellite data analysis .....	60
2.3.2.5	Core genotype analysis .....	61
2.3.3.	Sulphite tolerance assessment.....	61
<b>2.4.</b>	<b>Results .....</b>	<b>63</b>
2.4.1.	<i>B. bruxellensis</i> genotyping analysis and population structure .....	63
2.4.2.	Core genotype analysis .....	68

2.4.2.1	Core diploid data subset .....	68
2.4.2.2	Ancestral populations and inference of population structure .....	69
2.4.2.3	Population differentiation analysis .....	70
2.4.3.	Sulphite tolerance.....	71
<b>2.5.</b>	<b>Discussion.....</b>	<b>73</b>
<b>2.6.</b>	<b>Data availability .....</b>	<b>80</b>
<b>2.7.</b>	<b>Acknowledgments .....</b>	<b>81</b>
<b>Chapter 3. Competition experiments between <i>Brettanomyces bruxellensis</i> strains reveal specific adaptation to sulfur dioxide and complex interactions at intraspecies level .....</b>		<b>83</b>
<b>3.1.</b>	<b>Introduction.....</b>	<b>85</b>
<b>3.2.</b>	<b>Materials and methods .....</b>	<b>87</b>
3.2.1.	<i>Brettanomyces bruxellensis</i> strains .....	87
3.2.2.	Strains construction and selection of transformants .....	88
3.2.3.	Transformation validation.....	89
3.2.4.	Competition experiments – media and growth conditions .....	90
3.2.5.	Colony enumeration.....	93
3.2.6.	Wild type control .....	94
<b>3.3.</b>	<b>Results .....</b>	<b>94</b>
3.3.1.	Validation of transformants .....	95
3.3.2.	Competition experiments .....	97
<b>3.4.</b>	<b>Discussion.....</b>	<b>102</b>

<b>Chapter 4. Molecular diagnosis of <i>Brettanomyces bruxellensis</i>' sulfur dioxide sensitivity through genotype specific method.....</b>	<b>107</b>
<b>4.1. Introduction.....</b>	<b>109</b>
<b>4.2. Material and methods.....</b>	<b>110</b>
4.2.1. Strains .....	110
4.2.2. Sulphite tolerance assessment.....	113
<b>4.3. Results .....</b>	<b>114</b>
4.3.1. Growth behaviour in presence of SO <sub>2</sub> .....	114
4.3.2. SO <sub>2</sub> tolerance/resistance and relation to genetic group.....	115
<b>4.4. Discussion.....</b>	<b>118</b>
<b>Chapter 5. Discussion and perspectives .....</b>	<b>123</b>
<b>Chapter 6. Conclusions.....</b>	<b>129</b>
<b>Appendix 131</b>	
<b>Supplementary Figure S-2.1. ....</b>	<b>131</b>
<b>Supplementary Figure S-2.2 .....</b>	<b>132</b>
<b>Supplementary Figure S-4.1. ....</b>	<b>133</b>
<b>Written communications .....</b>	<b>139</b>
<b>References 145</b>	

## **Chapter 1. Bibliographical research**

### **1.1. Of yeast and men**

#### **1.1.1. Yeast and fermentation**

Humans consume and produce fermented beverages since millennia. It was through empirical experience that ancient people discovered the pleasant properties of the liquids resulting from fruit crushing and subsequent fermentation. Vessels containing traces of fermented beverage based on rice, honey and fruit (hawthorn or grape, elucidated *via* the presence of calcium salt of tartaric acid residues) were discovered in China and were dated back to 7000 BC (McGovern et al., 2004). The oldest chemical evidence of grape-wine mixed with *Pistacia atlantica* resin was found in Iran and was evaluated to 5400-5000 BC (McGovern et al., 1996) which coincides with the period when the first human population settlements and plant and animal domestication took place (McGovern et al., 1996). Thus, by inducing, managing, and favouring fermentation of fruit and other materials, human societies have (possibly unconsciously) interacted with microbial populations responsible of the fermentation process. Non-exhaustive list of fermented products made by humans and yeasts would include the most popular ones like wine, beer, and bread, but also yoghurt, chocolate, cheese, *etc.* This list is further broadened by fermented plants that are followed by distillation processes (tequila, whiskey, and others). Even if unconscious of the existence of yeasts performing the fermentation, people probably noticed that for example bread was lighter and better for consumption after addition of small quantity of wine (that was putatively a first form of leavened bread)(Mortimer, 2000a). Thus, unconsciously, since ancient time humans were in constant interaction with microorganisms. Nowadays, we know that fermented beverages present various types of ecological environments for the development of multiple genera, species, and strains of microorganisms – moulds, yeast, bacteria, and viruses. Indeed, it was not until the 1870s, following the work of Louis Pasteur, that it was demonstrated that small

unicellular organisms (yeasts) were responsible for the transformation of sugars contained in fruit to alcohol and carbon dioxide in the reaction called fermentation. Central role in the knowledge of yeasts and their metabolism is occupied by the species *Saccharomyces cerevisiae* (the so-called baker's yeast) which is in the origin of various fermented beverages.

### **1.1.2. Focus on wine yeast**

Wine is probably the most ancient fermented beverage consumed by people and this is partially due to fact that wine would occur “naturally” without addition of leaven. The provenance of yeast performing grape fermentation is still a controversy. Some authors suggest that the yeasts are already present on the grapes' surface and perform the fermentation once the grapes are crushed and their sugars released (Mortimer and Polsinelli, 1999). It was believed for long time that *S. cerevisiae* cells were found only in association with human environments. Some authors suggested that wine yeasts were present on the grapes surface and could also be transported in the winery through insects and other vectors, subsequently residing on the winemaking equipment (Naumov, 1996). Interestingly, the main yeast performing grape fermentation – *S. cerevisiae*, is detected on grape surface but at very low frequencies compared to other species (Goddard and Greig, 2015; Mortimer and Polsinelli, 1999). This observation led to another model suggesting that *S. cerevisiae* was a “nomad yeast” without particular niche (Goddard and Greig, 2015). Eventually, *S. cerevisiae* is present in probably all grape fermentations and it plays a central role in winemaking for several reasons: i) it is the only non-spoilage yeast species related to winemaking environment that is able to produce and survive to high concentrations of ethanol, ii) it is able to consume all sugars present in the grape must, thus reducing the risk of sluggish fermentation and spoilage during wine storage, iii) it is associated with enzymatic activities implied in the transformation of aromatic precursors contained in the grape. Thus, over the last decades, people have adapted their winemaking practices in order to favour the development of this particular species, often avoiding the occurrence of other microorganisms. The most straightforward example for this is the selection and production of

the first active dried yeast designed for controlled inoculation of grape must by winemakers in the 1970s (see Chambers and Pretorius, 2010). At present, the majority of conventional winemakers use this technique in order to avoid sluggish fermentations and generally to obtain better control of wine fermentation. Subsequently, multiple scientific articles focused on the selection and development of *S. cerevisiae* strains to obtain adapted sugar/ethanol yield, aromatic characteristics, and metabolic features corresponding to the winemaking environment (SO<sub>2</sub>, copper, and ethanol tolerance, flocculation capacity, and others) (see Chambers and Pretorius, 2010).

Nowadays, there is a high and rising interest for the so-called non-*Saccharomyces* yeast (or species other than the ones from *Saccharomyces* genus). Those species are generally developed in the early stages of grape fermentation process. Later, their population declines and is displaced by *S. cerevisiae*. Thus, their presence in the beginning of the fermentation is acting on wine properties (Fleet, 2003). From oenological point of view, the impact of non-*Saccharomyces* yeast could be related to the volatile metabolites production involved in wine aroma (Fleet, 2003). From a biological point of view, non-*Saccharomyces* yeasts are modifying the physical environment for *S. cerevisiae* (and other microorganisms). Also, some non-*Saccharomyces* yeast species are described as spoilage microorganisms in wine, leading to altered wine taste, aroma, and/or mouthfeel. Thus, nowadays the importance of non-*Saccharomyces* yeast in winemaking is undisputable (both as beneficial and spoilage microorganisms). The attempt to produce more complex and diverse wines has led to high and rising interest for various non-*Saccharomyces* yeast species for the controlled must inoculation. Among the commercialised species available for co-inoculation with *S. cerevisiae*, are *Torulaspota delbrueckii*, *Metschnikowia pulcherrima*, *Lachancea kluyveri*, *Lachancea thermotolerans* (Jolly et al., 2014; Masneuf-Pomarede et al., 2016).



### **1.1.3. Yeast as human commensals and pathogens**

Different yeast species are part of human body as commensals. However, in some cases strains can become pathogenic, especially in the case of immunocompromised patients (ex. HIV patients). Among others, popular human-pathogen yeast are several species from the *Candida* genus (ex. *C. albicans*, *C. glabrata*), as well as *Cryptococcus neoformans* (Hazen, 1995; Wertheimer et al., 2016). Interestingly, *Saccharomyces cerevisiae* is sometimes encountered as human pathogen (Wertheimer et al., 2016; Zhu et al., 2016).

### **1.1.4. Yeast as model organisms**

From a fundamental point of view, yeasts are model organisms for the study of higher eukaryote cell metabolism, human-cell ageing, and cancer (Botstein et al., 1997; Denoth Lippuner et al., 2014; Wassmann and Benezra, 2001), especially after the first genome sequence *S. cerevisiae* was published (Goffeau et al., 1996). This is mainly due to the genetic similarity with multicellular eukaryotes combined with the easiness of manipulating those unicellular organisms in laboratory. The ability to manipulate *S. cerevisiae* in the laboratory in a highly controlled manner allows studying of genome instability, which is a typical trait of cancer cells (Wassmann and Benezra, 2001). The short generation time of yeasts in the laboratory combined with the availability of genetic transformation tools (see Chambers and Pretorius, 2010 for review), makes of yeasts irreplaceable tools for revealing gene functions and evolution mechanisms for eukaryotes.

### **1.1.5. Yeast as “cell factories”**

The importance of yeasts is enhanced by the use of those unicellular microorganisms as “cell factories” for the production of different molecules of industrial and pharmaceutical interest with among others vanillin, insulin, and hepatitis B vaccine (Hou et al., 2012; Joan et al., 2009; Jørgen et al., 2010). Particularly interesting industrial application of yeasts from environmental

point of view is the production of ethanol, butanol, and isoprenoids that can be used as biofuel (Gírio et al., 2010; Hong and Nielsen, 2012).

Overall, the use of yeasts in beverage production and other industrial branches led to a gradual control of the fermentation processes on the behalf of people. On one hand, people are providing and modifying the environment in a way that would favour or limit the development of certain types of microorganisms. On the other hand, people would actively breed, modify, and select for organisms of interest. In industry, this is mostly driven by the idea of maintaining quality product as well as preventing production of inconsumable (and therefore non-sellable) products, thus guaranteeing optimal process efficiency. In this context, it is essential to i) know the biological material that is developing in fermentation conditions and ii) explore its behaviour in different conditions, in order to iii) be able to predict its impact on the product, and eventually adapt the used techniques according to that.

## **1.2. Yeast population genetics – an approach to study yeast evolution**

The qualitative analysis of microbial diversity of an ecosystem can be done on multiple levels – type of microorganisms (yeast, mould, bacteria), species level (for example, identify the yeast species present in a wine sample), and intraspecies level (identify the variability of strains present among the species). If we take the example of wine, studies of microbiome related to winemaking often explore the species diversity. However, many characteristics of those organisms could present high level of intraspecies variability with subsequent variable repercussions on wine qualities. In this chapter, different methods for the assessment of genetic intraspecies diversity among strains are going to be discussed with an accent on wine-related species (Table 1.1). Few of those methods have furthermore the advantage to elucidate genetic relations between strains (AFLP, MLST, WGS and SNPs analysis). In recent years, the advancements in the fields of both high throughput techniques (Next Generation Sequencing) and bioinformatics, made possible the analysis of large amount of genetic data. Further,

population genetics is the scientific enquiry that puts phenotypic variation in the context of genetic evolution (Chakravarti, 1999). Comparison between individuals and populations with their specific genetic characteristics, allows exploring the genetic bases behind phenotypic variation. Thus, population genetics brings insights into the evolutionary history of a species, and the factors playing major role in shaping its population structure (McDonald, 1997). Genetic relations between different populations are essential for the study of the evolutionary success of a species and the putative relation with human activity. Population genetics in microbiology can be studied through various methods that should have the ability to i) be discriminant, ii) reproducible, iii) and to highlight genetic relations between different genotype (or genome) profiles observed. Even if genome sequencing is the most complete and accurate method for this type of analysis, other methods have the advantage to be easier to apply and analyse, by still remaining accurate. Microsatellite markers (or SSR standing for single sequence repeats) have few very strong advantages, among others: codominance (meaning that if there are two or more different alleles present for a locus, they would be visible), neutrality (related to the fact that they are generally not subject of selective pressure), low risk of homoplasmy (they are generally specific and unique to the species), high variability among strains (Clark and Schreier, 2017; Guichoux et al., 2011; Vieira et al., 2016). Furthermore, microsatellite analyses allow the screening of a high number of isolates and establishment of genetic relations between individuals, and give indications on the ploidy level of the studied organism. Thus, the use of microsatellite analysis for population genetics studies allows covering a wide range of genetic diversity at intraspecies level.

**Table 1.1. Techniques for yeast differentiation at intraspecies level**

<b>Method<sup>a</sup></b>	<b>Species</b>	<b>References<sup>b</sup></b>
<b>CGH</b>	<i>Saccharomyces cerevisiae</i>	Dunn et al., 2005 Ayoub et al., 2006
<b>MLST</b>	<i>Saccharomyces cerevisiae</i>	Fay and Banavides, 2005 Munoz et al., 2009
<b>Karyotyping</b>	<i>Saccharomyces cerevisiae</i>	Schuller et al., 2004
<b>REA-PFGE</b>	<i>Brettanomyces bruxellensis</i>	Miot-Sertier and Lonvaud-Funel, 2007
<b>RAPD</b>	<i>Pichia guilliermondii</i>	Lopes et al., 2009
	<i>Starmerella bacilaris</i>	Tofalo et al., 2012
	<i>Saccharomyces cerevisiae</i>	Urso et al., 2008
	<i>Hanseniaspora uvarum</i>	Cadez et al., 2002
<b>RFLP</b>	<i>Saccharomyces cerevisiae</i>	Sturm et al., 2006
	Multiple species	Guillamon et al., 1998
<b>AFLP</b>	<i>Brettanomyces bruxellensis</i>	Curtin et al., 2012
	<i>Saccharomyces cerevisiae</i>	Lopandic et al., 2007 Salinas et al., 2010
<b>mtDNA</b>	<i>Saccharomyces cerevisiae</i>	Dubourdieu et al., 1987 Cubillos et al., 2009
<b>mt-RFLP</b>	<i>Saccharomyces cerevisiae</i>	Querol et al., 1992 Munoz et al., 2009
	<i>Brettanomyces bruxellensis</i>	Ibeas et al., 1996 Martorell et al., 2006
	<i>Pichia guilliermondii</i>	Martorell et al., 2006
<b>Microsatellite</b>	<i>Saccharomyces cerevisiae</i>	Legras et al., 2007 Richards et al., 2009 Almeida et al., 2015 Borlin et al., 2016
	<i>Saccharomyces kudrivzevii</i>	Erny et al., 2012
	<i>Hanseniaspora uvarum</i>	Albertin et al., 2016
	<i>Starmerella bacilaris</i>	Masneuf-Pomarede et al., 2015
	<i>Torulaspora delbureckii</i>	Albertin et al., 2014
	<i>Brettanomyces bruxellensis</i>	Albertin et al., 2014
	<i>Lachancea thermotolerans</i>	Hranilovic et al., 2017
<b>Inter-delta</b>	<i>Saccharomyces cerevisiae</i>	Legras et al., 2003 Schuller et al., 2004
<b>TRtRNA</b>	<i>Metschnikowia pulcherrima</i>	Barquet et al., 2012
<b>FT-IR</b>	<i>Hanseniaspora uvarum</i>	Grangeteau et al., 2015
<b>Genomics/SNP analysis</b>	<i>Saccharomyces cerevisiae</i>	Liti et al., 2009 Schacherer et al., 2009 Almeida et al., 2015 Gallone et al., 2016
	<i>Saccharomyces uvarum</i>	Almeida et al., 2014

<sup>a</sup>CGH (array-CGH or “microarray karyotyping”); MLST (multilocus sequence typing); REA-PGFE (restriction enzyme analysis with pulsed field gel electrophoresis); RAPD (rapid amplification of polymorphic DNA); RFLP (restriction fragment length polymorphism); AFLP (amplified fragment length polymorphism); mtDNA (mitochondrial DNA); mtRFLP (mitochondrial DNA restriction length polymorphism); TRtRNA (tandem repeat tRNA); FT-IR (fourier transform infrared spectroscopy); SNP (single nucleotide polymorphism) <sup>b</sup>(Albertin et al., 2014a, 2014b, 2016, Almeida et al., 2014, 2015, 2015; Ayoub et al., 2006; Barquet et al., 2012; Börlin et al., 2016; Cadez et al., 2002; Cubillos et al., 2009; C. Curtin et al., 2012b; Dubourdieu et al., 1987; Dunn et al., 2005; Erny et al., 2012; Fay and Banavides, 2005; Gallone et al., 2016; Grangeteau et al., 2015; Guillamón et al., 1998; Hranilovic et al., 2017; Ibeas et al., 1996; Legras et al., 2007; Legras and Karst, 2003; Liti et al., 2009; Lopandic et al., 2007; Lopes et al., 2009; Martorell et al., 2006; Masneuf-Pomarede et al., 2015; Miot-Sertier and Lonvaud-Funel, 2007; Muñoz et al., 2009; Querol et al., 1992; Richards et al., 2009; Salinas et al.,

2010; Schacherer et al., 2009; Schuller et al., 2004, 2004; Sturm et al., 2006; Tofalo et al., 2012; Urso et al., 2008)

Among unicellular eukaryotes, *S. cerevisiae* is the most broadly studied model species and this mirrors its importance as industrial yeast with high contribution to fundamental knowledge of micro- and macro-organisms. Population genetics of the species highlighted great genetic diversity and grouping according to type of industrial fermentation environment (Aa et al., 2006; Fay and Benavides, 2005; Gallone et al., 2016; Legras et al., 2007; Liti et al., 2009; Schacherer et al., 2009). Industrial fermentations are directly related to human activity; therefore this correlation is a strong indicator of domestication of *S. cerevisiae*. Indeed, population genomics studies led to the hypothesis that at least two lineages of *S. cerevisiae* population (namely, European/Wine and Sake group) were a subject of domestication (Liti et al., 2009; Schacherer et al., 2009). Precisely, wine strains were demonstrated to form a specific cluster, first by study of polymorphic sites at five unlinked loci (Fay and Benavides, 2005), and then, by microsatellite analysis (Legras et al., 2007) and genome sequencing (Liti et al., 2009; Schacherer et al., 2009). These findings were also supported by certain genetic signatures of domestication, related to human activity such as tolerance to sulphur dioxide, copper, and other chemical agents (Pérez-Ortín et al., 2002; Warringer et al., 2011). Recently, the domestication of beer *S. cerevisiae* strains was also elucidated (Gallone et al., 2016). At the beginning of the era of *S. cerevisiae* population structure analysis, natural isolates were missing. However, it was recently discovered that wild *S. cerevisiae* populations were present on oak barks and associated soil from the Mediterranean region (Almeida et al., 2015). This made it possible to compare the “wild” populations to the human-associated ones and highlighted a group of grape wine fermentation-related genes that were present among wine strains and absent among the wild oak ones (Almeida et al., 2015). This analysis confirmed the predictions of population diversification related to domestication.

### **Can a microorganism be “domesticated”?**

Domestication is a term generally used for the relationship between man and plants or animals. However, several yeast species from the *Saccharomyces* genus are also considered as domesticated by humans (see Liti, 2009, Schacherer, 2009, Libkind et al., 2011, Gallone et al., 2016; Baker et al., 2015; Almeida et al., 2015; Almeida et al., 2014; Sicard and Legras, 2011). Based on the studies cited above, there are few major characteristics that should be present for defining a species or sub-population of strains as “domesticated”: i) genetic fingerprint of domestication whose presence and expression contributes to adaptation to human-related environment; ii) phenotypic characteristics related to human activity; iii) niche specialisation or adaptation to artificial man-made environment which is related to the other two points; iv) presence of traits that are desirable for humans (e.g. production of aroma compounds of interest, fermentation efficiency, *etc.*); v) genetic distinction between human-related and natural isolates. A relevant example is a sub-group of beer *S. cerevisiae* industrial strains which are characterised by a decay of sexual reproduction, convergent evolution towards industrially favourable traits like maltotriose utilisation, stress resistance and non-production of off-flavours (Gallone et al., 2016).

Almeida et al., 2015, 2014; Baker et al., 2015; Gallone et al., 2016; Libkind et al., 2011; Liti et al., 2009; Schacherer et al., 2009; Sicard and Legras, 2011)

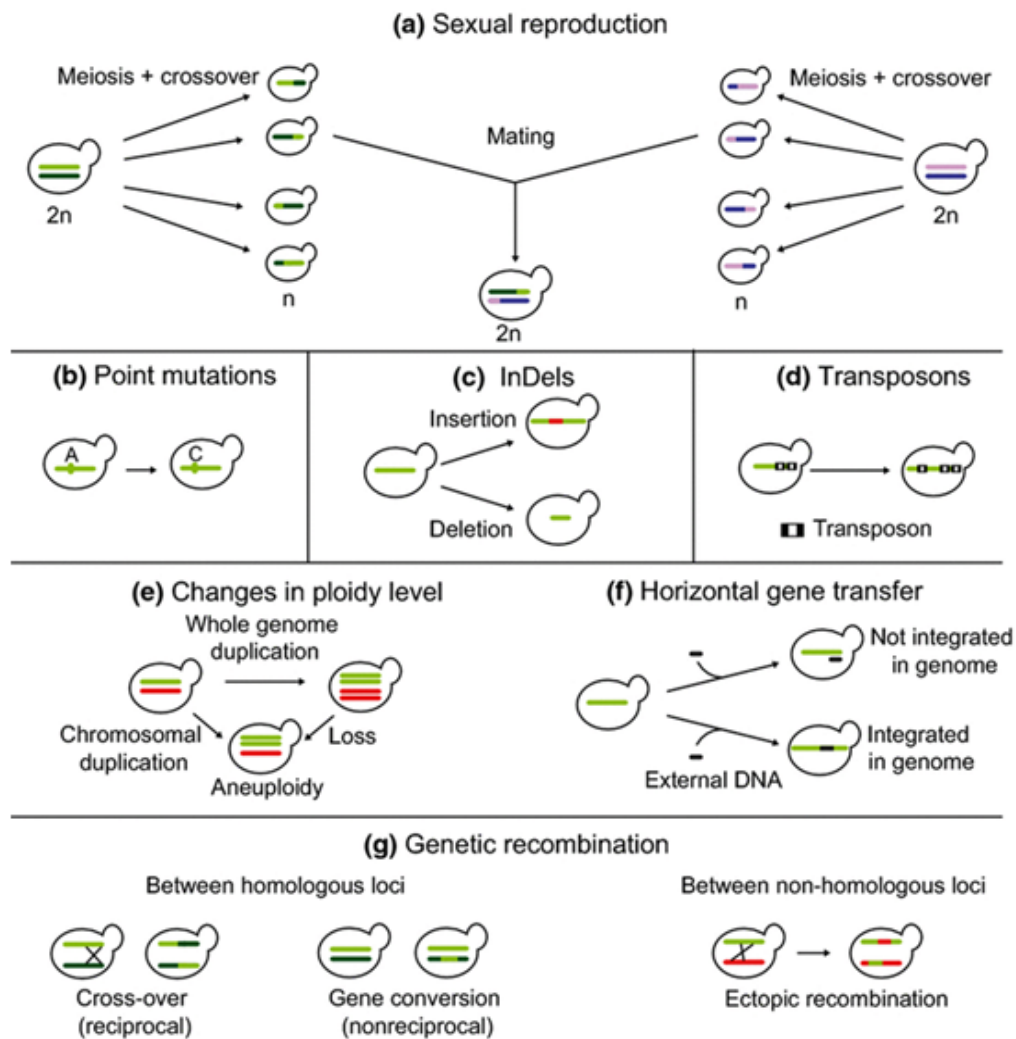
Several population genetics studies were performed on different wine yeast species that are also present in other beverages (Albertin et al., 2016, 2014b, 2014a; Hranilovic et al., 2017; Masneuf-Pomarede et al., 2015). The use of microsatellite analysis for those studies makes it possible to deduce ploidy level of the species, thus leading to hypotheses on their life cycle. Interestingly, not all studied wine-related yeast populations clustered according to the same factors as *S. cerevisiae*. For some species, populations cluster according to geographical origin (*S. bacillaris* (Masneuf-Pomarede et al., 2015), *H. uvarum* (Albertin et al., 2016)). This would suggest that the factors related to the geographical localisation are more important for those populations than factors related to the industrial fermentation environment. In other cases, niche type was demonstrated to be the determining factor for population structure. This was the

case for *T. delbrueckii* (Albertin et al., 2014a) and *L. thermotolerans* (Hranilovic et al., 2017) (both commercialised for must inoculation in combination with *S. cerevisiae*), for which a genetic differentiation between natural and industrial isolates was observed. Microsatellite genotype analysis was also applied for *Brettanomyces bruxellensis* (Albertin et al., 2014b) – wine spoilage yeast which is also isolated from other fermented products. For this species, clonal populations were shown to persist over decades in the same winery (Albertin et al., 2014b). The same study also highlighted the high dissemination of the species, as genetically close strains were detected at distant geographical locations (Albertin et al., 2014b). The significance of those population genetics studies is related to the contribution to a more holistic picture of the species, their adaptation and evolution in human-related environments.

### **1.3. Ploidy level among wine (and other) yeast of interest**

“One of the most striking features of genome structure is its lability.” (Otto, 2007)

Genetic variability is in the origin of genetic diversity and the subsequent adaptation capacity of a species. Yeast genomes can gain variability through different mechanisms including sexual reproduction (mix and shuffle of two parent genomes, single point mutations (ex. changes in single nucleotides), InDels (insertions or deletions events of relatively short pieces of DNA), transposons (mobile genetic elements that can cause mutations by insertion), genetic recombination (reorganisation of parts of the genome), or acquisition of exogenic DNA pieces by horizontal gene transfer (reviewed by Dequin and Casaregola, 2011; Steensels et al., 2014) (Figure 1.1). In the following section a particular attention will be attributed to ploidy variation as a source of genomic plasticity for few species of industrial and clinical importance.



**Figure 1.1. Origins of genetic variation in yeast.** Genetic variation can be caused by several different mechanisms. For sake of simplicity, only one chromosome per yeast cell is displayed (green or purple). Different color shades represent homologous chromosomes. In (e), a second chromosome is represented in red. (a) Sexual reproduction: after sporulation and concomitant meiotic cross-over events in the parental strains ( $2n$ ), genomes of two haploid ( $n$ ) segregants can hybridize, a process called mating. (b) Point mutations: changes in single nucleotides. These mutations can be synonymous or nonsynonymous: synonymous mutations do not change the amino acid sequence, while nonsynonymous mutations do. Nonsynonymous mutations are therefore more likely to alter the phenotype. (c) InDels: insertion and deletion events of relatively short pieces of DNA. (d) Transposons: insertion of transposable elements in the genome. (e) Changes in ploidy level: the whole genome, or large parts, is duplicated or lost, which can result in poly- or aneuploidies. (f) Horizontal gene transfer: transfer of genes by means other than regular sexual reproduction. (g) Genetic recombination: reorganization of parts of the genome. It can act on both homologous (cross-over and gene conversion) and nonhomologous loci (ectopic recombination). Homologous recombination such as gene conversion (nonreciprocal transfer of genetic material between highly homologous genes) occurs relatively frequently and can sometimes give rise to novel or modified traits. Ectopic recombination events such as TY-promoted chromosomal translocations are rarer, but can drastically rearrange the genome, and even generate novel genes. (Steensels et al., 2014)



### 1.3.1. What is polyploidy?

Polyploidy is the state of having more than one (for haploid organisms) or two (for diploid organisms) sets of chromosomes. To further discuss the incidence and impact of polyploidy, it is important to define different types of polyploidy as described in the relevant literature (see the box below).

#### **Different types of polyploidy**

**Diploidy:** The state of being diploid; that is, containing two complete sets of chromosomes (or genomes).

**Aneuploidy:** the state of having chromosome number that is not the exact multiple of the typical haploid set for a species.

**Polyploidy:** the state of having more than one (for haploid organisms) or two (for diploid organisms) complete sets of chromosomes

**Autopolyploidy:** the state of polyploidy resulting from genome doubling that arises within a species; it may involve a single individual or crossing between individuals from genetically distinct lineages within a species.

**Allopolyploidy:** polyploidy formed through the combined processes of interspecific hybridisation and mutation of chromosome number.

according to (Chen, 2010; Soltis et al., 2015)

### 1.3.2. How does polyploidy occur?

Generally, polyploidy is linked to impaired chromosome segregation that can be due to various genetic and environmental factors (Otto, 2007). Polyploidy can be achieved by inhibition of some or all aspects of mitosis in variant cell cycles (Frawley and Orr-Weaver, 2015). Thus, polyploids arise when a rare mitotic or meiotic catastrophe causes the formation of gametes with more than one set of chromosomes. Further, diploid gametes can fuse with haploid ones, and produce triploids that can either be sterile, or further give polyploid gametes (Ramsey and Schemske, 1998). Also, the fusion of diploid zygotes would result in tetraploid daughter cells.

Thus, a whole genome duplication (WGD) caused by “abnormal” cell division could lead to autopolyploidy (Peer et al., 2017). Whereas autopolyploids formation results “simply” from mutation of chromosome number, allopolyploidy is the result of concurrent hybridisation and mutation in chromosome number (Comai, 2005). Furthermore, polyploidy is prevalent among hybrid taxa which is possibly related to meiotic pairing (Otto, 2007). Diploid hybrids are prone to form unreduced gametes, which have the same number of chromosomes as the somatic cells and thus the rate of polyploids is often increased in hybrid lineages (Otto, 2007).

These phenomena generally have a fitness cost on the respective organisms due to the difficulty to maintain imbalanced chromosome number during cell division, the propensity of polyploid mitosis and meiosis to produce aneuploid cells and the associated epigenetic instability, as well as other effects related to nuclear and cell enlargement (Comai, 2005). From a structural and regulatory point of view, increasing genomic content of the cell can lead to increased nucleus and cell volume (especially in the case of somatic polyploidy which concerns multicellular organisms and is not discussed in details in this work) (Melaragno et al., 1993). Consequently, the surface to volume ratio of the cell is modified, and could lead to dosage imbalance, regulatory repercussions (Comai, 2005), and/or lower growth rate depending on the environment (Mable, 2001).

Because of those disadvantages related to polyploidy, it is generally accepted that the polyploid state is maintained only if it confers selective advantage to the cell and/or respective population in a particular environment condition (Wertheimer et al., 2016). Immediate advantages of polyploidy are related to increased genetic variation and possible changes in gene expression (especially in the case of allopolyploids resultant from diverged lineages (Otto, 2007) and epigenetic remodelling (Peer et al., 2017) (Figure 1.2, from Rancati and Pavelka, 2013). Genome plasticity of polyploids could be a result of gene redundancy (presence of the same gene in multiple copies). This phenomenon has masking effect on mutations that could

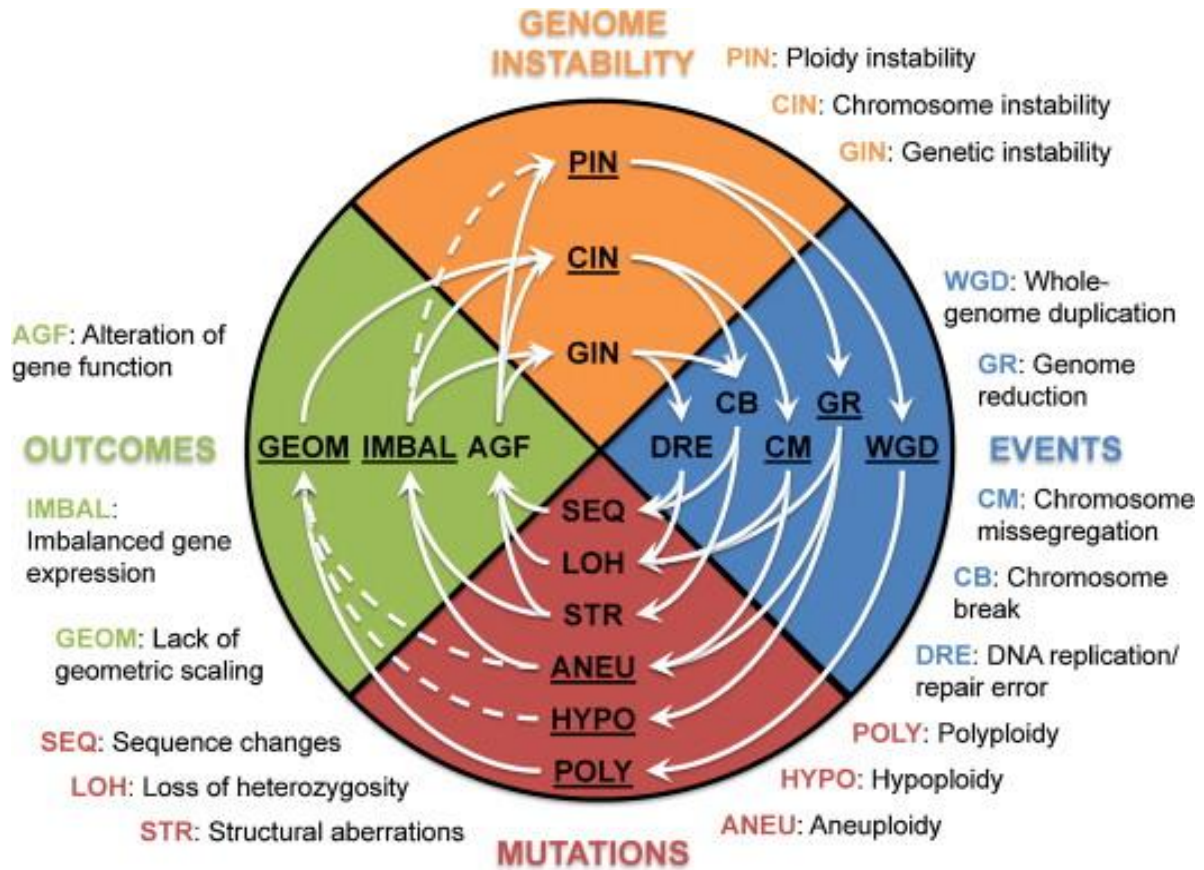
otherwise be deleterious, but also provides field for evolutionary experimentation through neo-functionalisation of the respective genes (Comai, 2005; Peer et al., 2017). In allopolyploids, successful genetic combinations could be favoured through heterosis (Otto, 2007). Thus, by changing the genomic context of certain genetic features (or genome repatterning), polyploidy can lead to increased variability. This variable genetic background is especially important for small populations which are result from bottleneck phenomena. In those cases, it would be the balance between fitness cost and survival novelty of the newly formed genetic configuration that would determine the prosperity of the polyploid lineage.

### Several famous polyploids

Actually, most of the crops that sustain humanity are polyploids (Paterson and Wendel, 2015) and this highlights the industrial importance of polyploidy, especially for plants. In many cases, characteristics of polyploids were of interest for the production managed by people and they selected for them consciously or unconsciously. Some popular examples are the potato (*Solanum tuberosum*;  $2n=4x=48$ ), bread wheat (*Triticum aestivum*,  $2n=6x=42$ ), maize (*Zea mays*;  $2n=4x=20$ ), bread wheat (*Triticum aestivum*,  $2n=6x=42$ ), upland cotton (*Gossypium hirsutum*;  $2n=4x=52$ ), oilseed rape (*Brassica napus*;  $2n=4x=38$ ), sisal (*Agave sisalana*;  $2n=5x= 80$ ), banana (*Musa*;  $2n=3x=33$ ), coffee (*Coffea arabica*;  $2n=4x=44$ ) (Chen, 2010; Leitch and Leitch, 2008).



**Sample of polyploid agricultural crops.** Showing oil from oilseed rape, bread from bread wheat, rope from sisal, coffee beans, banana, cotton, potatoes, and maize. (Leitch and Leitch, 2008)



**Figure 1.2. Karyotypic changes as catalysers of genetic variation.** Graphical representation of the potential snowballing effects of karyotype changes (underlined in the blue, Events section) on genome alterations. For instance, whole-genome duplication events leading to polyploidy result in geometric imbalances underlying chromosome instability. The ensuing chromosome missegregation events lead to loss of heterozygosity and/or aneuploidy, which in turn, through alteration of gene function and/or imbalanced gene expression, elevate all forms of genome instability, further perpetuating the vicious cycle of ever-increasing accumulation of various types of mutations. For completeness, the figure illustrates also the role of genetic instability and sequence mutations and how the various types of mutations and genome instability are intertwined with each other. Solid arrows: documented links. Dashed arrows indicate hypothetical links. (Rancati and Pavelka, 2013)

For this state to remain stable, it is required that the conditions wouldn't allow displacement by the polyploids' diploid relatives (Otto, 2007). If this condition is satisfied, the polyploid population can eventually establish leading to adaptation. Higher polyploid states are often associated with reproductive incompatibility with parent diploid cells and therefore post-zygotic reproductive isolation. Generally, adapted polyploids further enter an evolutionary path of diploidisation during which duplicated genes can be lost, retained or undergo sub- or neo-functionalisation (Comai, 2005). The increased number of gene copies can fuel new beneficial

mutations and lead to specific adaptations. Thus, polyploidisation may favour long-term diversification, evolutionary success, and possibly speciation.

### **1.3.3. Polyploidy in yeast**

In fungi, aneuploidy and polyploidy have been shown to confer selective advantage in extreme conditions, such as high osmotic pressure, presence of drugs, low temperature, and others (Albertin et al., 2009; Albertin and Marullo, 2012; Mulla et al., 2014; Todd et al., 2017; Wertheimer et al., 2016). Polyploids are often observed among yeast species in particular. Many yeast species are commensals related to humans but can become pathogenic in some occasions, especially in the case of immunocompromised patients (Odds, 1988; Todd et al., 2017; Wertheimer et al., 2016; Zhu et al., 2016). The most prevalent yeast related to fungal infections is *Candida albicans* (Wertheimer et al., 2016). This opportunistic yeast is known to have a complex life cycle with alternation of haploid and diploid populations (Hickman et al., 2013). Antibiotics from the azole family are the most popular method to treat candidiasis but cases of fluconazole resistance have often been reported among isolates (White et al., 1998). The latter antibiotic triggers an enzyme involved in the ergosterol pathway which is coded by the gene *ERG1* (see White et al., 1998 for review). It was experimentally demonstrated that resistant strains were aneuploid for a region of the left arm of chromosome 5 that contained the drug-trigger gene, as well as other genes related to drug efflux and transcription factors that positively regulate a subset of efflux pump genes (Selmecki et al., 2006). Therefore, this aneuploidy contributes to resistant phenotype by both i) higher synthesis rate of the fluconazole trigger and ii) higher drug efflux rate. Another fungal species – *Cryptococcus neoformans*, which is generally an environmental saprophyte, is also reported to lead to meningoencephalitis in humans, especially in the late 1980s when the incidence of HIV patients increased (May et al., 2016). *C. neoformans* is most often found in haploid state and can reproduce both sexually and asexually. Strikingly, during infections, *C. neoformans* cells can gain virulence through the formation of “titan cells” with ploidy ranging from  $4n$  to  $>64n$  (Feldmesser et al., 2001;

Okagaki et al., 2010) (reviewed by Todd et al., 2017). Higher ploidy of those cells is associated to volume increase and subsequent protection from phagocytosis by immune cells (Okagaki et al., 2010). Furthermore, when treated with fluconazole, polyploid strains gave rise to different aneuploid daughter cells (Gerstein et al., 2015). Thus, aneuploid formation contributes to rapid generation of diversity to *C. neoformans*. As a consequence, the population is more prone to rapidly correspond to the changes in host-related environment, leading to successful virulence.

Strikingly, recent study has demonstrated that 70% among 132 *S. cerevisiae* clinical isolates were aneu- or polyploid (Zhu et al., 2016). It was suggested that the higher ploidy level of those strains contributes to the transition from industrial to human pathogen lifestyle (Zhu et al., 2016). Indeed, previous population genomics studies suggested that *S. cerevisiae* clinical isolates originate from industry-related strains that have gained the ability to colonise human tissues (Schacherer et al., 2009). In a clinical context, genetic flexibility following polyploidisation can promote tumorigenesis in mammalian cells (Fujiwara et al., 2005). Thus, *S. cerevisiae* is an important model for polyploid behaviour and evolution. Indeed, *in vitro* evolution experiments with isogenic haploid, diploid, and tetraploid *S. cerevisiae* strains highlighted that polyploidy can, not only promote, but also accelerate adaptation (Selmecki et al., 2015). Actually, yeast polyploidy is far from being an exceptional event, as polyploidy is in the origin of the whole *Saccharomyces* genus which was the subject of whole genome duplication (WGD)(Wolfe and Shields, 1997) that occurred 100 million years ago. Among other features, WGD implied duplication of glycolytic genes (Conant and Wolfe, 2007) and subsequent enhanced sugar metabolism and ethanol make-accumulate-consume strategy (Rozpędowska et al., 2011; Thomson et al., 2005). This event coincides with the period when fleshy fruit colonised the Earth surface thus providing sugar-rich environment. Therefore, rapid sugar consumption associated with production of ethanol (which is toxic for microorganisms concurrent in the same niche), associated with ethanol accumulation and subsequent consumption, possibly gave selective advantage to *Saccharomyces* yeast (Thomson et al.,

2005). This ancient WGD event is considered a key event in *Saccharomyces* clade leading to adaptation of the species to environmental changes. Polyploid *S. cerevisiae* strains are also related to industrial environments. For instance, autotetraploid *S. cerevisiae* strains are shown to be specifically related to baking environment (Albertin et al., 2009). It was suggested that the autotetraploid state of the respective populations conferred adaptation to baking-specific conditions through high osmotic pressure tolerance and high metabolic efflux (Albertin et al., 2009). Stable *S. cerevisiae* autopolyploid populations were also isolated from millet beer (Safadi et al., 2010) and sherry-type wines (Guijo et al., 1997; Naumov et al., 2000). The occurrence of autopolyploid *S. cerevisiae* in those specific environments suggests the putative industrial interest of this genomic state, which would be related to their high metabolic flux leading to high process efficiency.

Apart from autopolyploids resulting from WGD, allopolyploid populations, and precisely hybrid species, are also encountered in human-related industrial environments. Populations resulting from hybridisation phenomena were often reported among the *Saccharomyces* genus and are wittingly or unwittingly utilised by people for the production of fermented products (Table 1.2, reviewed by Marsit and Dequin, 2015; Morales and Dujon, 2012).

**Table 1.2. Few examples of hybrid species related to wine, beer, and cider fermentations**

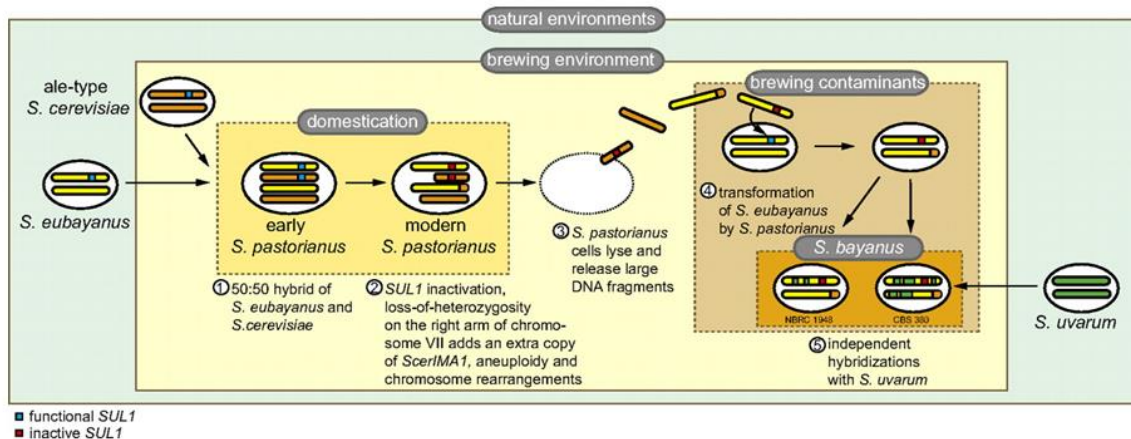
Parental species	Industrial fermentation environment	Industrial relevance	References <sup>a</sup>
<i>S. cerevisiae</i> x <i>S. kudravzevii</i>	Beer	Low-temperature fermentation; Adaptation to fluctuating conditions; Production of glycerol and aroma compounds	González et al., 2008; Belloch et al., 2008
	Wine	Efficient glucose and fructose fermentation; Ethanol production; Aromatic profile/Ester production; Low temperature fermentation	Bradbury et al., 2006; Gangl et al., 2009; Gonzalez et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998; Borneman et al., 2012; Erny et al., 2012; Schutz et al., 1994; Arroyo et al., 2009;
<i>S. cerevisiae</i> x <i>S. eubayanus</i>	Beer (Lager)	Low-temperature fermentation	Libkind <i>et al.</i> , 2011
<i>S. cerevisiae</i> x <i>S. bayanus</i>	Beer		González et al., 2008
	Wine		Naumov et al., 2000;
<i>S. cerevisiae</i> x <i>S. uvarum</i>	Wine		Le Jeune et al., 2007;
<i>S. cerevisiae</i> x <i>S. kudravzevii</i> x <i>S. bayanus</i>	Wine		Borneman et al., 2012; Gonzalez et al., 2006
	Cider		Masneuf et al., 1998; de Barros Lopes et al., 2002;

<sup>a</sup>(Arroyo-López et al., 2009; Belloch et al., 2008; Borneman et al., 2012; Bradbury et al., 2006; de Barros Lopes et al., 2002; Erny et al., 2012; Gangl et al., 2009; González et al., 2008, 2007, 2006; Libkind et al., 2011; Lopandic et al., 2007; Masneuf et al., 1998; Schütz and Gafner, 1994)

Popular example is allotetraploid *S. pastorianus* (used for the elaboration of lager-style beers) which is the result of hybridisation between *S. cerevisiae* parent and cryotolerant species that was recently elucidated to be *S. eubayanus*, and was reported to be present in natural environments in Patagonia (Libkind et al., 2011), Tibet (Bing et al., 2014), North America (Peris et al., 2014) and New Zealand (Gayevskiy and Goddard, 2016). Through the combination of ecological studies and comparative genomics, it was demonstrated that *S. eubayanus* genome sequence was 99.5% identical to the non-*S. cerevisiae* portion of *S. pastorianus*' genome and suggested related changes in sulphite and sugar metabolism that are important for lager-beer related environment (Libkind et al., 2011) (Figure 1.3). Thus, the



resultant *S. pastorianus* is well adapted to lager beer styles which are characterised by bottom fermentation at cold temperatures. Other hybrids were also elucidated in the brewing environments, and some of the ale strains also appear to be hybrids (Rainieri et al., 2008). Another type of hybrid between *S. cerevisiae* x *S. kudriavzevii* was elucidated for Trappist beer isolates (González et al., 2008) (Table 1.2). Hybrids resulting from the latter combination of parent species (*S. cerevisiae* x *S. kudriavzevii*) were also isolated from wine-related environments (Belloch et al., 2008; González et al., 2006; Lopandic et al., 2007) where they were well adapted to low-temperature fermentation (Belloch et al., 2008) and were associated with interesting ester production profile (Lopandic et al., 2007). In wine, hybrids between *S. cerevisiae* x *S. bayanus* are also encountered (Belloch et al., 2008; González et al., 2006; Lopandic et al., 2007; Masneuf et al., 1998). Strikingly, triple hybrids between the species *S. bayanus* x *S. kudriavzevii* x *S. cerevisiae* were also described in both cider (Masneuf et al., 1998), and wine (González et al., 2006). Those cases taken together suggested that establishment of hybrid populations is a common phenomenon among *Saccharomyces* yeast related to beer, wine, and cider fermentations. Interestingly, *S. bayanus* itself is considered a hybrid species with contributions from *S. uvarum*, *S. eubayanus* and *S. cerevisiae* (Libkind et al., 2011; Nguyen et al., 2000). Furthermore, even inter-family hybrid between *Hanseniaspora vineae* and *S. cerevisiae* (Cappello et al., 2010) , and *Zygosaccharomyces bailii* and *S. cerevisiae* (Novo et al., 2009) were formed through horizontal gene transfer in grape environment. The evolutionary success of hybrids originates in bringing together characteristics of two (or more) divergent species, thus leading to beneficial combination of metabolic, morphological, and genetic features, which would allow survival in specific conditions and occupation of related environments.



**Figure 1.3. A model of the formation of *S. pastorianus* and the hybrid strains of *S. bayanus*.** (Libkind et al., 2011) A model of the formation of *S. pastorianus* and the hybrid strains of *S. bayanus*. First, wild *S. eubayanus* and ale-type *S. cerevisiae* hybridized to form an allotetraploid that gave rise to *S. pastorianus*. Second, domestication imposed strong selective pressure for strains with the most desirable brewing properties. Third, in the brewing vats with high densities of *S. pastorianus*, cell lysis releases large DNA fragments that occasionally transform, fourth, contaminating wild strains of *S. eubayanus* because of the lack of pure culture techniques. Fifth, multiple hybridization events with wild strains of *S. uvarum* gave rise to CBS 380T and NBRC 1948. This model does not exclude prior or parallel involvement of *S. uvarum* in brewing or contamination.

Successful polyploid state and hybridisation related to industrial fermentation environments is not limited to the *Saccharomyces* genus. An example is the species *Brettanomyces bruxellensis*, for which triploids resulting from least two independent hybridisation events were reported (Borneman et al., 2014). Allopolyploid strains of this wine-spoilage species happen to correspond to a highly disseminated genotype among wine *B. bruxellensis* isolates from Australia, representing 92% of the total population (Curtin et al., 2007). For this species, the putative advantages of the allopolyploid state are still to be elucidated.

## 1.4. *Brettanomyces bruxellensis*

### 1.4.1. History of the species *Brettanomyces bruxellensis*

*Brettanomyces bruxellensis* is a yeast species isolated from various fermented beverages and is often associated with its wine spoilage capacity and contribution to some specialty beer aroma profile. The etymology *Brettanomyces* comes from the Greek words Brettano [British brewer]

and Myces [fungus]. This name appeared for the first time in scientific literature in the year 1904 (Claussen, 1904). It was Claussen, who first used this name to describe the species, which he attributed to the *Torula* genus and considered an essential contributor to typical aroma profile of English Ales (Claussen, 1904) (and thus, the word “*Brettano*” in the name that he has given to the species). The industrial importance of the species was underscored since its first description, as *B. bruxellensis* was the first microorganism ever to be patented (UK patent number GB190328184). Indeed, in his work Claussen insisted that “...the action of *Brettanomyces* is absolutely necessary to bring English stock beers into proper cask and bottle condition, and to impart to them that peculiar and remarkably fine flavour which in a great measure determines their value.” (Claussen, 1904). Actually, following Claussen’s report, other scientists declared that they had previously isolated yeast with similar morphology and aromatic characteristics in Kalinkin brewery in Russia and Guinness’ Chemist Laboratory in Ireland in the years 1889 and 1899 respectively (see Gilliland, 1961). Further, in 1921 Kufferath and Van Laer isolated *Brettanomyces* from Belgian Lambic beer and named it *Brettanomyces bruxellensis* (Kluyver and Custers, 1921). Lambic is the result of complex spontaneous open-tank fermentation which lasts for one to three years. During that time, multiple families, genera and strains of microorganisms act sequentially to contribute to the peculiar organoleptic characteristics of the final beverage. Indeed, the most characteristic property of Lambic beer is its aroma profile and mouthfeel that are believed to be directly related to the microorganisms involved in the fermentation, with *B. bruxellensis* being detected at the end of the process (Spitaels et al., 2014). Later on, *B. bruxellensis* was also isolated from wine (Cocolin et al., 2004; Curtin et al., 2007; Esteve-Zarzoso et al., 2001; Krumbholz and Tauschanoff, 1933; Peynaud and Domercq, 1956; Walt and Kerken, 1960; Wright and Parle, 1974). There, this microorganism was described as a spoilage factor related to high acidity and unpleasant aromas (Peynaud and Domercq, 1956). In the 1990s, *B. bruxellensis* was shown to be responsible for the production of ethyl-phenols in wine (Heresztyn, 1986; Chatonnet, 1992).

Those molecules are associated to aromatic descriptors like horse leather, barnyard, and medicinal (Chatonnet, 1992). This characteristic is the main property that defines *B. bruxellensis* as wine spoilage yeast. Additionally, it is also associated to mousiness and high acidity (Grbin and Henschke, 2000; Peynaud and Domercq, 1956). Intense research on the species *B. bruxellensis* related to wine followed after the 90s (possibly due to the fact that it was demonstrated to be involved in ethyl phenols production) with rising interest over the last 15 years (the role of *B. bruxellensis* in wine will be detailed in the next sections). Over the decades, *Brettanomyces* genus enlarged and other species were included and excluded from the yeast taxonomy books (Sam Crauwels, 2015; Steensels et al., 2015). Up to date, there are five species – *B. bruxellensis*, *B. anomalus*, *B. custerianus*, *B. naardenensis*, and *B. nanus*. The name *Dekkera bruxellensis* was also introduced for the teleomorph form of *B. bruxellensis* following the observation of spore formation reported by Walt and Kerken back in 1960. Therefore the designation *Brettanomyces/Dekkera bruxellensis* was often used in scientific publications. However, to our knowledge no further prove of sporulation was reported. This, combined with the fact that by the rules of the Melbourne code species should be designated with only one valid name, as well as the tendency to end dual nomenclature for fungi (Hibbett and Taylor, 2013) leads to the prioritisation of the name *Brettanomyces bruxellensis* for the species over the last years, even if *Dekkera* is still used by some authors. Interestingly, microbiome descriptive studies on multiple fermented food and beverages highlighted presence of *B. bruxellensis*. Isolates belonging to the species were found on grapes (Renouf and Lonvaud-Funel, 2007), in cider (Coton et al., 2006; Morrissey et al., 2004), kombucha tea (Coton et al., 2017; Teoh et al., 2004), kefir (Laureys and De Vuyst, 2014), olives (Coton et al., 2006), bioethanol production plants (Basílio et al., 2008; Beckner et al., 2011; de Souza Liberal et al., 2007; Passoth et al., 2007; Souza et al., 2012), agave fermentation for tequila production (Lachance, 1995; Lappe-Oliveras et al., 2008), soft drinks (Deak and Beuchat, 1995; Put et al., 1976; Yarrow and Ahearn, 1971), sourdough (Hammes et al., 2005; Meroth et al., 2003),

yoghourt (Kosse et al., 1997), *etc.* Particularly interesting case is the one of *B. bruxellensis* occurrence in bioethanol production plants (Basílio et al., 2008; Beckner et al., 2011; de Souza Liberal et al., 2007; Passoth et al., 2007; Souza et al., 2012). Bioethanol production through microorganism fermentation allows ethanol synthesis from organic matter, thus presenting an eco-friendly process for the production of ethanol which can be used as fuel. This process involves highly controlled large-scale fermentations mainly held by selected *S. cerevisiae* strains. The fermentation conditions are characterised by low pH, presence of inhibitor factors, abundance of complex sugars, *etc.* Even in those harsh conditions, contaminant *B. bruxellensis* strains were isolated (Basílio et al., 2008; Beckner et al., 2011; de Souza Liberal et al., 2007; Souza et al., 2012). Furthermore, they were able to displace *S. cerevisiae* population during controlled fermentation (Souza et al., 2012). Thus, even if mainly considered spoilage yeast in bioethanol production (Basílio et al., 2008; Beckner et al., 2011; de Souza Liberal et al., 2007), *B. bruxellensis* is also seen as potential microorganism for the fermentation of molasses for bioethanol production (Blomqvist and Passoth, 2015; Passoth et al., 2007). Still in the industrial context, *B. bruxellensis* was suggested as the most efficient organism among five other species for the synthesis of resveratrol (Kuo et al., 2017). Those aspects highlight the industrial impact of the yeast *B. bruxellensis*.

#### **1.4.2. Genetics of *Brettanomyces bruxellensis***

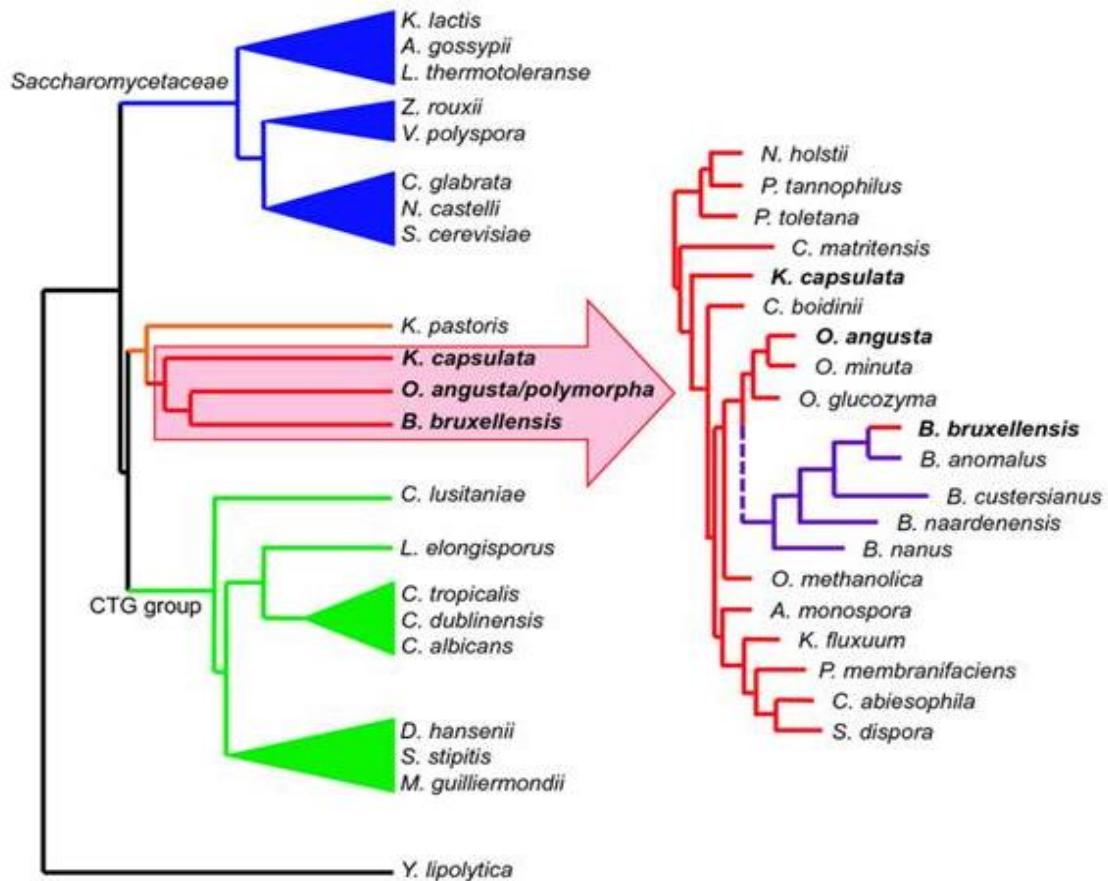
Since the first pioneer scientific articles on *Brettanomyces bruxellensis*, the words “diversity” and “variability” often accompanied the description of the species. Indeed, still in the 1960s and specifically in the wine context, Peynaud and Domercq spoke about the variability of different strains in means of sugar consumption (Peynaud and Domercq, 1956). Further, other phenotypic aspects were highlighted as variable among strains, such as growth capacity (Agnolucci et al., 2009; Barbin et al., 2008; Fugelsang and Zoecklein, 2003; Oelofse et al., 2009; Romano et al., 2008; Vigentini et al., 2008a), sugar metabolism (Conterno et al., 2006; Crauwels et al., 2017, 2015; Galafassi et al., 2011), nitrogen source utilisation (Borneman et

al., 2014; Crauwels et al., 2015), ethyl phenols production (Agnolucci et al., 2009; Conterno et al., 2006; Crauwels et al., 2017; Di Toro et al., 2015; Martorell et al., 2006; Renouf, 2009; Vigentini et al., 2008a), behaviour in viable but not cultivable state (Capozzi et al., 2016; Longin et al., 2016a), and response to abiotic factors like temperature (Barata et al., 2008; Conterno et al., 2006), pH (Blomqvist et al., 2010; Conterno et al., 2006), oxygen availability (Capusoni et al., 2016; Du Toit et al., 2005a; Uscanga et al., 2003) and sulfur dioxide (SO<sub>2</sub>) (Agnolucci et al., 2010; A. Barata et al., 2008; Conterno et al., 2006; Crauwels et al., 2017; C. Curtin et al., 2012b; Vigentini et al., 2013). To seek explanation for these variations, different scientific teams have explored the genetic diversity of the species. Despite several studies on the genetic diversity of this species using fingerprinting techniques such as Random Amplified Polymorphism DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), pulsed field electrophoresis (REA-PFGE), and mtDNA restriction analysis (Agnolucci et al., 2009; Campolongo et al., 2010; Conterno et al., 2006; Curtin et al., 2012a; Curtin et al., 2007; Di Toro et al., 2015; Joseph et al., 2013; Martorell et al., 2006; Oelofse et al., 2009; Vigentini et al., 2012), our understanding of the *B. bruxellensis* global population structure and the factors that drive it remains limited. Some of those genotypic studies suggested a correlation with geographical origin of the isolates (Conterno et al., 2006). Others highlighted correlations between genotypic profile and phenotypic characteristics (e.g. SO<sub>2</sub> tolerance and ethyl phenols production (Conterno et al., 2006)). For example, Conterno *et al.*, 2006 highlighted a particularly “dangerous” genotypic group correlated with high ethyl phenol production and SO<sub>2</sub> tolerance (Conterno et al., 2006; Curtin et al., 2012a). Meanwhile, other scientific teams were also interested in the genetic specificities of *B. bruxellensis*. Woolfit *et al.*, 2007 made a first attempt to obtain partial genome sequence of the strain CBS 2499, and highlighted a proteome enriched in transporters and genes involved in nitrogen and lipid metabolism (Woolfit et al., 2007). In this work, it was suggested that those characteristics could be related to the environment from which the isolate was obtained (namely wine) and could confer survival in

this medium characterised by low nutrient availability and high ethanol content. In this first genomic work on *B. bruxellensis*, it was suggested that this strain was haploid following the observation that compared to *Saccharomyces cerevisiae* there was a small number of lineage-specific duplicated genes (Woolfit et al., 2007). Therefore, at this stage, it was assumed that *B. bruxellensis* was a haploid species. However, very quickly this assumption was changed by another study which was published two years later by the same scientific team. Indeed, Hellborg and Piškur, 2009 discussed the high karyotype variability among 30 *B. bruxellensis* strains with different geographical origin (Hellborg and Piškur, 2009). By applying PFGE analysis (Pulsed Field Gel Electrophoresis), the authors underscored a remarkable karyotype variability (ranging from 4 to 9 chromosomes), a characteristics that is not common for eukaryotes (Hellborg and Piškur, 2009). Thus, the simple haploid organisation was excluded, and new hypothesis of polyploid state of the species arose (Hellborg and Piškur, 2009). Further confirmation of the polyploid state of the species was provided by the partial sequence analysis of five genes that showed heterozygosity and presence of different haplotypes for the same strain (Hellborg and Piškur, 2009). Those haplotypes were virtually re-grouped by their sequence similarities, and it was noticed that there was more resemblances between haplotypes from the same group but different strains, than in between haplotypes from the same strain (Hellborg and Piškur, 2009). This led to the hypothesis that hybridisation events occurred during the evolutionary history of the species (Hellborg and Piškur, 2009). This information, taken together with the high karyotype variability led to the assumption that the species was possibly asexual. Based on those findings, two major hypotheses were made on the evolutionary history of the species: i) hybridisation with closely related species led to asexuality of the progenitors and mutations accumulation in the resultant descendant population, ii) a diploid progenitor existed, that became asexual and accumulated mutations that led to high intraspecies diversity. Hints on the plausibility of those hypotheses were provided by the whole genome sequencing of few *B. bruxellensis* isolates that was published

during the following years (Piškur et al., 2012). Actually, up to date, the sequences of ten isolates from different fermentation and geographic regions are available (Borneman et al., 2014; Crauwels et al., 2014; Curtin et al., 2012; Fournier et al., 2017; Olsen et al., 2015; Piškur et al., 2012; Valdes et al., 2014). Intriguingly, the first genomic sequences of strains belonging to the species *B. bruxellensis* highlighted diploid (Piškur et al., 2012) and triploid (Curtin et al., 2012) strains. Also, *B. bruxellensis* was actually genetically distant from other food-related yeast species (Figure 1.4). Indeed, previous work highlighted that *B. bruxellensis* and *S. cerevisiae* (baker's yeast) evolved separately and their lineages separated 200 million years ago (Rozpędowska et al., 2011) (or 100 million years before the whole genome duplication (Woolfit et al., 2007) that occurred in *S. cerevisiae* lineage). However, both lineages developed, independently, similar survival strategies based on make-accumulate-consume metabolism. For *S. cerevisiae*, whole genome duplication (WGD) led to duplication of genes related to the adaptation of high-sugar environment possibly in response to the environmental changes related to the concomitant abundance of flowering plants. The fact that *B. bruxellensis* has gained the same adaptation mechanism based on make-accumulate-consume strategy without WGD makes of those two species an excellent model for convergent evolution.

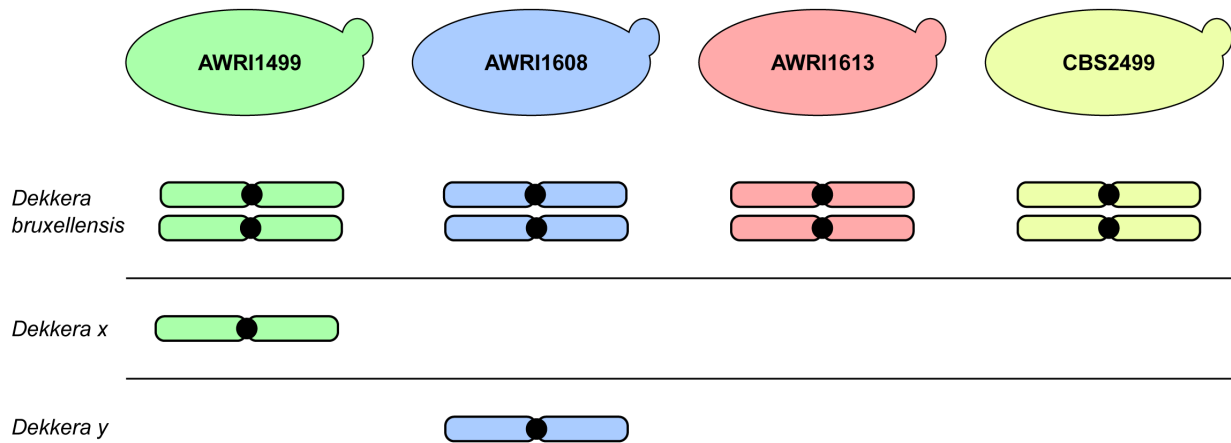




**Figure 1.4. Schematic overview of *Brettanomyces bruxellensis* phylogenetic relationship to other yeast species.** ‘Whole-genome’-based phylogenies place *B. bruxellensis* in an intermediate evolutionary group with methylotrophic species *Komagetalla pastoris*, *Kuraishia capsulata* and *Ogataea angusta/O. polymorpha* (Left). A multi-gene phylogeny expands upon the relationship between these species and places *K. pastoris* outside of the *B. bruxellensis* containing clade (Right, red branches). The relative positions of other *Brettanomyces* species have been estimated based upon a separate multi-gene phylogeny (Right, purple branches). From (Curtin et al., 2015)

Indeed, the genome sequence of the strain CBS 2499 provided insights to the mechanisms of its high ethanol production and tolerance which lays in the lineage-specific duplication of *ADH* gene-family (Piškur et al., 2012) (a trait also typical of *S. cerevisiae* but which evolved independently in *B. bruxellensis*). Those genes are also related to the synthesis of higher alcohols and aromatic esters precursors and it was suggested that this could partially be the cause of *B. bruxellensis*’ peculiar aromatic profile. Another striking feature of *B. bruxellensis*’

genome was revealed by the *de-novo* sequence assembly of the wine strain AWRI1499, which presented a triploid genome consisting of moderately heterozygous diploid genome and associated haploid genome from another closely related species (Curtin et al., 2012). Strikingly, the triploid strain was a representative of a highly dispersed and SO<sub>2</sub> tolerant genotype among Australian isolates. This led to the first hypothesis that this polyploid state could actually confer selective advantage in winemaking conditions to strains of this group (Borneman et al., 2014). It seems that this phenomenon is not only present in Australia, as strains with similar microsatellite profile were isolated from wines from France and South Africa (Albertin et al., 2014b). In the next years, few other genomes became available to the scientific community (Borneman et al., 2014; Crauwels et al., 2014; Fournier et al., 2017; Olsen et al., 2015; Valdes et al., 2014). Comparative genomics study revealed that there are at least two independent hybridisation events leading to two divergent triploid populations (Borneman et al., 2014) (Figure 1.5). This scenario is similar to the one of *Saccharomyces* genus where interspecific hybrids are often formed and combine characteristics of two parent species to confront environmental changes. Indeed, the parallel between those two lineages was underscored at the time of the first partial genome analysis published by Hellborg and Piškur (Hellborg and Piškur, 2009). Eventually, it was even suggested that it is possible that *B. bruxellensis*, rather than being one species, is actually a consortium of species (Curtin et al., 2015). However, this hypothesis remains to be discussed among the scientific community.



**Figure 1.5. Schematic representation of four *D. bruxellensis* strain genomes.** Each of the *D. bruxellensis* strains is predicted to contain a conserved diploid set of chromosomes. In addition, AWRI1499 and AWRI1608 are predicted to both contain a third full set of chromosomes that have been inherited from more distantly related strains or a closely related species that is unique to each strain (*Dekkera x* and *Dekkera y*). From (Borneman et al., 2014)

Another sequence of a strain isolated from beer became available in 2014 (Crauwels et al., 2014). This diploid beer strain was compared with the two available sequences of diploid wine strain (CBS 2499) and triploid wine strain (AWRI1499). Analysis of SNPs (Single Nucleotide Polymorphisms), CNVs (Copy Number Variations) and indels (insertions and/or deletions of genomic fragments) highlighted at least two regions that were present in the wine strains but missing in the beer isolate (Crauwels et al., 2014). Those regions were related to nitrogen and carbon metabolism and, in this work, it was suggested that they could be useful for wine-type environment. Hypothetically, presence of those regions could be associated with fitness cost in beer environment and were therefore eliminated by selective pressure in the latter (Crauwels et al., 2014). In this study, another intriguing observation was made. LSU rRNA analysis allowed the establishment of genetic relations between strains and it was interesting to notice that soft drinks isolates clustered separately from wine and beer strains (Crauwels et al., 2014). The authors therefore suggested that this was a hint for possible niche adaptation among the species *B. bruxellensis* (Crauwels et al., 2014). Indeed, this hypothesis was partially supported by phenotypic test performed with eight strains from wine, beer, and soft drink grown in different types of beverages (Crauwels et al., 2017). The results of this study reported that only wine

strains were able to grow in wine medium suggesting niche adaptation for those isolates (Crauwels et al., 2017).

Recently, another remarkable feature of *B. bruxellensis*' genome was elucidated. Apart from the differences in ploidy level between strains (Borneman et al., 2014), it was also demonstrated that the diploid strain CBS 2499 possesses “atypical” centromere loci that are prone to induce ploidy changes (Ishchuk et al., 2016). Insertions of the centromeric regions *CEN1* and *CEN2* led to ploidy shifts and phenotypic switch – development of fluffy colonies with 3 times more biofilm production (Ishchuk et al., 2016). This led to the hypothesis that this genetic feature can be related to adaptation to low-nutrient environment (Ishchuk et al., 2016) (such as wine). Furthermore, from a fundamental point of view this study demonstrated for the first time the presence of miniature inverted repeat transposable element (MITE) – genetic feature that is encountered among animals and plant species but was described for the first time in yeast. This characteristic enhances the importance of *B. bruxellensis* as model yeast species for the study of genome plasticity in eukaryote organisms. The availability of high-quality sequences that is on the path of intense development (Fournier et al., 2017; Olsen et al., 2015) will pave the way for future population genomics studies that would possibly elucidate more remarkable adaptation features of the species *B. bruxellensis*.

At present, there are some answers to the questions that were raised by the first genomic study performed back in 2007 (Woolfit et al., 2007): i) the remarkable karyotype variation could be explained by the incidence of polyploid strains and high genome plasticity related to specific centromeric loci structure leading to ploidy shifts, ii) hybridisation events were highlighted for the species and this gives indications for the putative evolutionary strategy of the species. However, the incidence of polyploid state remains to be elucidated among strains from various substrates and geographic origins, and the sexuality of the species remains an open question.

### 1.4.3. Occurrence of *Brettanomyces bruxellensis* in wine

*Brettanomyces bruxellensis* was first isolated from winemaking-related environment in the 1930s from a French wine (Krumbholz and Tauschanoff, 1933). In this work, the species was defined as *Mycotorula intermedia* but it was later re-classified as *B. bruxellensis* (Peynaud and Domercq, 1956). This case is a good example of change of species name that has occurred multiple times for *Brettanomyces* species (see Steensels et al., 2015). Here, the name *Brettanomyces bruxellensis* will be used for all species that were firstly published with other names but are re-defined as *B. bruxellensis* to date. Further report of *B. bruxellensis* isolation from grape must in France dates back to 1956, when Peynaud et Domercq analysed different *B. bruxellensis* strains from Bordeaux region for their morphology, sugar consumption, and impact on wine organoleptic properties (Peynaud and Domercq, 1956). These authors also mentioned that previous *B. bruxellensis* isolations occurred in the 1950s from highly acid wine from Italy, from Jura wine associated with yeast film developed on the surface, and a sparkling wine from Germany (see Peynaud and Domercq, 1956). They described the cells as slow growing, elongated, and not forming spores. They were associated with peculiar aromatic profile described as fruity/aldehyde-like, but also sour, acetamide-like repugnant aroma (the latter was described as “mousiness”) (Peynaud and Domercq, 1956). Four years after this work, *B. bruxellensis* was also described as being part of the wine yeasts of the Cape, South Africa (Walt and Kerken, 1960). Later on, *B. bruxellensis* was treated in relation to New Zealand wine industry (Wright and Parle, 1974), where *Brett* contamination was widespread for the vintage 1971, and interestingly it was of higher incidence among fortifying spirit production sites (Wright and Parle, 1974). Over the last decades, *B. bruxellensis* presence was further reported in Spain, Australia, USA, Chile, etc. (Agnolucci et al., 2009; Curtin et al., 2007; Ganga and Martínez, 2004; Ibeas et al., 1996). The importance of this yeast for the winemaking industry is mostly related to the production of ethyl phenols, associated with unpleasant aromas, provoking consumers’ rejection and subsequent economic loss for the producers (Loureiro and

Malfeito-Ferreira, 2003; Wedral et al., 2010). *B. bruxellensis*' spoilage potential is further enhanced by its variability, scavenger metabolism (low nutrient requirements), and tolerance to sulfur dioxide, which will be discussed in the following sections.

#### **1.4.4. Impact of *Brettanomyces bruxellensis* on organoleptic wine qualities**

In the 1990s, it was demonstrated that *B. bruxellensis* was able to convert hydroxycinnamic acids (HCAs) in ethyl phenols (EPs) (namely 4-ethylphenol and 4-ethylguaiacol, 4-EP and 4-EG) (Heresztyn, 1986; Chatonnet et al., 1992). The latter molecules are associated to descriptors like horse sweat, medicinal, barnyard, leather (Chatonnet et al., 1995a, 1992). At that time, the perception threshold for those molecules was fixed at 425  $\mu\text{g.L}^{-1}$ , and it was generally accepted that *Brett* character was not preferred by consumers (Chatonnet et al., 1995a, 1992; Curtin et al., 2015). It was demonstrated that those molecules can also affect wine taste associated to metallic characters (Lattey et al., 2010). Interestingly, previous studies showed that Beaujolais wines with ethyl phenol concentration well above the sensory threshold (around 2000  $\mu\text{g.L}^{-1}$ ) were preferred by consumers (Etievant et al., 1989). This result, however, remains an exception from the general trend, and wine with high ethyl phenols are usually not appreciated by consumers (Curtin et al., 2015). Even if EPs seem to have an important role for wine perception, the role of those molecules for the cell remains unknown. For now, the main hypothesis is that 4-EP and 4-EG synthesis is involved in i) maintaining of redox balance (Du Toit et al., 2005a; Fugelsang and Zoecklein, 2003; Liti et al., 2009), ii) detoxification through hydroxycinnamic acids conversion (Carmona et al., 2016; Harris et al., 2008), iii) attraction of insects which act as dissemination vectors (Dweck et al., 2015). Despite those hypotheses, the clear role of EPs synthesis for the cell and related population remains to be elucidated. There is also a controversy concerning the intraspecies variability for 4-EP and 4-EG production, some authors reporting that the production is the same among isolates (Curtin et al., 2013; Joseph et al., 2013) and others claiming that there is a certain degree of variability (Conterno et al., 2006; Vigentini et al., 2008b). It is neither clear whether cells which are not actively proliferating are

able to synthesise vinyl phenols and/or ethyl phenols (Agnolucci et al., 2010; Serpaggi et al., 2012) or not (André Barata et al., 2008; Dias et al., 2003; Longin et al., 2016a; Romano et al., 2008; Vigentini et al., 2008b), with different studies claiming controversial observations on the subject.

Additional to ethyl phenols production, *B. bruxellensis* has other incidence on wine organoleptic qualities. Namely, mousiness (or “goût de souris”) was firstly reported by (Peynaud and Domercq, 1956) and explored more thoroughly by (Grbin and Henschke, 2000). Furthermore, isovaleric acids related to rancid aromas (Curtin et al., 2013) and high acidity (Peynaud and Domercq, 1956; Romano et al., 2008; Vigentini et al., 2008b) were associated to *B. bruxellensis*. Interestingly, isobutyric and isovaleric were shown to have masking effect on the detection of EPs (Romano et al., 2009). Other authors suggest that the differences related to the latter characteristics are not perceptible if EPs are present above their sensory threshold (Curtin et al., 2013), which defines EPs as the major factor related to *B. bruxellensis* wine spoilage. The negative impact on the organoleptic qualities of wine is intensified by the masking effect of those molecules of the fruity aromas of wine (Tempere et al., 2016).

#### **1.4.5. *Brettanomyces bruxellensis* spoilage prevention and elimination**

##### **1.4.5.1 *Prediction and prevention methods in wine***

For a long time *Brettanomyces bruxellensis* spoilage was thought to be result of inefficient winery sanitation. Even if the correlation between wine hygiene and *B. bruxellensis* spoilage is still a controversy, it is generally recommended to keep good hygiene of the winery equipment. Nowadays, different types of software allow the prediction of putative *B. bruxellensis* spoilage. They often take into account wine physicochemical properties (ethanol content, pH, *etc.*), winemaking practices (SO<sub>2</sub> addition dose and frequency, filtration, *etc.*), and winery-related environmental factors (e.g. temperature). Thus, winemakers can evaluate a risk for *B.*

*bruxellensis* spoilage (high, low, or inexistent) and can adapt winemaking practices according to the risk of spoilage.

Prevention techniques often include adapted SO<sub>2</sub> addition (discussed below), and in some cases control of the quantity of EP precursors (Benito et al., 2009) (that is expected to lower the risk of EPs formation by *B. bruxellensis*). However, the latter technique does not lead to *B. bruxellensis* elimination from wine if it is already present.

#### **1.4.5.2 Detection of *Brettanomyces bruxellensis* in wine**

Probably the most straightforward method for *Brettanomyces bruxellensis* detection is to smell *Brett* character related to the wine. However, more precise and efficient detection methods can be used both in the winery and with the assistance of specialised laboratories.

Microscope observation is sometimes used as simple way for detecting *B. bruxellensis* but is of controversial reliability. Direct observation of wine sample by optical microscopy was believed to allow *B. bruxellensis* cells identification thanks to their specific elongated shape and the susceptibility to form pseudohyphal structures (Peynaud and Domercq, 1956). However, now it is known that *B. bruxellensis* cell morphology is of heterogeneous nature and depends on multiple factors. Thus, this type of observations should be interpreted with care. Also, if *B. bruxellensis* cells are not observed by microscope when analysing wine sample, this doesn't mean that they are not present at all for the reasons described above. In this case, it is advised to apply other detection techniques. Flow cytometry is a culture-independent method that allows quality- and quantity- analysis of resident population. Coupled with fluorescence *in situ* hybridisation (FCM-FISH) this technique becomes a valuable tool for the specific counting of *B. bruxellensis* population present in wine sample and evaluating of cell physiological state and viability (Longin et al., 2016a; Serpaggi et al., 2010). However, this method is difficult to apply in the winery and demands highly specialised equipment and manipulators.



“Classic” method for *B. bruxellensis* detection consists in plating on selective medium and enumeration of viable and cultivable population (culture-dependant method). Different types of media were developed for the selective isolation of *B. bruxellensis* containing antibiotics eliminating other microorganisms (moulds, bacteria, *Saccharomyces*, and non-*Saccharomyces* yeasts) and/or containing p-coumaric acid as EP precursor and which would help identification by aroma detection by the person performing the analysis. This method has the advantage to be relatively cheap, and easy to apply by untrained personnel. However, *B. bruxellensis* being a slow-growing organism, the response is obtained after 5-10 days and thus does not allow immediate reaction to the contamination. Another recurrent problem of culture-dependant detection of *B. bruxellensis* is the fact that this species can enter into a viable but non cultivable (VBNC) (Agnolucci et al., 2010; Capozzi et al., 2016; Longin et al., 2016a; Serpaggi et al., 2012), and therefore cells could be present without being detected on selective medium. The spoilage potential of VBNC cells will be discussed further. Culture independent methods are possible alternative to enumeration on selective medium. These techniques are based on the amplification of *B. bruxellensis* species specific DNA fragments by PCR reaction. Species-specific PCR can be only qualitative (showing *B. bruxellensis* presence or absence in the sample), or quantitative (qPCR) giving an idea of the population level in the sample (Longin et al., 2016b; Phister and Mills, 2003; Tessonière et al., 2009). Another technique consists in cells detection through plasmon resonance biosensors (Manzano et al., n.d.). The advantage of those methods is mainly the time efficiency – a sample can be taken and the result can be obtained in few hours. However, special equipment and trained personnel are needed for the test application. Furthermore, those techniques have the disadvantage to sometimes detect DNA of cells that are inactive or dead, possibly leading to false positives.

#### **1.4.5.3 Elimination methods for *Brettanomyces bruxellensis***

When prevention methods weren't applied or were inefficient, it is possible to act against the already present *B. bruxellensis* population. Elimination methods can be roughly grouped in

chemical and physical methods. Further, physical methods can act by directly removing the cells present in the wine, or by altering their physiological state.

The most broadly used chemical method is sulfur dioxide (SO<sub>2</sub>) addition. Efficient SO<sub>2</sub> utilisation includes both efficient dose and frequency of SO<sub>2</sub> application to must and wine. The major advantage of this method is the fact that SO<sub>2</sub> also has other beneficial properties on wine quality as an antioxidant and antioxidasic agent (meaning that it inhibits the action of oxidation enzymes like laccase) (Ribéreau-Gayon et al., 2017). Furthermore, it is a popular and cheap method that is easy to apply. However, sulfur dioxide overdose could have repercussions on wine organoleptic qualities and its concentration in final wine is a subject of regulation. Thus, it is important to determine the optimal SO<sub>2</sub> dose in order to obtain efficacy without interfering with wine quality. When deciding SO<sub>2</sub> adjustment, pH and ethanol content of the wine should be taken into account. An additional challenge is the variability among strains in means of SO<sub>2</sub> sensitivity. The latter two aspects will be discussed in details in further sections. Other additives used in winemaking are chitosan – a hydrophilic biopolymer with antimicrobial properties (Petrova et al., 2016; Taillandier et al., 2015), and DMDC (dimethyl dicarbonate) (Delfini et al., 2002). Killer toxins produced by non-*Saccharomyces* yeast were also studied over the last few years and their effect on *B. bruxellensis* and possible application in winemaking remains to be investigated (Comitini and Ciani, 2011; Mehlomakulu et al., 2014, 2015)

Physical removal of *B. bruxellensis* cells can be done by filtering (Duarte et al., 2017; Renouf, 2009; Umiker et al., 2013). The choice of filter can be done according to the material of the membrane and its porosity – often 1 or 0.45 µm (and more rarely 0.22 µm) of diameter. This method has the advantage to be easy to apply and the possibility to be coupled with chemical techniques like SO<sub>2</sub> addition. However, it should be taken into account that *B. bruxellensis* cell size has been shown to be variable depending on the physiological state (Serpaggi et al., 2012).

Thus, it is possible that some filters would still allow the passage of some cells that could give rise to higher population level later on. Apart from filtration, other physical methods can be used, such as pulsed electric field (Delsart et al., 2016), low electric current (Lustrato et al., 2010), high hydrostatic pressure (González-Arenzana et al., 2016), high temperature treatment (Fabrizio et al., 2015), high power ultrasonics (Luo et al., 2012), and others. All these methods have the advantage to avoid chemicals addition to wine. However, their efficacy, price, and application mode should be further studied in order to expand their use in the wine industry.

#### **1.4.5.4 Post-spoilage curative methods**

One of the biggest problems concerning *B. bruxellensis* is the fact that often winemakers become aware of the contamination when EPs are already produced above the olfactory detection threshold. Unfortunately, in those cases, even if the contaminant population is removed by one of the methods cited above, the *Brett* aroma character remains present in the wine. To address this problem, a few techniques were developed for the removal of EPs from wine. Those methods are based on reverse osmosis for the removal of EPs from wine (Ugarte et al., 2005) or addition of polymers that bind selectively to EPs (Carrasco-Sánchez et al., 2015; Garcia et al., 2015; Teixeira et al., 2015; Larcher et al., 2012). Some authors even suggested that there was a post-bottling solution to the problem of EPs in wine, showing that suberin in cork closure has an EP-binding capacity (Gallardo-Chacón and Karbowski, 2015). However, those methods need further validation and do not solve the problem with the contamination itself, which means that *B. bruxellensis* could develop in the winery during the next vintages.

To sum up, different methods for prevention, detection, and elimination of *B. bruxellensis* are available on the market. However, sulfur dioxide addition remains undoubtedly the most broadly used antimicrobial agent in winemaking.

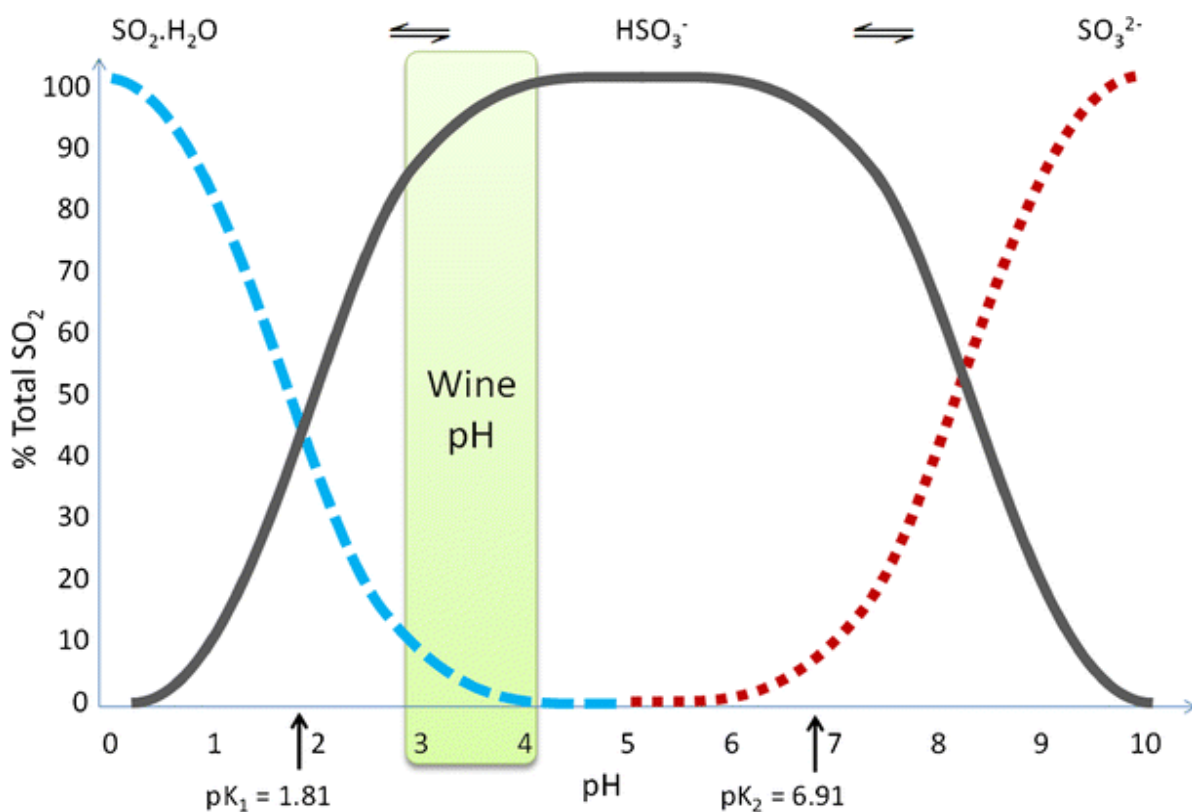
### 1.4.5.5 Focus on sulfur dioxide use in wine

#### 1.4.5.5.1 Use of sulfur dioxide in wine

Sulfur dioxide is the most broadly used antimicrobial agent in wine. Its success is related to multiple beneficial physicochemical properties when it comes to wine quality. Sulfur dioxide has not only antimicrobial, but also antioxidant, and antioxidasic effect on wine (Pascal Ribéreau-Gayon et al., 2017). The reactive molecule of SO<sub>2</sub> actually binds to some oxidases like tyrosinase and laccase that could otherwise be detrimental for wine quality. Despite the beneficial properties of SO<sub>2</sub>, its use should be done with care, as excessive SO<sub>2</sub> addition could have negative effect on wine aroma and colour intensity (Bakker et al., 1998; Pascal Ribéreau-Gayon et al., 2017). As a chemical additive, SO<sub>2</sub> (often added in wine as potassium metabisulphite solution or sulfur tablets that are burnt in the barrels) is a subject of regulation. Thus, the final doses of total sulfur dioxide in wine for most European countries are 150 mg.L<sup>-1</sup> for red wines, 200 mg.L<sup>-1</sup> for white and rosé wines, 300 mg.L<sup>-1</sup> for red, white, and rosé wines with more than 4 g.L<sup>-1</sup> of reducing substances, and 400 mg.L<sup>-1</sup> for sweet wines (OIV, Office Internationale de la Vigne et du Vin, 2015 <http://www.oiv.int/public/medias/4086/e-coei-1-soudio.pdf>). The legislation often refers to total form of sulfur dioxide due to the variability of free and bound forms according to the physicochemical properties of the wine (described below).

The most popular shapes of sulfur dioxide addition are the burning of sulfur tablets for barrel sanitation and the addition of potassium metabisulfite solution (K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> or PBS). In aqueous solution, sulfur dioxide is present in three different chemical species depending on the pH of the solution – bisulphite ion (HSO<sub>3</sub><sup>-</sup>), molecular SO<sub>2</sub>.H<sub>2</sub>O, and sulphite ion SO<sub>3</sub><sup>-</sup> (Figure 1.6). The chemical equilibrium between those species is dependent on pH and at the moderately acidic pH of wine (around pH 3.5) the most abundant SO<sub>2</sub> form is HSO<sub>3</sub><sup>-</sup> (Figure 1.6). In wine, the balance between those two forms is further complicated by the fact that SO<sub>2</sub> can bind

different molecules present in the medium like acetaldehyde, glutathione, glucose, anthocyanins, *etc.* Thus, in the winemaking context, several different forms should be differentiated: i) bound form of  $\text{SO}_2$  – the one comprising  $\text{SO}_2$  linked to other molecules, ii) free  $\text{SO}_2$  – the fraction of  $\text{SO}_2$  that is not bound to other molecules, and iii) molecular  $\text{SO}_2$  that is part of the free  $\text{SO}_2$  and represents the fraction of free  $\text{SO}_2$  that is in neutral  $\text{SO}_2 \cdot \text{H}_2\text{O}$  form. The combination of bound and free  $\text{SO}_2$  is referred to as total  $\text{SO}_2$ . From a microbiological point of view, the molecular  $\text{SO}_2$  ( $\text{mSO}_2$ ) is the one that has antimicrobial action (Du Toit et al., 2005a; Macris and Markakis, 1974). In practice, the free  $\text{SO}_2$  can be measured *via* titration methods, and further the  $\text{mSO}_2$  fraction can be deduced from the measured free  $\text{SO}_2$  concentration according to the wine's pH, temperature, and alcohol content ( $\text{mSO}_2$  fraction decreases with higher pH, lower ethanol content, and lower temperature).



**Figure 1.6.  $\text{SO}_2$  species in aqueous solution and their representation in percentage of total  $\text{SO}_2$  throughout the pH range.**  $\text{pK}_1$  is the dissociation constant of the chemical reaction  $\text{SO}_2 \cdot \text{H}_2\text{O} \leftrightarrow \text{HSO}_3^- + \text{H}^+$  and  $\text{pK}_2$  that of the reaction  $\text{HSO}_3^- \leftrightarrow \text{SO}_3^{2-} + \text{H}^+$ . The effective pH range of wine is highlighted. From (Divol et al., 2012)

The antimicrobial action of  $\text{mSO}_2$  is considered to occur on multiple levels. Molecular  $\text{SO}_2$  is a small molecule with no charge that can enter the cell passively (Stratford et al., 1987), or *via* specific membrane transporters (Macris and Markakis, 1974; Park and Bakalinsky, 2004; Pilkington and Rose, 1988). Since the first contact with the cell, it could lead to morphological and physiological changes linked to the binding to cell wall and membrane components (Anacleto and van Uden, 1982). Another morphological effect on the cell, induced by  $\text{SO}_2$  is cell disruption and subsequent leakage of metabolites, as well as possible binding and inactivating of the membrane ATPases leading to decrease in intracellular ATP concentration (reviewed by Divol et al., 2012). Once inside the cell, the molecule of  $\text{SO}_2$  faces a change in pH (the intracellular pH being around 6.5). Therefore, the fraction of  $\text{mSO}_2$  lowers, whereas  $\text{HSO}_3^-$  and  $\text{SO}_3^-$  concentrations increase (Figure 1.6). Subsequently, to attain osmotic balance between different  $\text{SO}_2$  species, more  $\text{mSO}_2$  molecules can enter the cell from the extracellular environment. Inside the cell, bisulphite ion ( $\text{HSO}_3^-$ ) can block essential metabolic pathways through binding with various cell metabolites. Among others,  $\text{SO}_2$  can bind to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) a key enzyme of the glycolysis (Hinze and Holzer, 1986), leading to lower ATP generation and a decrease of the energy available for the cell. Sulfur dioxide can also bind to acetaldehyde (Rankine and Pocock, 1969), consequently blocking the synthesis of ethanol and therefore decrease of NADH regeneration rate. Furthermore,  $\text{SO}_2$  can bind to metabolism substrates like glucose, leading to lower nutrients available for the cell (Divol et al., 2012). Finally,  $\text{SO}_2$  has been reported to act on genetic level by causing point mutations, thus altering cell function (Shapiro, 1977).

To sum up,  $\text{SO}_2$  is an essential contributor quality in conventional winemaking with beneficial effects on multiple levels. Its antimicrobial activity is undisputable but complex phenomenon. However, some yeast species have managed to develop tolerance to  $\text{SO}_2$ . The related mechanisms have been thoroughly studied for *Saccharomyces cerevisiae* and will be reviewed in the following section.

#### 1.4.5.5.2 Sulfur dioxide tolerance in *Saccharomyces cerevisiae*

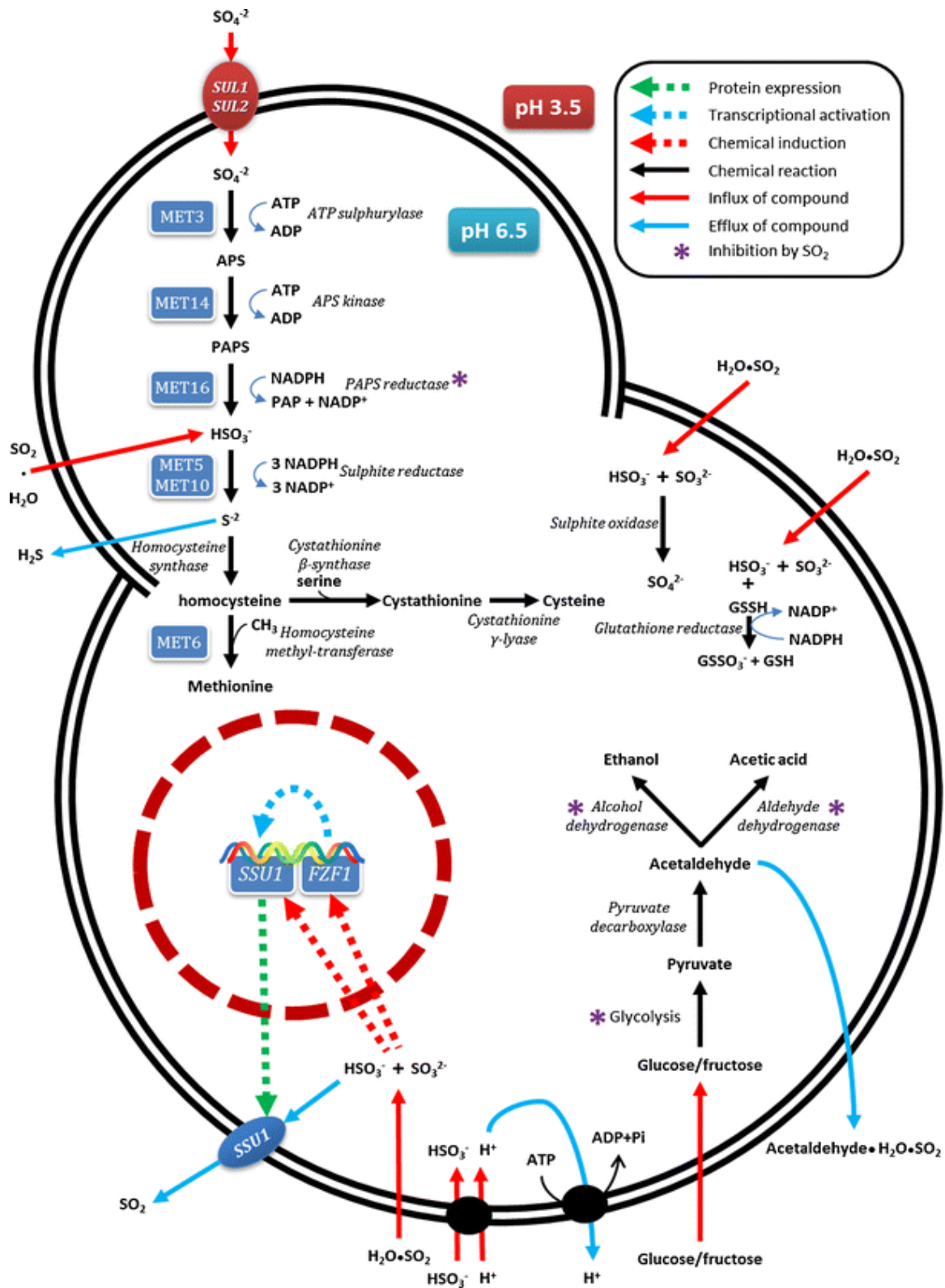
Nowadays, *Saccharomyces cerevisiae* is often inoculated to wine must because of its high fermentation efficiency (sugar to ethanol yield), and capacity to consume all the sugars present in the must without residuals. This property is essential in winemaking because even low quantities of residual sugars can present source of nutrients for microorganisms during wine storage, and lead to subsequent wine spoilage. Thus, the capacity to tolerate SO<sub>2</sub> without repercussions on cell metabolism is an essential element for the selection of commercial *S. cerevisiae* strains for wine production. Subsequently, this characteristic has been thoroughly studied for this species.

As described in the previous section, SO<sub>2</sub> acts on multiple physiological, morphological, and genetic levels. Therefore, it is expected that SO<sub>2</sub> tolerance would be the result of different cell properties combined together. Indeed, there are various mechanisms that have been described in the literature for being related to SO<sub>2</sub> tolerance. Briefly they can be grouped in i) sulfur reduction mechanisms, ii) sulfur oxidation, iii) sulphitolysis, and iv) active efflux of SO<sub>2</sub> molecule (reviewed by Divol et al., 2012 and represented on Figure 1.7). Reduction mechanisms for coping with high concentrations of SO<sub>2</sub> involve utilisation of HSO<sub>3</sub><sup>-</sup> ions in the sulphur amino acid biosynthesis (SAAB) pathway. As shown on Figure 1.7, HSO<sub>3</sub><sup>-</sup> ions are intermediaries in this pathway; therefore increased SAAB can be a way to utilise HSO<sub>3</sub><sup>-</sup> ions and make them beneficial for the cell metabolism. SAAB pathway is downregulated by the concentration of the final products of the reaction, like methionine, and it was indeed demonstrated that higher concentration of methionine was associated to lower SO<sub>2</sub> resistance of yeast cells (Aranda et al., 2006). Sulfur dioxide oxidation is a mechanism that has been described in higher eukaryotes but for now has not been elucidated in yeast. Similarly, sulphitolysis of bisulphite anions through chemical reaction with the oxidised form of glutathione is a controversy and has not been clearly demonstrated for unicellular eukaryotes (Divol et al., 2012). However, yeast cells can have another “trick” to cope with the excess of

intracellular SO<sub>2</sub> – acetaldehyde (ethanal) synthesis. Acetaldehyde production is particularly interesting in the context of winemaking, as this molecule has strong impact on wine organoleptic qualities being associated with bruised-apple off-flavour when present at high concentrations. Acetaldehyde is strongly reactive molecule and can therefore bind SO<sub>2</sub> (as well as other molecules present in wine). However, demonstrating whether acetaldehyde is a response to or consequence of SO<sub>2</sub> intoxication, is a challenge (Divol et al., 2012).

The most studied (and probably most efficient) trick of the cell for coping with SO<sub>2</sub> intoxication is the active efflux through a specific plasma membrane pump – Ssu1p (Park and Bakalinsky, 2004). This mechanism was thoroughly explored over the last 15 years, and some evolutionary pathways of its acquisition were highlighted. Overall, the *SSU1* gene was shown to exist in at least two alleles, one of which is (*SSU1-R*) related to resistance and is subject of high heterozygosity between strains (Aa et al., 2006). Actually, this allele was shown to be the result of specific translocation between chromosomes VIII-XVI (Nardi et al., 2010; Pérez-Ortín et al., 2002). Furthermore, this translocation has been shown to have adaptive importance to winemaking conditions (where SO<sub>2</sub> is broadly used), as it was very common for wine strains and missing in *S. cerevisiae* strains from other industrial fermentation environments (Pérez-Ortín et al., 2002). Surprisingly, another translocation event of similar nature was also highlighted between chromosomes XV and XVI (Zimmer et al., 2014). Again, this translocation was specific to wine strains and showed to confer selective advantage in presence of SO<sub>2</sub> *via* shorter lag phase compared to other strains. The latter study highlighted that genome plasticity and namely translocation events are important evolutionary element for the SO<sub>2</sub> tolerance of *S. cerevisiae* wine strains.





**Figure 1.7. A summary of the sulphate assimilation pathway and the cellular and molecular responses of *S. cerevisiae* to the presence of  $\text{SO}_2$ . SAAB sulphur amino acid biosynthesis, SR sulphur reduction (Divol et al., 2012)**

Several authors highlighted a general cell response to  $\text{SO}_2$ , described as viable but not cultivable (VBNC) state. VBNC is a common term used for bacteria but it was only recently

that it started to be used for unicellular eukaryote organisms as well. VBNC describes a state for which cells are viable but don't grow on culture medium as a result of stress-response to environmental factor. Particular trait of VBNC is the reversibility of this state meaning that the cells are able to "resuscitate" if the stress-factor is removed from the medium. Here, the word "reversible" is important, as VBNC state is demonstrated *via* experimental inducing of this state but should also involve resurrection when the stress agent is removed. Indeed, it was suggested that *S. cerevisiae* enters into a VBNC state after SO<sub>2</sub> addition and was able to re-gain cultivability when mSO<sub>2</sub> fraction was lowered in the medium through pH increase. The existence of the VBNC state is important in oenological context as it could lead to false negatives when microbiological analysis is performed through culture-dependant methods. Particularly, the importance of VBNC state was highlighted for *B. bruxellensis* – a species that is characterised by variable SO<sub>2</sub> tolerance.

## Resistance/Tolerance

When referring to survival strategies of microorganisms in presence of SO<sub>2</sub>, both the terms “tolerance” and “resistance” are often used without clearly defining the meaning behind them. The same ambiguity is encountered among clinical scientific studies<sup>a</sup>, where usually, tolerance is related to survival, whereas resistance refers to the capacity to actively grow at the presence of an antibiotic (which is often associated to specific molecular mechanisms and is inherited). Typical method for defining efficient drug dose for the eradication of resistant microorganisms is MIC (minimum inhibitory concentration)<sup>a,b</sup>. However, this method is not well adapted to tolerant microorganisms, as they are able to survive (even if not actively growing) at transient drug concentrations higher than the MIC. Recently, MDK (minimum duration for killing) was suggested as quantitative indicator for tolerance<sup>b</sup>. In clinical context, those differences are important as they can lead to a misclassification of tolerant pathogens as resistant or vice versa, subsequently leading to inefficient treatment. In the context of winemaking and the use of SO<sub>2</sub> as antimicrobial agent in particular, these terms are even more difficult to define, as the kinetics of different forms of SO<sub>2</sub> is complex (Figure 1.6) and highly variable in the wine matrix. The molecular form of SO<sub>2</sub> (mSO<sub>2</sub>) constantly varies in wine due to SO<sub>2</sub> additions and re-adjustments, wine practices related to contact of wine with oxygen, binding of SO<sub>2</sub> to various molecules present in wine, and other physicochemical variations. Therefore, the definition of MIC or MDK is difficult in these conditions, because they are variable according to wine characteristics and wine storage conditions. However, it is important to keep in mind that different mechanisms at the cellular and population level could be in the origin of the survival and growth of spoilage of microorganism in the presence of SO<sub>2</sub>. Subsequently, related microorganisms should potentially be treated with different concentration and frequencies of antimicrobial addition.

<sup>a</sup>(Rex et al., 1995), <sup>b</sup>(Brauner et al., 2016)

### 1.4.5.5.3 Sulfur dioxide tolerance in *Brettanomyces bruxellensis*

Generally, non-*Saccharomyces* yeast species are considered as SO<sub>2</sub> sensitive, meaning that they are not viable after SO<sub>2</sub> addition in winemaking conditions. However, some cases of SO<sub>2</sub> tolerant species have been recently discussed namely for *Zygosaccharomyces bailii* and *Brettanomyces bruxellensis* (Zuehlke et al., 2013). *B. bruxellensis* presents a major problem for

winemakers all over the world and is broadly discussed, especially over the last two decades. Subsequently, intense research was done on those properties of *B. bruxellensis* that are directly related to winemaking spoilage on one hand, and survival of this yeast in winemaking conditions, on the other. Interestingly for scientists, but sadly for winemakers, *B. bruxellensis*' tolerance to SO<sub>2</sub> seems highly variable. Indeed, different studies report concentrations ranging from 0.2 to 1.1 mg.L<sup>-1</sup> for preventing *B. bruxellensis*' growth (Curtin et al., 2015). Further, those variations were defined as strain-dependant, and in some cases were linked to genotype. Conterno et al., 2006 highlighted that among several groups defined by 26S rDNA analysis, one was with particularly high spoilage potential because of its survival rate in presence of SO<sub>2</sub> and ethyl phenols (EPs) production profile. Another striking example is the study of Curtin et al., 2012a which demonstrated that the most prevalent genotype among Australian isolates (as defined by AFLP analysis), was also associated with high tolerance to SO<sub>2</sub> (>0.6 mg.L<sup>-1</sup> mSO<sub>2</sub>). It was further shown that a strain from this genotypic group was actually an allopolyploid (triploid to be precise) resulting from interspecific hybridisation event with a species closely related to *B. bruxellensis*. This led to a hypothesis for revealing the mechanisms behind *B. bruxellensis*' variable tolerance to SO<sub>2</sub>, as strains that were SO<sub>2</sub> sensitive had different genetic configuration. Indeed, it was suggested that the triploid state of AWRI1499 strain could be involved in the tolerance to SO<sub>2</sub>. Furthermore, it was recently demonstrated *via* microsatellite analysis, that triploid strains from the same genotypic group as AWRI1499 are also present in France and South Africa (Albertin et al., 2014b) highlighting that the occurrence of this genotype is not limited to Australia. To support the hypothesis of SO<sub>2</sub> tolerance for the isolates that are genetically close to AWRI1499 it would be interesting to correlate genotype and phenotype for more strains from various origins.

Overall, variability of SO<sub>2</sub> tolerance for the species *B. bruxellensis* presents a problem for winemakers, as doses which are efficient and at the same time not excessive are difficult to define. The issue is even more complex than that, as initial population level was also

demonstrated to have a significant effect on SO<sub>2</sub> efficacy with higher initial population levels leading to a decrease of SO<sub>2</sub> efficacy (Longin et al., 2016a).

Interestingly, similarly to *S. cerevisiae*, VBNC state was reported for *B. bruxellensis*. It was first suggested by du Toit *et al.* who observed an initial loss of cultivability after SO<sub>2</sub> addition, followed by growth recovery after oxygen administration (Du Toit et al., 2005a). Intrigued by those observations, and the recurrent problem of SO<sub>2</sub> tolerance of *B. bruxellensis*, other teams studied the phenomena of cultivability loss and growth recovery associated to SO<sub>2</sub>. Agnolucci *et al.* measured both viability *via* trypan blue cell staining, and cultivability on agar plates after SO<sub>2</sub> exposure for seven different strains (Agnolucci et al., 2010). Based on those experiments, they claimed that different concentrations of sulfur dioxide were necessary for the induction of VBNC state among strains, and reported the production of vinyl phenols during this putative metabolically inactive state (Agnolucci et al., 2010). Later, Serpaggi *et al.* reported ethyl phenol production during VBNC state in *B. bruxellensis* (Serpaggi et al., 2012). Proteomics analysis of the VBNC state was performed in the context of this study, and the difference in proteins related to glycolytic flux and redox balance was shown to be modified as a result from SO<sub>2</sub> exposure (Serpaggi et al., 2012). The main role of redox balance- and carbon catabolism-related genes expression associated with VBNC state in *B. bruxellensis* was further confirmed by transcriptomics analysis (Capozzi et al., 2016). This approach also demonstrated a true “resuscitation” of the cells (Capozzi et al., 2016) after stress removal induced by pH increase. The resuscitation rather than re-growth of small part of the population was supported by the observed repression of DNA replication genes during VBNC state (Capozzi et al., 2016). This study furthermore reported the intraspecies variability among strains concerning the entry in VBNC. Whereas EP production by cells in VBNC was observed by Serpaggi *et al.*, another study claimed the lack of EP synthesis during this state (Longin et al., 2016a). The latter also evaluated the state of the cell membrane after SO<sub>2</sub> exposure and highlighted increase cell membrane permeability related to this stress factor (Longin et al., 2016a).

Overall, the current knowledge on *B. bruxellensis* SO<sub>2</sub> tolerance phenomenon reveals great variability among strains. This characteristic renders the definition of the optimal and efficient SO<sub>2</sub> dose for *B. bruxellensis* spoilage prevention a challenge. Promising link between SO<sub>2</sub> tolerance and genotype was underscored by the screening of 41 *B. bruxellensis* isolates from Australia, highlighting a widely spread SO<sub>2</sub> tolerant genotype among the continent (Curtin et al., 2012a). The presence of this genotypic family was further shown to have specific genetic configuration – triploid state resultant from hybridisation event, and isolates from this genetic group were furthermore detected in France and South Africa (Albertin et al., 2014b). The link between genotype and phenotype is therefore a potential way to evaluate SO<sub>2</sub> tolerance among *B. bruxellensis* and merits further exploration at finer scale.



## **Chapter 2. *Brettanomyces bruxellensis* population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution**

Marta Avramova<sup>1,8,\*</sup>, Alice Cibrario<sup>1</sup>, Emilien Peltier<sup>1</sup>, Monika Coton<sup>2</sup>, Emmanuel Coton<sup>2</sup>, Joseph Schacherer<sup>3</sup>, Giuseppe Spano<sup>4</sup>, Vittorio Capozzi<sup>4</sup>, Giuseppe Blaiotta<sup>5</sup>, Franck Salin<sup>6</sup>, Marguerite Dols-Lafargue<sup>1,7</sup>, Paul Grbin<sup>8</sup>, Chris Curtin<sup>9</sup>, Warren Albertin<sup>1,10</sup>, Isabelle Masneuf-Pomarede<sup>1,11</sup>

<sup>1</sup>Univ. Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, 33140 Villenave d'Ornon, France <sup>2</sup>Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France <sup>3</sup>Université de Strasbourg, Centre National de la Recherche Scientifique, Génétique Moléculaire, Génomique, Microbiologie, Unité Mixte de Recherche, 7156, Strasbourg, France <sup>4</sup>Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy <sup>5</sup>Department of Agricultural Sciences, Division of Vine and Wine Sciences, University of Naples Federico II, Viale Italia - 83100 Avellino (Italy) <sup>6</sup>INRA, UMR Biodiversité Gènes et Ecosystèmes, PlateForme Génomique, 33610 Cestas, France <sup>7</sup>Bordeaux INP ISVV EA 4577, F-33140 Villenave d'Ornon, France <sup>8</sup>School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia <sup>9</sup>Department of Food Science and Technology, Oregon State University, 100 Wiegand Hall, Corvallis, Oregon 97331-6602, USA <sup>10</sup>ENSCBP, Bordeaux INP, 33600 Pessac, France <sup>11</sup>Bordeaux Sciences Agro, 33170 Gradignan, France

Status: Submitted in *Scientific Reports*





## 2.1. Abstract

*Brettanomyces bruxellensis* is a unicellular fungus of increasing industrial and scientific interest over the past 15 years. Previous studies revealed high genotypic diversity among *B. bruxellensis* strains as well as strain-dependent phenotypic characteristics. Genomic assemblies revealed that some strains harbour triploid genomes and based upon prior genotyping it was inferred that a triploid population was widely dispersed across Australian wine regions. We performed an intraspecific diversity genotypic survey of 1488 *B. bruxellensis* isolates from 29 countries, 5 continents and 9 different fermentation niches. Using microsatellite analysis in combination with different statistical approaches, we demonstrate that the studied population is structured according to ploidy level, substrate of isolation and geographical origin of the strains, underlying the relative importance of each factor. We found that geographical origin has a different contribution to the population structure according to the substrate of origin, suggesting an anthropic influence on the spatial biodiversity of this microorganism of industrial interest. The observed clustering was correlated to variable stress response, as strains from different groups displayed variation in tolerance to the wine preservative sulfur dioxide (SO<sub>2</sub>). The potential contribution of the triploid state for adaptation to industrial fermentations and dissemination of the species *B. bruxellensis* is discussed.



## 2.2. Introduction

Grape derived wine is one of the most popular alcoholic beverages and has been produced by humans since ancient times. It is the result of grape juice fermentation by yeasts which consume the fruit sugars and mainly release ethanol and carbon dioxide. Even though microorganisms are an essential part of the winemaking process, they must cope with a very hostile and variable environment, characterised by high initial sugar content and subsequent high ethanol content, low pH, presence of antimicrobial agents, and lack of nutrients. Despite these stressful conditions, some opportunistic microorganisms manage to survive and multiply during and after alcoholic fermentation. A striking example is the wine spoilage yeast *Brettanomyces bruxellensis* (teleomorph *Dekkera bruxellensis*) that is typically detected during wine aging but also at lower frequency during the early stages of the winemaking process (grapes and must) (Renouf, 2009; Renouf and Lonvaud-Funel, 2007). When it grows in wine, *B. bruxellensis* produces odorant molecules (namely volatile phenols), which are associated with unpleasant aromas described as barnyard, horse sweat, Band-aid<sup>®</sup> (Chatonnet et al., 1995a, 1992; Heresztyn, 1986). Therefore, the presence of *B. bruxellensis* in wine often provokes rejection by consumers and serious economic losses for winemakers (Wedral et al., 2010).

The wider industrial relevance of this yeast is highlighted by the fact that it is isolated from various fermented beverages and products. For example, *B. bruxellensis* is an essential contributor to the elaboration of some specialty Belgian and American beers, which are the result of complex spontaneous fermentations performed by various genera of bacteria and yeasts (Bokulich et al., 2012; Steensels et al., 2015). Indeed, *B. bruxellensis* was the first microorganism to be patented for its contribution to English 'stock' ales (Claussen, 1904), in 1904. This yeast has also been isolated from other fermented beverages and food like kombucha, kefir, cider, and olives (Coton et al., 2017; Schifferdecker et al., 2014; Steensels et al., 2015). Interestingly, *B. bruxellensis* was reported to be a common contaminant in

bioethanol production plants (Passoth et al., 2007; Souza et al., 2012), and under the right conditions can take the place of the industrial *Saccharomyces cerevisiae* strains and perform molasses fermentation (Souza et al., 2012).

The recurrent problem of *B. bruxellensis* in wine and its potential use for beer and bioethanol industrial fermentations has led to high and rising interest in this yeast species. Various studies highlighted great phenotypic diversity of *B. bruxellensis* regarding growth capacity (Agnolucci et al., 2009; Barbin et al., 2008; Fugelsang and Zoecklein, 2003; Oelofse et al., 2009; Romano et al., 2008; Vigentini et al., 2008a), sugar metabolism (Conterno et al., 2006; Crauwels et al., 2017, 2015; Galafassi et al., 2011), nitrogen source utilisation (Borneman et al., 2014; Crauwels et al., 2015), volatile phenols production (Agnolucci et al., 2009; Conterno et al., 2006; Crauwels et al., 2017; Di Toro et al., 2015; Martorell et al., 2006; Renouf, 2009; Romano et al., 2008), behaviour in viable but not cultivable state (Capozzi et al., 2016), and response to abiotic factors like temperature (Barata et al., 2008; Conterno et al., 2006), pH (Blomqvist et al., 2010; Conterno et al., 2006), oxygen availability (Capusoni et al., 2016; Du Toit et al., 2005a; Uscanga et al., 2003) and sulfur dioxide (SO<sub>2</sub>) (Agnolucci et al., 2010; Barata et al., 2008; Conterno et al., 2006; Crauwels et al., 2017; Curtin et al., 2012a; Vigentini et al., 2013). This phenotypic variation makes it difficult to predict the spoilage potential of *B. bruxellensis* and is therefore a major concern for winemakers. For example, across several studies the concentration of molecular SO<sub>2</sub> (mSO<sub>2</sub>) required to stop *B. bruxellensis*' growth ranged from 0.2 to 1.1 mg.L<sup>-1</sup> (Curtin et al., 2015). This observed variability was at least partly due to the use of different strains. However, only a few studies have attempted to correlate SO<sub>2</sub> tolerance to a genotypic profile (Conterno et al., 2006; Curtin et al., 2012a). A striking example is the study of 41 *B. bruxellensis* wine isolates from Australia showing that the most common genotype (92% of studied isolates) was correlated with SO<sub>2</sub> tolerance, thus suggesting that SO<sub>2</sub> usage patterns may have created a selective pressure on this population (Curtin et al., 2012a).

Despite several studies that have explored genetic diversity of this species using fingerprinting techniques such as Random Amplified Polymorphism DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), pulsed field electrophoresis (REA-PFGE), and mtDNA restriction analysis (Agnolucci et al., 2009; Campolongo et al., 2010; Conterno et al., 2006; Curtin et al., 2012a; Curtin et al., 2007; Di Toro et al., 2015; Joseph et al., 2013; Martorell et al., 2006; Oelofse et al., 2009; Vigentini et al., 2012), our understanding of the *B. bruxellensis* global population structure and the factors that drive it remains limited. Several studies highlight an important intraspecific diversity of *B. bruxellensis* (Agnolucci et al., 2009; Conterno et al., 2006; Curtin et al., 2007; Vigentini et al., 2012) which makes the prediction of its occurrence and behaviour in industrial fermentations difficult. Further, recent genetic studies on a limited number of strains (Albertin et al., 2014b; Borneman et al., 2014; Curtin et al., 2012) have suggested that polyploidy and hybridisation may play a significant role in microevolution of the species, along with plasticity in chromosomal structure due to “untraditional” centromeres (Ishchuk et al., 2016). The role of polyploidy in adaptive changes to suit environment and/or lifestyle has been observed in other organisms (Albertin and Marullo, 2012; Comai, 2005; Selmecki et al., 2015; Wertheimer et al., 2016), notably for *S. cerevisiae* which shares similar fermentation niches to those occupied by *B. bruxellensis*.

To enhance our knowledge of the global *B. bruxellensis* population, here we used a recently developed microsatellite profiling method (Albertin et al., 2014b) to genotype 1488 isolates from various fermentation niches across five continents. Typing based on microsatellite markers is a rapid, reliable and discriminant genotyping approach that has been successfully used to decipher complex population structures (Guichoux et al., 2011; Vieira et al., 2016) and provide insight into the ploidy-state (Albertin et al., 2014b). The performed research work aimed to determine the population structure of a large *B. bruxellensis* collection and test for a

link between the identified subpopulations and their adaptive ability, with a focus on tolerance to sulfur dioxide.

## **2.3. Materials and methods**

### **2.3.1. Yeast strains**

*B. bruxellensis* strains used in this study were collected from different origins: i) from CRB Oenologie collection (Centre de Ressources Biologiques Oenologie, Institut des Sciences de la Vigne et du Vin, France), ii) sent from other laboratories, and iii) isolated from wines for the purpose of this work. Overall, the collection of *B. bruxellensis* used in this study contained 1488 isolates (Supplementary Table online and available upon request) which were further analysed by genotyping.

Strain isolation from contaminated wines was performed by spreading 100  $\mu\text{L}$  of wine sample on solid YPD medium containing 10  $\text{g.L}^{-1}$  yeast extract (Difco Laboratories, Detroit M1), 10  $\text{g.L}^{-1}$  bactopectone (Difco Laboratories, Detroit M1), 20  $\text{g.L}^{-1}$  D-glucose (Sigma-Aldrich) and 20  $\text{g.L}^{-1}$  agar (Sigma-Aldrich). This medium was supplemented with antibiotics in order to limit the growth of bacteria (5  $\text{g.L}^{-1}$  chloramphenicol Sigma-Aldrich), moulds (7.5  $\text{g.L}^{-1}$  biphenyl, Sigma-Aldrich), and yeast of the *Saccharomyces* genus (50  $\text{g.L}^{-1}$  cycloheximide, Sigma-Aldrich). The samples were then incubated at 30 °C for 5 to 10 days. Ten colonies were then picked randomly and analysed by PCR using the DB1/DB2 primers (Ibeas et al., 1996) (Eurofins MWG Operon, Les Ulis, France) for species identity confirmation (DNA extraction was performed as described below for the microsatellite analysis). Putative *B. bruxellensis* colonies were streaked and grown on selective YPD medium twice consecutively in order to insure the strain purity. Colonies that gave a positive result by PCR DB1/DB2 were stored at -80 °C in 50% YPD/glycerol medium.

## **2.3.2. Genotyping by microsatellite analysis**

### **2.3.2.1 DNA extraction**

For DNA extraction, strains were grown on YPD solid medium at 30 °C for 5 to 7 days and fresh colonies were lysed in 30 µL of 20 mM NaOH solution heated at 99 °C for 10 minutes using iCycler thermal cycler (Biorad, Hercules, CA, USA).

### **2.3.2.2 Microsatellite loci identification and primers design**

Twelve pairs of primers were designed on the basis of the *de-novo* genome assembly of the triploid *B. bruxellensis* strain AWRI1499 ( Curtin et al., 2012) as previously described by (Albertin et al., 2014b). Four pairs of primers were added to the eight that were previously described in order to improve the discriminative power of the test and to insure its robustness (Supplementary Table S-2.1 online and in Appendix).

### **2.3.2.3 Microsatellites amplification**

In order to reduce the time and cost of analysis, some of the PCR reactions were multiplexed as shown in the T<sub>m</sub> column in Supplementary Table S-2.1 online and in Appendix. By this procedure the number of PCR reactions per sample was reduced from 12 to 9.

PCR reactions were performed in a final volume of 15 µL containing 1 µL of DNA extract (extraction performed as described above), 0.05 µM of forward primer, 0.5 µM of reverse primer and labelled primer (or 1 µL in the case of duplex PCR reactions), 1x Taq-&GO (MP Biomedicals, Illkirch, France). The forward primers were tailed on their 5' end with M13 sequence as described by (Schuelke, 2000). Universal M13 primers were labelled with FAM-, HEX-, AT565- (equivalent to PET) or AT550- (equivalent to NED) fluorescent dyes (Eurofins MWG Operon, Les Ulis, France). This method allows labelling of several microsatellite marker primers with the same fluorochrome marked primer (M13) instead of marking each of the 12 forward primers and thus reduces significantly the analysis cost.



Touch-down PCR was carried out using an iCycler thermal cycler (Biorad, Hercules, CA, USA). The program consisted of an initial denaturation step of 1 min at 94 °C followed by 10 cycles of 30 s at 94 °C, 30 s at  $T_m+10$  °C (followed by a 1 °C decrease per cycle until  $T_m$  is reached) and 30 s at 72 °C, then 20 cycles of 30 s at 94 °C, 30 s at  $T_m$  and 30 s at 72 °C, and a final extension step of 2 min at 72 °C.

Amplicons were first analysed by a microchip electrophoresis system (MultiNA, Shimadzu) and the optimal conditions for PCR amplifications were assessed. Then, the exact sizes of the amplified fragments were determined using the ABI3730 DNA analyser (Applied Biosystems) (a core facility of INRA, UMR Biodiversité Gènes et Ecosystèmes, PlateForme Génomique, 33610 Cestas, France). Prior to the ABI3730 analysis, PCR amplicons were diluted (1800-fold for FAM, 600-fold for HEX, 1200-fold for AT565 and 1800-fold for AT550) and multiplexed in formamide. The LIZ 600 molecular marker (ABI GeneScan 600 LIZ Size Standard, Applied Biosystems) was diluted 100-fold and added to each multiplex. Before loading, diluted amplicons were heated 4 min at 94 °C. Allele size was recorded manually using GeneMarker Demo software V2.2.0 (SoftGenetics).

#### **2.3.2.4 *Microsatellite data analysis***

To investigate the genetic relationships between strains, the microsatellite dataset was analysed using the Poppr package (Kamvar et al., 2014) in R (3.1.3 version, <https://www.r-project.org>). A dendrogram was established using Bruvo's distance (Bruvo et al., 2004) and Neighbour Joining (NJ) clustering (Paradis et al., 2004). Bruvo's distance takes into account the mutational process of microsatellite loci and is well adapted to populations with mixed ploidy levels and is therefore suitable for the study of the *B. bruxellensis* strain collection used in this work. Supplementary tests were applied to the same dataset in order to confirm the clusters obtained by Neighbour Joining. First, an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis was compared with NJ. Then, the partition method (Prosperi et al.,

2011) was applied in order to confirm the reliability of the nodes obtained by NJ. Also, a principal component analysis (PCA) by the ade4 package in R (<https://www.r-project.org>) was applied to the same dataset and finally, the function ‘*find.clusters*’ available in the adegenet R package was used to identify clusters by successive K-means (Jombart, 2008). Further, AMOVA (analysis of molecular variance) was used to assess the relative importance of geographical localisation and substrate origin regarding *B. bruxellensis* genetic diversity. To confirm the results obtained by the AMOVA analysis, the link between genetic divergence and geographic distance was further evaluated by MANTEL test.

### **2.3.2.5 Core genotype analysis**

Among the 124 alleles included in the initial dataset, 70 were found to be significantly associated with the triploid isolates ( $\chi^2$  test,  $p < 0.01$ ) and were excluded to create a new dataset comprising alleles common to all groups and representative of the core genotype (*i.e.* the genotype common to all groups).

For the inference of population structure with this dataset, LEA package was used (Frichot and François, 2015) in combination with the TESS tool to map the geographical cluster assignments of the ancestral populations as defined by Höhna *et al.* (2016) (Höhna *et al.*, 2016). Further, a differentiation test analysis was performed by calculating the fixation index ( $F_{ST}$ ) for the core diploid genotype.

### **2.3.3. Sulphite tolerance assessment**

The assay was performed in liquid medium containing 6.7 g.L<sup>-1</sup> of YNB (Difco™ Yeast Nitrogen Base, Beckton, Dickinson and Company), 2.5 g.L<sup>-1</sup> D-glucose, 2.5 g.L<sup>-1</sup> D-Fructose, 5% (v/v) ethanol and increasing concentrations of potassium metabisulphite (PMB, K<sub>2</sub>S<sub>2</sub>O<sub>5</sub>) (Thermo Fischer Scientific) in order to obtain 0, 0.2, 0.4 and 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub> final concentrations. For the calculation of mSO<sub>2</sub> it was considered that K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> corresponds to about

50% of total SO<sub>2</sub> (therefore a solution of 10 g.L<sup>-1</sup> K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> corresponds to approximately 5 g.L<sup>-1</sup> total SO<sub>2</sub>). In order to deduce the final mSO<sub>2</sub> concentration, the free SO<sub>2</sub> concentration was assessed by aspiration/titration method. Then, the mSO<sub>2</sub> was estimated by taking into account the final pH, temperature and alcohol content of the medium (resource available at <http://www.vignevin-sudouest.com/services-professionnels/formulaires-calcul/so2-actif.php>).

Final pH was adjusted to 3.5 with phosphoric acid (1M H<sub>3</sub>PO<sub>4</sub>) and the four media (corresponding to the 4 different concentrations of SO<sub>2</sub>) were filtered separately with 0.22 μm pore filter (Millipore).

Small-scale fermentations were performed in sterile 4 ml spectrophotometer cuvettes containing a sterile magnet stirrer (Dutscher, France). The cells were grown on YPD agar and inoculated into the YNB-based medium without SO<sub>2</sub>. After 96 h of pre-culture (the point at which all strains reached stationary phase), the cells were inoculated at OD<sub>600</sub> 0.1 in a final volume of 3 ml. The inoculated medium was then covered with 300 μL of sterile silicone oil (Sigma-Aldrich) to avoid oxidation of the medium which could favour the free SO<sub>2</sub> consumption. Then, the cuvette was capped with a plastic cap (Dutscher) and sealed with parafilm. A sterile needle was added by piercing the cap to allow CO<sub>2</sub> release. The “nano-fermenters” were then placed in a spectrophotometer cuvettes container box and on a 15 multi-positions magnetic stirrer plate at 25 °C (the final temperature in the “nano-fermenters” was therefore 29 °C due to the stirrer heating). Optical density (OD<sub>600</sub>) was measured every 24h during at least 300h to follow cell population growth until stationary phase was reached.

For each growth curve, the following three parameters were calculated: maximal OD was the maximal OD reached at 600 nm, the lag phase (in hours) was the time between inoculation and the beginning of cell growth (5% maximal OD increase), and finally, the maximal growth rate was calculated (maximal number of division per hour based on the OD measurement divided

by time). A non-parametric Kruskal-Wallis test was used at  $\alpha=5\%$  to identify the means that were significantly different.

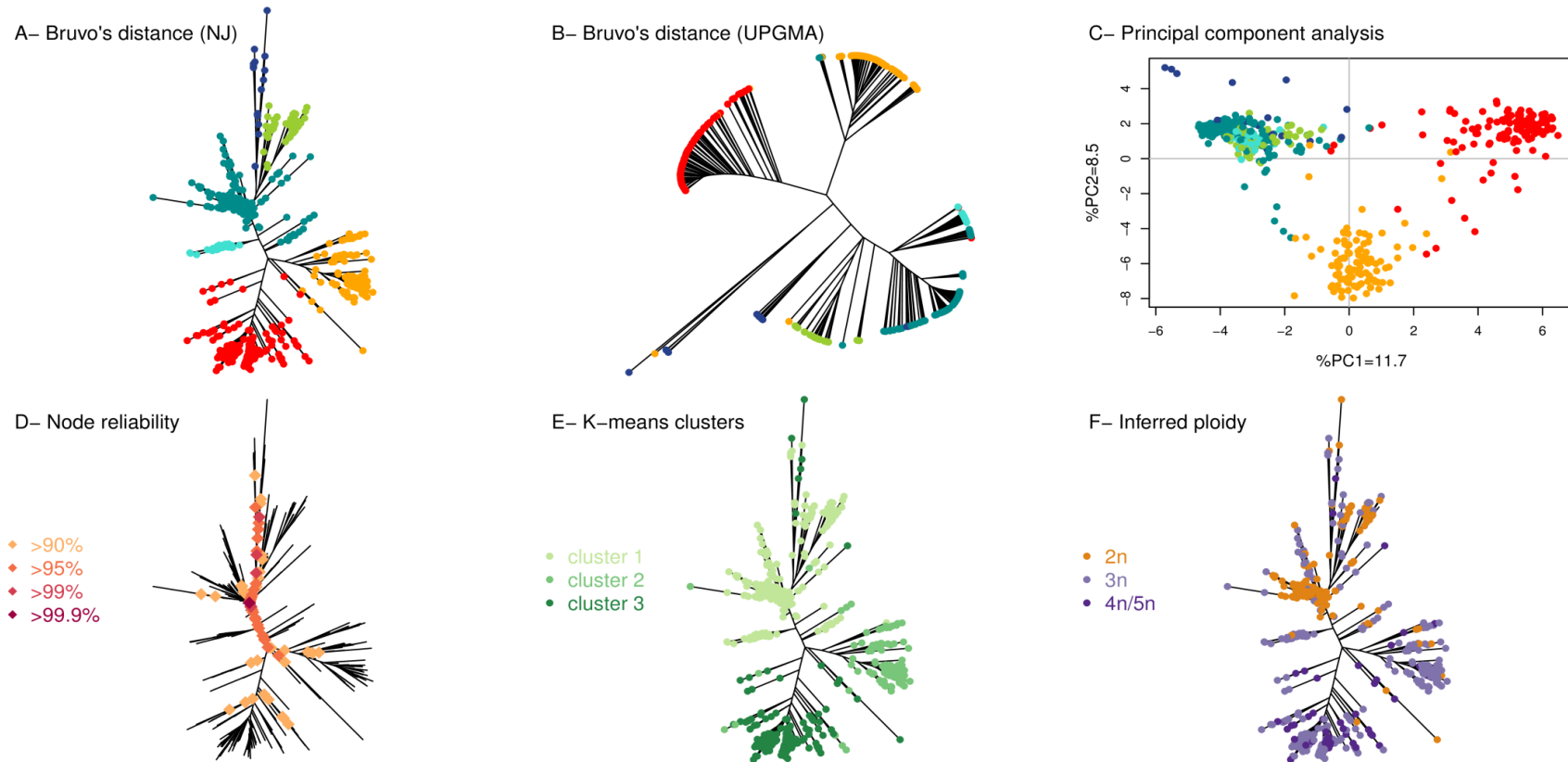
## **2.4. Results**

### **2.4.1. *B. bruxellensis* genotyping analysis and population structure**

The *B. bruxellensis* collection used in this study comprised 1488 isolates from 29 countries and 9 different substrates, the majority of strains (87%) originating from wine (Supplementary Table online and available upon request). The 1488 isolates were genotyped with 12 primer pairs amplifying microsatellite regions, including four new loci in addition to the eight previously published (Albertin et al., 2014b). Characteristics of the different loci and number of alleles are given in Supplementary Table online and available upon request. One locus out of the four additional loci (D1) displayed a high allelic diversity, presenting 18 different alleles. All isolates were shown to be heterozygous for at least one locus. Many isolates were shown to have more than 2 alleles per locus. About half of the isolates had up to 3 alleles per locus (792 isolates) and some had up to 4 and 5 alleles per locus (67 and 1 isolates, respectively). The high number of isolates with up to 3 alleles per locus suggests the existence of triploidy in the studied population. The same observation was reported previously by (Curtin et al., 2012) and (Borneman et al., 2014) who performed *de-novo* sequencing and comparative genomics respectively, highlighting two triploid strains having different triploidisation origins.

The raw data obtained by the microsatellite analysis corresponds to the alleles (*i.e.* the size of the amplified microsatellite sequences) per locus and per strain (Supplementary Table online). This data was further used for the construction of a dendrogram reflecting the genetic proximity between strains (Figure 2.1 A). The method was based on Bruvo's distance and Neighbour Joining (NJ) and was chosen for being reliable and suitable for populations with mixed ploidy levels. The population clusters in 3 main genetic groups (Figure 2.1 A).

Additional methods, including complementary tests and Bayesian approaches were applied to verify the reliability of the clustering obtained by NJ (Figure 2.1). The NJ tree showed three main branches that were almost perfectly conserved with UPGMA method (Figure 2.1 A and B). Then, a principal component analysis (PCA) was performed using R ade4 package (Figure 2.1 C). The PCA analysis showed that the three main groups were almost identical to the clusters previously defined. Furthermore, the partition method (Prosperi et al., 2011) was applied on the same dataset. This algorithm identifies monophyletic clusters for which the individuals are more closely related than randomly selected individuals. The reliability of the node is then computed and nodes with reliability higher than 90% are considered (Figure 2.1 D). The partition method also confirmed the three main clusters obtained with NJ as reliable. Finally, clusters were identified using successive K-means (ade4 package, function *'find.clusters'*). This function implements the clustering procedure used in Discriminant Analysis of Principal Components (DAPC) (Jombart et al., 2010), where successive K-means are run with an increasing number of clusters (k), associated with a statistical measure of goodness of fit. This approach identified 3 clusters, once again very similar to those obtained by NJ (Figure 2.1 E). Overall, the five approaches taken together confirmed the reliability of the three main clusters observed in the studied *B. bruxellensis* population.

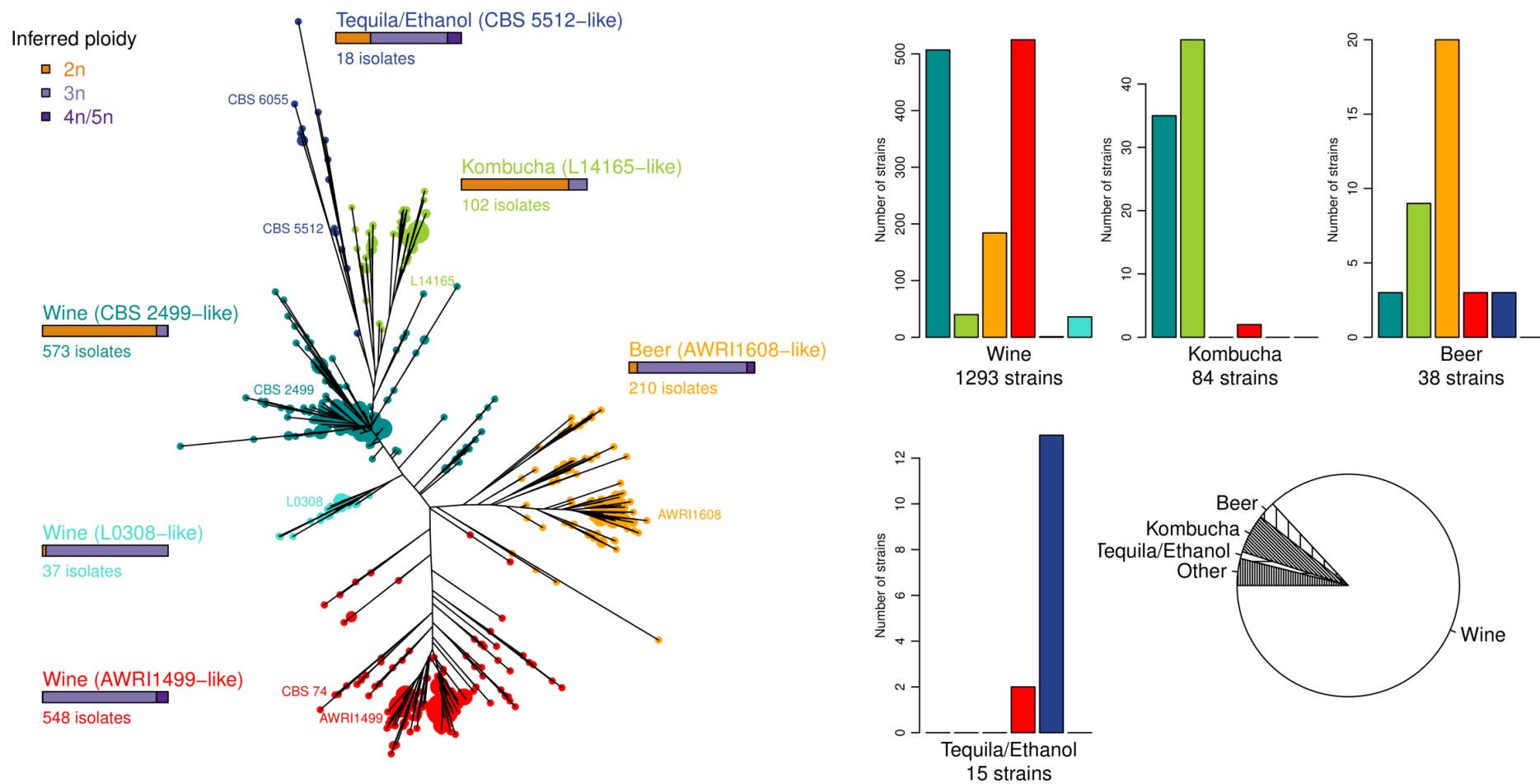


**Figure 2.1. *B. bruxellensis* population clusters identification by combining different tools and parameters.** A- Dendrogram using Bruvo's distance and NJ clustering. The figure was produced using the *poppr* package in R. B- Dendrogram using Bruvo's distance and UPGMA clustering. The figure was produced using *poppr*. Isolates are shown in the same colours as in A. C- Principal component analysis (PCA) based on microsatellite genotyping. The PCA was computed using the *ade4* package in R. For isolates with incomplete genotyping, the missing data was inferred from the closest neighbour using Bruvo's distance. Isolates are shown with the same colours as in A. D- Node reliability using the partition method (Prosperi et al., 2011). Only the nodes with reliability >90% are shown on the NJ tree. E- Cluster identification using successive K-means. The *find.cluster* function from the *adeget* package in R was applied, using within-groups sum of squares (WSS) statistics and the default criterion *diffNgroup*. This tool identifies an optimal number of 3 clusters, represented on the NJ tree using different arbitrary colours. F- Inferred ploidy. The maximum number of alleles per locus was computed. Isolates with up to 2 alleles/locus were considered as diploid (2n). Isolates with up to 3 alleles/locus were considered as triploid (3n), and the number of loci showing up to 3 alleles was recorded (1-2 loci, or more than 2 loci showing up to three alleles). Finally, isolates with up to 4 or 5 alleles/locus were noted as 4n/5n. The inferred ploidy is represented on the NJ tree.

Since *B. bruxellensis* is known to exhibit different ploidy levels (Borneman et al., 2014; Curtin et al., 2012), we inferred putative ploidy level based on the microsatellite genotyping. Isolates with up to 2 alleles per locus were considered diploid and noted 2n (Figure 2.1 F). Isolates with up to 3 alleles/locus were considered triploid (3n). Finally, isolates with up to 4-5 alleles/locus were noted as 4n/5n. The ploidy level coincided clearly with the three main branches of the dendrogram, the red and orange groups being mostly triploid and the blue-green mostly diploid. Within this last cluster, two triploid sub-groups based on the substrate origin and ploidy level of the strains were defined, marked with blue and cyan colours. Finally, the combination of different methods and factors defined of 3 main groups, the ‘diploid’ one being further divided into 3 subgroups (Table 2.1 and Figure 2.2).

**Table 2.1. Clusters considered as a result of the microsatellite analysis and cluster validation with five different clustering methods**

Group name	Number of isolates	Number of genotypes	Putative ploidy (for the majority of the isolates in the group)	Substrate
AWRI1499-like	548	197	Triploid	Mostly from wine
AWRI1608-like	210	127	Triploid	Beer and Wine
CBS 2499-like	573	208	Diploid	Wine
L0308-like	37	26	Triploid	Wine
CBS 5512-like	18	16	Triploid	Bioethanol and tequila
L14165-like	108	58	Diploid	Kombucha



**Figure 2.2. Dendrogram of 1488 isolates of *B. bruxellensis* using 12 microsatellite markers.** The dendrogram was drawn *via* the *poppr* package, using Bruvo's distance and NJ clustering. Five clusters were considered and are represented by different colours. Isolates displaying identical genotypes are represented by a unique tip whose size is proportional to the number of isolates. Inferred ploidy was made as described in Figure 2.1 F. The histograms represent the distribution of isolates depending on the substrate and the five considered clusters. The pie chart illustrates the proportion of the strains originating from different types of sources.



To assess the relative importance of geographical localisation, substrate origin and ploidy level on *B. bruxellensis*' population structure, an analysis of molecular variance (AMOVA) was performed. The three factors were shown to be significant (p-value<0.0001). Ploidy level explained 46.9% of the variance, whereas the geographical origin and substrate factors explained only small proportions of the total variation (around 5% for each) (Table 2.2). However, when considering non-wine isolates, the geographical origin explains 54.8% of the total variance, suggesting that wine genotypes are highly disseminated across the regions studied in comparison with other substrates. The correlation between genetic and geographic distance matrix (MANTEL test) was also significant (p-value=0.0009), confirming that the genetic variation of the total population is significantly related to geographical localisation. The MANTEL test, performed only on the wine strains (p-value=0.0040), also confirmed the results obtained with AMOVA, suggesting a different population structure amongst wine strains compared to those from the other niches.

**Table 2.2. Impact of geographical localisation, substrate origin and ploidy on the population variance (AMOVA test)**

Factor	%Variance	p-value
Country	4.89	<0.0001
Country (wine isolates)	3.7	<0.0001
Country (non-wine isolates)	54.8	<0.0001
Substrate	5.93	<0.0001
Ploidy	46.9	<0.0001

## 2.4.2. Core genotype analysis

### 2.4.2.1 Core diploid data subset

Most classical population genetic analyses cannot be performed using our initial microsatellite dataset since *B. bruxellensis* population include diploid and polyploid isolates, and most traditional analyses are not available for mixed ploidy levels. To overcome such difficulties, we

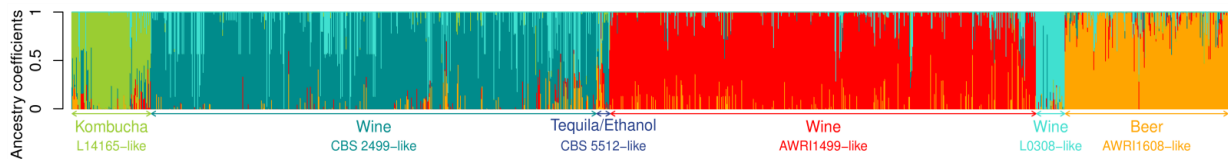
excluded the alleles identified as specific to the isolates showing more than 3 alleles for at least one locus. Among the 124 alleles included in the initial dataset, 70 were found to be significantly associated with the triploid isolates ( $\chi^2$  test, p-value<0.01), and were excluded to create a new dataset comprising alleles representative of the core genotype (*i.e.* the genotype common to all groups). This approach is justified as previous comparative genomics studies showed that *B. bruxellensis* isolates shared a core diploid genome (Borneman et al., 2014).

The obtained core genotype dataset showed up to 2 alleles per locus for most individuals (1350 out of 1488) and only 138 remaining individuals had loci with 3 or 4 alleles. This indicates that the removal of specific triploid alleles allowed us to have access to the core diploid genome common to all *B. bruxellensis* isolates. Loci with more than 2 alleles were considered as missing data and only concerned 138 individuals, of which 130 only had one locus with 3 alleles.

#### **2.4.2.2 Ancestral populations and inference of population structure**

LEA package and the *snmf* function in R were used to infer population structure for the ‘core diploid’ dataset. The number of ancestral populations tested ranged from K=1 to K = 15 (100 repetitions), and entropy criterion was computed to choose the number of ancestral populations explaining the genotypic data in the best way (Supplementary Figure S-2.1 online and in Appendix). Entropy was minimal for K=5 ancestral populations (K=3, 4, 5, 6 shown on Supplementary Figure S-2.2 online and in Appendix). Such Bayesian analysis shows that these 5 ancestral populations are congruent with previous analyses that considered the complete dataset (Figure 2.3): the AWRI1499-like (wine, red) and AWRI1608-like (beer, orange) groups were associated with only one ancestral population. Likewise, most of the blue-green subgroups (wine CBS 2499-like, wine L0308-like, kombucha L14165-like) previously defined were associated with only one ancestral population. Finally, only the tequila/ethanol group (CBS 5512-like) seemed to be associated with more than one ancestry. Altogether, the

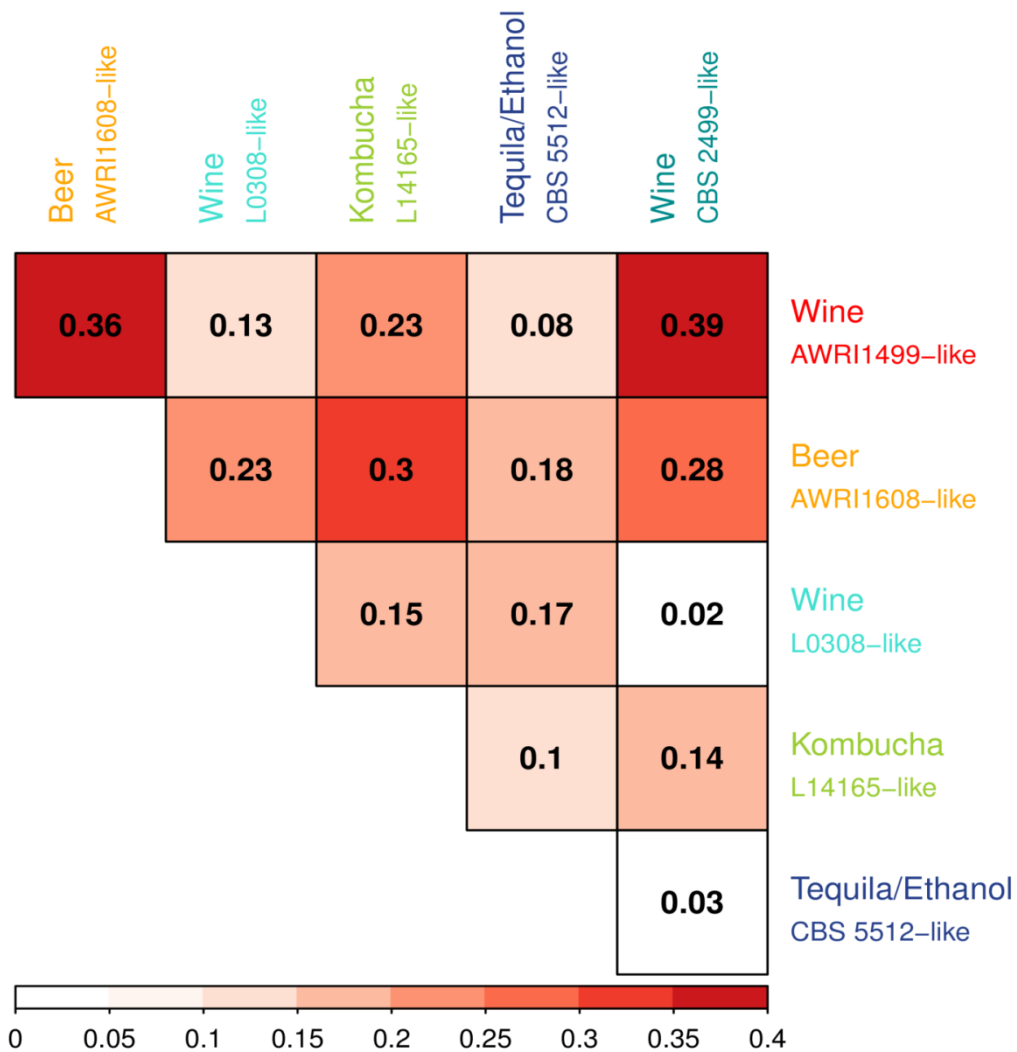
population structure analysis on the core diploid genotype confirmed the previous clustering and suggested the existence of only one ancestral population for each current population.



**Figure 2.3. Ancestral populations of 1488 *B. bruxellensis* strains.** STRUCTURE plots for K=5 (the number of ancestral population with lowest entropy, see Supplementary Fig. S1 online). Each bar represents an isolate and the colour of the bar represents the estimated ancestry proportion of each of the K clusters. The same colour code is kept as in Figure 2.1 and 2.2.

#### 2.4.2.3 Population differentiation analysis

A population differentiation analysis was performed by calculating the fixation index ( $F_{ST}$ ) on the core diploid genotype dataset (Figure 2.4). The wine AWRI1499-like population is highly differentiated from beer AWRI1608-like and wine CBS 2499-like groups (with  $F_{ST}$  0.36 and 0.39 respectively). This confirms the grouping obtained by the previous analyses. In addition, the pairwise  $F_{ST}$  values showed high differentiation between beer AWRI1608-like and wine CBS 2499-like populations ( $F_{ST}$  0.28). The L14165-like kombucha population seems to be mostly differentiated from the 1608-like beer population and is closer to CBS 5512-like tequila/ethanol group. Finally, it is interesting to point out that the CBS 5512-like group is not highly differentiated from all other groups, which is congruent with the fact that population structure analysis inferred multiple ancestries populations for that group.



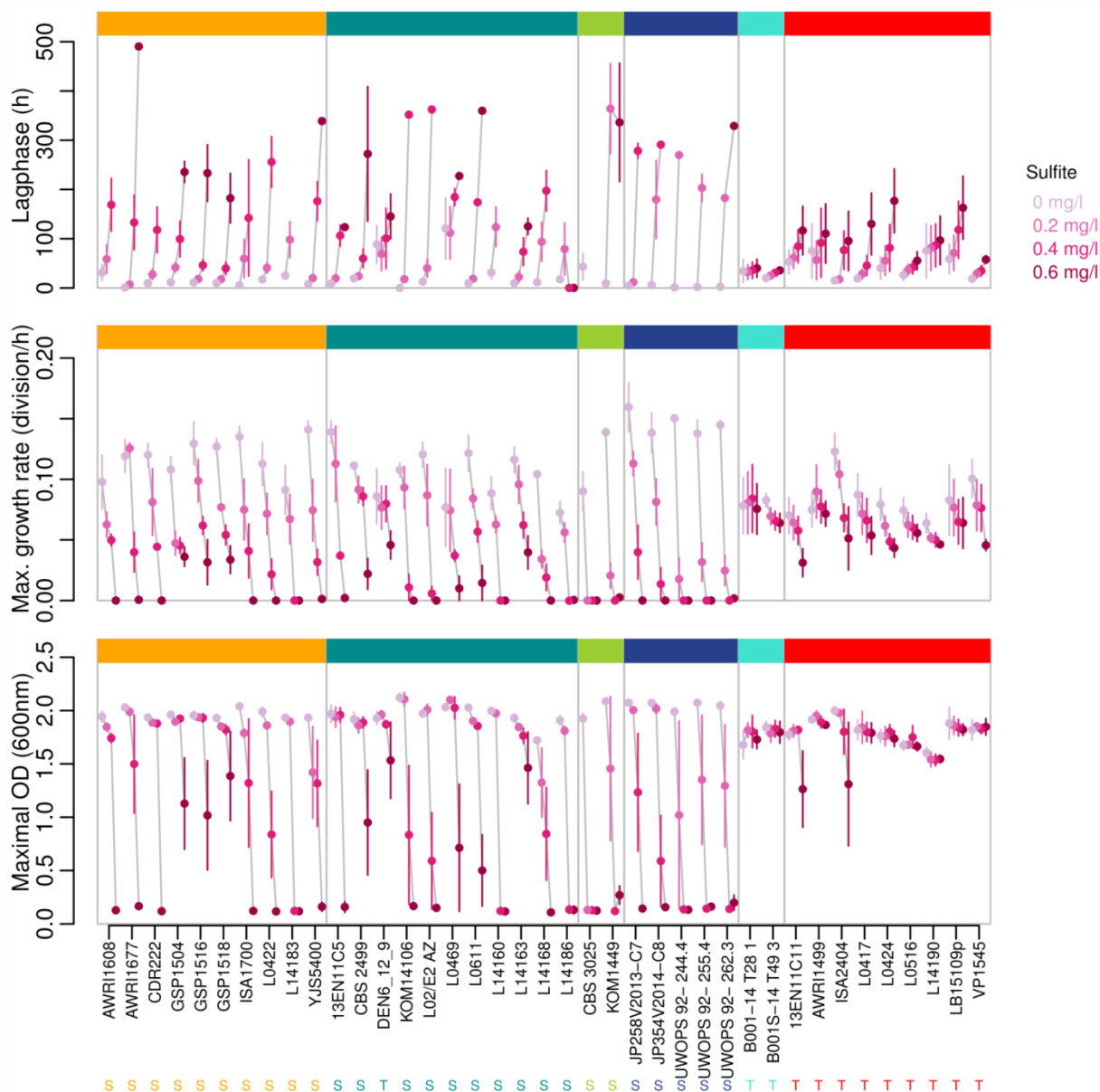
**Figure 2.4. Population differentiation represented by fixation index ( $F_{ST}$ ) of *B. bruxellensis* genetic groups between each other.** The range of  $F_{ST}$  is from 0 to 1, 1 meaning that the two populations do not share any genetic diversity.

### 2.4.3. Sulphite tolerance

Sulfur dioxide tolerance was assayed for a subset of *B. bruxellensis* (a total of 39 strains). The chosen strains were selected according to their various geographical origins, substrates and different genetic groups. Some isolates showing identical microsatellite genotypes were included to evaluate possible sulfur tolerance variation between strains with undifferentiated genotypic patterns (13-EN11C11=L0417=L0424; UWOPS 92- 244.4=UWOPS 92- 262.3; L0469=L14186). Each strain was grown in medium with increasing SO<sub>2</sub> concentration (ranging

from 0 to 0.6 mg.L<sup>-1</sup> molecular SO<sub>2</sub>) in biological triplicates, so that more than 480 fermentations were monitored.

Three growth parameters (lag phase, maximum growth rate, maximal OD) in the presence of four different concentrations of mSO<sub>2</sub> were followed until stationary phase was reached or for a maximum of 300h when growth was slow or absent. The isolates presented different behaviour according to mSO<sub>2</sub> concentration (Figure 2.5). Based on the growth parameters of the strains when exposed to increased concentrations of mSO<sub>2</sub>, two main groups were identified: 1) sensitive strains (S) characterised by an altered growth with i) a significant lag phase prolongation, ii) a significant decrease in maximum growth rate, and/or iii) significant decrease in maximum OD<sub>600</sub> (e.g. the sensitive strain L0422 had a lag phase of 17.2 h, 40.7 h, 255.8 h and growth absence, growth rate values were 0.11, 0.07, 0.02 divisions/h and growth absence for and OD<sub>600</sub> 2, 1.9, 0.8 and no growth at 0, 0.2, 0.4 and 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub> respectively); 2) tolerant strains (T) that showed unmodified growth rate and maximum OD<sub>600</sub> but sometimes a significant prolongation of lag phase was observed (e.g. the tolerant strain AWRI1499 had a maximal growth rate of 0.07, 0.09, 0.08 and 0.07 divisions/h, OD<sub>600</sub> 1.9, 2.0, 1.9 and 1.9, lag phase of 75, 56.5, 91.5 and 110.3 h at 0, 0.2, 0.4 and 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub> respectively for the same strain) (mean values of those parameters for each strain are shown in Supplementary Table S-2.4 online in Appendix). A clear relation between genetic group and SO<sub>2</sub> tolerance was highlighted (Figure 2.5). The isolates from groups AWRI1608-like, CBS 5512-like, CBS 2499-like and L14165-like were mostly identified as sensitive (S), whereas the triploid AWRI1499-like and triploid L0308-like groups were mostly classified as tolerant (T). Furthermore, the isolates with an identical microsatellite profile presented similar behaviour in means of growth parameters in the different conditions studied here (Figure 2.5 and Supplementary Table S-2.4 online in Appendix).



**Figure 2.5. Growth parameters of *B. bruxellensis* strains at different concentrations of  $\text{SO}_2$ .** 39 strains belonging to the 6 genetic groups defined previously were tested in small scale fermentations and growth ( $\text{OD}_{600}$ ) was measured in media containing different concentrations of sulfur dioxide (0, 0.2, 0.4, and 0.6  $\text{mg}\cdot\text{L}^{-1}$   $\text{mSO}_2$ ) and in biological triplicates. Three parameters were considered: lag phase (h): end of lag phase considered when  $\text{OD}$  above initial  $\text{OD}\cdot 5\%$ ; maximal growth rate ( $r$ ) = number of cellular divisions per hour; maximal  $\text{OD}$ ; S and T stand for sensitive and tolerant (Kruskal-Wallis test,  $\alpha=5\%$ ). Genetic groups are represented in the same colours as on Figure 2.2.

## 2.5. Discussion

The yeast *B. bruxellensis* has gained importance for its impact not only in wine industry, but also in beer- and bioethanol-associated fermentation processes. Subsequently, many

independent studies were held and results were obtained on different *B. bruxellensis* collections but without leading to a holistic picture of the *B. bruxellensis* species. In this study, a large collection of *B. bruxellensis* strains (1488 isolates) from various substrates (9, the majority of strains (87%) being isolated from wine) and geographic origins (5 continents) was genotyped. The use of a reliable and robust method (microsatellite analysis) determined a general picture of the species' genetic diversity and population structure. The analysis of the complete genotype dataset highlighted 3 main genetic clusters in the *B. bruxellensis* population represented by the AWRI1499-like group, AWRI1608-like and CBS 2499-like group correlating with ploidy level and substrate of isolation. Three sub-clusters were also defined for their ploidy level and substrate of isolation, namely tequila/ethanol CBS 5512-like group, wine L0308-like, and kombucha L14165-like group. Our results are consistent with comparative genomics analysis showing that the AWRI1499, AWRI1608 and AWRI1613 (genetically close to the strain CBS 2499) strains are genetically distant and that the AWRI1499 and AWRI1608 strains are triploid while AWRI1613 is diploid (Borneman et al., 2014).

Heterozygosity for at least one out of the 12 microsatellite loci was shown for all *B. bruxellensis* isolates. This observation supports the assumption that a simple haploid organisation of the genome is excluded, which is congruent with previous results based on the Southern analysis of single gene probes of 30 *B. bruxellensis* strains from different geographical origins (Hellborg and Piškur, 2009). In comparison, using microsatellite analysis, Legras *et al.* (2007) reported 102 out of 410 *S. cerevisiae* isolates (about 25%) and 75% of *Saccharomyces uvarum* strains (among 108 isolates from various geographical and substrates origins) to be homozygous (Legras et al., 2007). In general, highly homozygous strains are associated with sporulation and selfing phenomena (Mortimer et al., 1994). So, this could suggest that in the case of *B. bruxellensis* these mechanisms are non-existent or very rare amongst isolates from industrial fermentation environments. Indeed, there is only one study to

our knowledge (Walt and Kerken, 1960), which reports spore formation for *B. bruxellensis* (and therefore its teleomorph form *Dekkera bruxellensis*). In the scenario of rare or non-existent sexual reproduction, a large proportion of heterozygous strains would promote higher phenotypic diversity and therefore colonisation of new niches and adaptation to new environments (Magwene, 2014).

Our results confirm on a large scale the assumption that the *B. bruxellensis* population is composed of strains with different ploidy level (Albertin et al., 2014b; Borneman et al., 2014; Curtin et al., 2012; Hellborg and Piškur, 2009), as 57.8% of the isolates were shown to have more than 2 alleles for at least one locus. Moreover, polyploid strains were associated with various fermentation niches and geographical regions. A strong correlation between genetic clustering and ploidy level was highlighted, with some clusters predicted to be diploid (CBS 2499-like) while others were composed of mainly triploid isolates (e.g. AWRI1499- and AWRI1608-like). The latter two clusters derive from distinct ancestral populations and thus, presumably from different triploidisation events. The polyploid state typically has a high fitness cost on the eukaryote cell due to the difficulty to maintain imbalanced number of chromosomes during cell division as well as other effects caused by nucleus and cell enlargement (Comai, 2005). Thus, it is presumed that a stable polyploid or aneuploid state is maintained when it confers advantage for the survival of the cell in particular conditions (Wertheimer et al., 2016). Indeed, aneuploidy and polyploidy contribute to genome plasticity and have been shown to confer selective and fitness advantages to fungi in extreme conditions, such as the presence of high concentrations of drugs, high osmotic pressure, low temperature, and others (see Albertin and Marullo, 2012; Mulla et al., 2014; Wertheimer et al., 2016 for review). Similar observations have been made in clinical microbiology, for example, 70% of 132 completely sequenced *S. cerevisiae* clinical isolates with different geographic origins were shown to be poly- or aneuploid (Zhu et al., 2016). It has been suggested that the aneuploid state



contributes to the transition from commercial (industrial fermentations) to clinical (human pathogen lifestyle) environments. Aneuploidy was also reported for another human pathogen – *C. albicans*, for which an aneuploidy of an isochromosome [i(5L)] is shown to confer resistance to fluconazole (Selmecki et al., 2006). In the industry, stable autotetraploid *S. cerevisiae* strains have been described among isolates from a bakery environment and it was suggested that their prevalence in sour dough fermentation could be the result of human selection for tolerance to high osmotic pressure and high metabolic flux – highly favourable characteristics for baking (Albertin et al., 2009). In the case of *B. bruxellensis*, however, polyploidy seems to be not only due to a “simple” duplication of chromosomes and/or regions of chromosomes but is the result of independent hybridisation events with closely or distantly related unknown species (Borneman et al., 2014), which result in allotriploid strains. Efficient hybrid species are not rare in human related fermentations (Albertin and Marullo, 2012; Querol and Bond, 2009; Steensels et al., 2014) and often the hybridisation with a genetically close species is believed to confer tolerance to specific stress factor in a given environment. This is the case of *S. pastorianus*, used for lager beer fermentations characterised with low temperatures. This yeast has recently been shown to be a hybrid between *S. cerevisiae* and *S. eubayanus* – a cryotolerant species isolated from forests in Patagonia (Libkind et al., 2011), Tibet (Bing et al., 2014) and recently from New Zealand (Gayevskiy and Goddard, 2016). Thus, presumably sterile hybrids were naturally generated and they multiplied clonally, accumulating mutations which enhanced the adaptability of the new “species” (Libkind et al., 2011). Hybrids are also a widespread state among wine yeast, where natural or laboratory obtained combinations between two species could have interesting technological properties (Le Jeune et al., 2007; Masneuf et al., 1998; Naumov et al., 2000; Sipiczki, 2008; Steensels et al., 2014). Other form of genome dynamics was also highlighted for the diploid CBS 2499 strain possessing specific centromeric loci configuration that enables genome rearrangements and

ploidy shifts (Ishchuk et al., 2016). Based on the body of knowledge concerning other polyploid micro- and macro-organisms and the prevalence of polyploid strains highlighted in this study, we assume that *B. bruxellensis* has adapted to environmental stress factors by the means of genome plasticity, namely polyploidy.

Our study showed that at least one group, the AWRI1499-like triploid wine group, is composed of wine isolates that are highly tolerant to SO<sub>2</sub> and that are clearly divergent from other *B. bruxellensis* clusters ( $F_{ST}$  higher than 0.35 when compared with AWRI1608-like and CBS 2499-like groups). Nevertheless, for some wine samples, isolates from both AWRI1499-like triploid group and the CBS 2499-like diploid group were identified. Coexistence of diploid and polyploid (auto- and allopolyploid) “microspecies” has often been reported for plants, in which the polyploids are widely distributed as opposed to the diploids that have a more restricted distribution (Stebbins, 1940). Babcock and Stebbins were the first to name this coexistence of populations a diploid-polyploid complex (Babcock et al., 1938) for a *Crepis* species defined as a group of interrelated and interbreeding species that also have different levels of ploidy. These authors claimed that such polyploid complex can arise when there are at least two genetically isolated diploid populations and auto- and allopolyploid derivatives that coexist and interbreed. In the case of *B. bruxellensis*, the sexual cycle of this yeast is not yet elucidated and interbreeding remains to be evidenced. However, we propose that *B. bruxellensis* could be described as a diploid-triploid complex, in which sub-populations with different ploidy levels coexist.

To obtain a deeper understanding of the factors shaping *B. bruxellensis* population structure, we explored the impact of geographical localisation and industrial fermentation environment of origin on the total genetic variance. Contribution of the “geographic origin” factor to the population structure was shown to be significant yet only explained a relatively small proportion of variation. However, the variance proportion explained by this factor is much

higher when considering non-wine isolates, suggesting that wine strains are highly dispersed worldwide. This dispersal could easily reflect exchange of material and human transport associated with winemaking, followed by adaptation to local winemaking practices (Curtin et al., 2007). Exchange of material also happens between different industries, which would facilitate local transfer of microorganisms between beverages. For example, some beers are aged in oak barrels previously used for winemaking (Sanna and Pretti, 2015). Also, in the past, beer fermentation is thought to have been initiated by the addition of a small amount of wine (Mortimer, 2000b). Such exchanges could be a possible explanation for the low (but significant) contribution of the “substrate of isolation” factor to the total genetic variance in the studied population (5.93%,  $p$ -value $<0.0001$ ). Substrate of isolation and geographic origin contributed to a similar extent to the total genetic variance of the population. However, this percentage remained low (5%) compared to *S. cerevisiae* for which geographic origin was shown to contribute to 28% of the genetic variance (Legras et al., 2007), and *Candida albicans* for which 39% were reported (Fundyga et al., 2002). For *S. cerevisiae*, a significant contribution of geographic origin to the genetic variance is often perceived as a sign of local domestication (Almeida et al., 2015; Legras et al., 2007). Like *S. cerevisiae*, *B. bruxellensis* is isolated from human-conducted fermentations including beer and wine. However, until now there are no *B. bruxellensis* isolates from “natural” non-human related habitats contrary to the case of *S. cerevisiae* (Sampaio and Gonçalves, 2008; Sniegowski et al., 2002; Wang et al., 2012). A recent comparative study of strains with different industrial origins and their growth capacities in various type of media (wine, beer, and soft drink) suggests adaptation of *B. bruxellensis* strains to different fermented beverages (Crauwels et al., 2017). In our study, a low but significant contribution of substrate of isolation to the total genetic variance of the species was highlighted (5.93 %,  $p$ -value $<0.0001$ ), which is an indicator for the adaptation of certain sub-groups to different human-related niches (e.g. winemaking conditions, kombucha

fermentation, and others). This structuration is further accompanied by a specific genetic configuration, some groups being mostly diploid and others polyploid.

The hypothesis that the triploid state of *B. bruxellensis* is maintained for some genetic groups because of its contribution to adaptation to a certain type of environment or stress factors is strongly supported by the sulphite tolerance assay performed in our study. This indicated that strains representative of the globally dispersed wine triploid AWRI1499-like group are highly tolerant to SO<sub>2</sub>. Sulfur dioxide is the most common antimicrobial agent used in winemaking. However, very tolerant *B. bruxellensis* strains have been reported (Curtin et al., 2015). Particularly, in Australia 92% of the isolates are genetically close to a strain that has been shown to be triploid by genome sequencing and highly tolerant to SO<sub>2</sub> (normal growth at more than 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub>)(Curtin et al., 2012a). Here, we show that isolates from this genetic group are highly represented worldwide, namely in France, Italy, Portugal, Southern Argentina and Chile. Furthermore, we confirmed on a larger scale (39 strains from different geographical and fermentation niches) that even high SO<sub>2</sub> doses could not guarantee the absence of growth of these strains and therefore their potential to spoil wine. In this context, it is worth noting that isolates from substrates other than wine, were all sensitive to SO<sub>2</sub> which suggests a direct link between SO<sub>2</sub> exposure in wine and tolerance to this compound. Survival in the presence of SO<sub>2</sub> has been broadly studied in *S. cerevisiae* but is still not fully elucidated. SO<sub>2</sub> is a small and reactive molecule that could enter the cell passively or *via* selective transport (Divol et al., 2012). Once inside the cell, SO<sub>2</sub> can interact with different enzymes and molecules thus having an impact on the basic metabolic pathways of the cell, such as glycolysis. Strategies to tolerate SO<sub>2</sub> are also numerous, like its action on the cell: through the production of molecules that bind SO<sub>2</sub> (acetaldehyde, pyruvate, and others), SO<sub>2</sub> oxidation and SO<sub>2</sub> active efflux by sulphite pump (*SSUI*)(Divol et al., 2012). Even if in *B. bruxellensis* these mechanisms are not elucidated, SO<sub>2</sub> tolerance could be linked to different aspects – presence of gene(s) coding for a

sulphite transporter or presence of this gene (or genes) in multiple copies and therefore overexpression, differences in the gene regulation leading to more efficient response to SO<sub>2</sub> toxicity, or morphological and physiological state of the cell that would give it the ability to tolerate this antimicrobial agent (cell membrane structure, growth, *etc.*). The fact that all the highly tolerant *B.bruxellensis* strains are triploid indicates that this genetic configuration could contribute to SO<sub>2</sub> tolerance. As mentioned in the previous paragraphs, polyploid states are maintained when they confer a selective advantage. In this case, we can hypothesise that the allotriploid AWRI1499-like strains combine genetic and physiological characteristics from the parent genomes that confer to them the ability to survive in the presence of SO<sub>2</sub>.

A possible strategy to cope with the issue of highly tolerant strains would be the increase of SO<sub>2</sub> concentration added to the must and wine. However, the strong legislation and consumer pressure to reduce any kind of wine additives makes it undesirable to produce wines with high concentrations of SO<sub>2</sub> which would be needed for the prevention of AWRI1499-like strains growth. Therefore, the genetic content of *B. bruxellensis* has to be considered when choosing spoilage prevention and treatment methods in the winery in order to obtain optimal effect with minimum intervention. Overall, our results show that polyploid strains are widely disseminated and suggest that *B. bruxellensis* is a diploid-triploid complex whose population structure has been influenced by the use of sulfur dioxide as a preservative in winemaking. Thus, we highlight the importance of *B. bruxellensis* species as a non-conventional model microorganism for the study of polyploidy as an adaptation mechanism to human-related environments.

## **2.6. Data availability**

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

## **2.7. Acknowledgments**

The authors would like to thank the CIVB (Conseil Interprofessionnel du Vin de Bordeaux) and Région Aquitaine for the financial support. Also, the authors thank Lucile Pic (ICV, France), Viriginie Serpaggi (Inter Rhône, France), Manuel Ferreira (Technical University of Lisbon, Portugal), Marcos Morais (Universidade Federal de Pernambuco, Brazil), Morvan Koarer (IFV, France), Microflora (France), Marc-André Lachance (University of Western Ontario, Canada), Marina Bely (ISVV, France), Cécile Thibon (ISVV, France), Hervé Alexandre (Université de Bourgogne, France), Cédric Longin (Université de Bourgogne, France), Iavor Tchobanov (University of Food Technology, Plovdiv, Bulgaria), and all organisations, researchers and winemakers who provided isolates and contaminated wine samples for the purpose of this study.



# **Chapter 3. Competition experiments between *Brettanomyces bruxellensis* strains reveal specific adaptation to sulfur dioxide and complex interactions at intraspecies level**

Marta Avramova<sup>1,2</sup>, Paul Grbin<sup>2</sup>, Anthony Borneman<sup>3</sup>, Cristian Varela<sup>3</sup>, Warren Albertin<sup>1,4</sup>,  
Isabelle Masneuf-Pomarède<sup>1,5</sup>

<sup>1</sup>Univ. Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, 33140 Villenave d'Ornon, France <sup>2</sup>School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia <sup>3</sup>The Australian Wine Research Institute, PO Box 197, Glen Osmond (Adelaide), Urrbrae, South Australia 5064, Australia <sup>4</sup>ENSCBP, Bordeaux INP, 33600 Pessac, France <sup>5</sup>Bordeaux Sciences Agro, 33170 Gradignan, France

Status: in preparation





### 3.1. Introduction

*Brettanomyces bruxellensis* is a microorganism that is gaining high and rising industrial importance over the last years. Comparative genomics studies have revealed a complex genetic structure of the species, as some strains are diploid (e. g. CBS 2499), whereas others are allotriploid (e. g. AWRI1499 and AWRI1608), resulting from independent hybridisation events (Borneman et al., 2014). In a recent study, we demonstrated that whole genetic clusters (namely CBS 2499-like, AWRI1499-like and AWRI1608-like) correlate with ploidy level of the strains and substrate of isolation (Avramova et al., submitted). Indeed, this yeast species is isolated from various human-related fermentation environments (see Avramova et al., submitted) and was suggested to have strong niche adaptation corresponding to different types of fermented beverages (Crauwels et al., 2017; Avramova et al., submitted), especially for the wine strains (Crauwels et al., 2017; Avramova et al., submitted). In wine, *B. bruxellensis* is considered a spoilage species, mainly due to the production of ethyl phenols (Chatonnet, 1992), which confer unpleasant aromas described as barnyard, horse sweat, and medicinal (Chatonnet et al., 1995b; Heresztyn, 1986). The spoilage potential of this yeast is further enhanced by its tolerance to sulfur dioxide (SO<sub>2</sub>) (Agnolucci et al., 2013, 2010; Curtin et al., 2012a) – the most broadly used antimicrobial agent in winemaking. Several scientific works have treated this issue over the last decade (Agnolucci et al., 2013; A. Barata et al., 2008; Capozzi et al., 2016; Curtin et al., 2012a; Vigentini et al., 2013; Zuehlke and Edwards, 2013; Avramova et al., submitted), and have underscored variable tolerance to SO<sub>2</sub> that correlates with genetic grouping of *B. bruxellensis* (Agnolucci et al., 2009; C. Curtin et al., 2012b). Strikingly, 92% of the Australian isolates (C. Curtin et al., 2012b) and high percentage of the Bordeaux isolates (Cibrario *et al.*, in preparation) were reported to have genotypes previously correlated with high SO<sub>2</sub> tolerance (C. Curtin et al., 2012b) Avramova et al., submitted. Ironically, it was suggested that SO<sub>2</sub> use applied constant selective pressure on *B. bruxellensis* wine population, thus

leading to the establishment of tolerant genotypes (C. Curtin et al., 2012b) and rendering common doses of SO<sub>2</sub> inefficient against this spoilage microorganism. Sulfur dioxide tolerance is accepted to be a hallmark of domestication for *Saccharomyces cerevisiae* wine strains (Legras et al., 2007) and thus it was recently suggested that *B. bruxellensis* was a species closely related to human activity (Avramova et al., submitted). The problem becomes even more complex, as the most tolerant strains have a allopolyploid configuration (Avramova et al., submitted; Borneman et al., 2014; Curtin et al., 2012) and co-exist with diploid populations in a diploid-triploid complex (Avramova et al., submitted). These characteristics make of *B. bruxellensis* an excellent model organism for the study of polyploidy as an adaptation strategy in eukaryotes. Indeed, the striking correlation between genetic configuration (namely, allotriploid state corresponding to AWRI1499-like group) and SO<sub>2</sub> tolerance, taken together with the high dispersal of triploid genotypes (C. Curtin et al., 2012b), Avramova et al., submitted, Cibrario *et al.*, in preparation) led to the hypothesis that the genetic configuration of AWRI1499-like strains is conferring selective advantage in winemaking conditions (Borneman et al., 2014) and exposure to SO<sub>2</sub> (Avramova et al., submitted). The aim of the present study is to verify this hypothesis. For that purpose, competition experiments were designed to evaluate the relative fitness of an allotriploid AWRI1499 strain versus a reference diploid strain (CBS 2499) and a divergent allotriploid (AWRI1608) in means of growth capacity. In order to differentiate the competitor and reference strain in mixed culture, competition trials with yeast at intraspecies level often involve constructing strains with auxotrophic or antibiotic resistance markers through genetic transformation. However, genetic manipulation of *B. bruxellensis* is still not a common laboratory practice, and directed genetic transformation presents a challenge due to the putative asexuality of this organism (Curtin and Pretorius, 2014; Avramova et al., submitted) and its mixed ploidy level (Borneman et al., 2014; Schifferdecker et al., 2016; Avramova et al., submitted). To address this problem, non-directed methods were made

available to the scientific community over the last years, based on both auxotrophic (Schifferdecker et al., 2016) and antibiotic resistance (Miklenić et al., 2015, 2013) markers. Here, we used an optimised version of the protocol published by (Miklenić et al., 2013) (Varela *et al.*, in preparation) and we constructed strains with antibiotic resistance cassettes, as those markers were shown to be better adapted for competition experiments (Baganz et al., 1997). The strains AWRI1499, AWRI1608 and AWRI1626 (=CBS 2499) were chosen as representatives of the three main *B. bruxellensis* genetic groups, associated with different genetic background, ploidy level, and SO<sub>2</sub> tolerance (Avramova et al., submitted). Thus, we tested the hypothesis of selective advantage of AWRI1499-like strains over AWRI1608-like or CBS 2499-like strains in presence of SO<sub>2</sub> through competition experiments. Additionally, our experimental design brings new insights on *B. bruxellensis*' interactions at intraspecies level and the impact of polyploidy on the adaptation capacities of the species.

## 3.2. Materials and methods

### 3.2.1. *Brettanomyces bruxellensis* strains

Three *B. bruxellensis* wine strains - AWRI1499 (allotriploid, SO<sub>2</sub> tolerant), AWRI1608 (allotriploid, SO<sub>2</sub> sensitive) and AWRI1626 (equivalent to CBS 2499, diploid, SO<sub>2</sub> sensitive) were chosen as representatives of the three major genetic groups of the *B. bruxellensis* population (Albertin et al., 2014b; Borneman et al., 2014) (see Avramova et al., submitted) and their different tolerance to sulfur dioxide (SO<sub>2</sub>) (see Avramova et al., submitted) (strain details in Table 3.1, and growth profiles in Supplementary Figure S-3.1 in Appendix).

**Table 3.1. Strains used in this study**

Strain name	Source of isolation	Country of isolation	Ploidy level	Sequence reference	SO <sub>2</sub> tolerance
AWRI1499	Wine	Australia	3n	Curtin <i>et al.</i> , 2012	Tolerant
AWRI1608	Wine	Australia	3n	Borneman <i>et al.</i> , 2014	Sensitive
AWRI1626 (=CBS 2499)	Wine	France	2n	Piškur <i>et al.</i> , 2012	Sensitive

### 3.2.2. Strains construction and selection of transformants

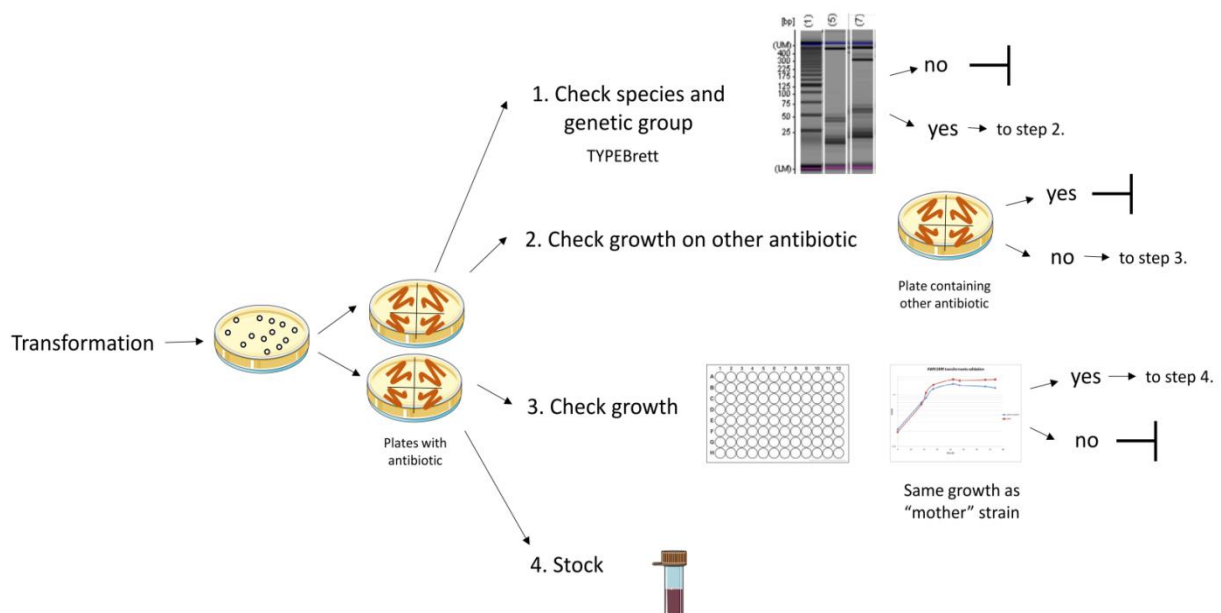
To differentiate AWRI1499, AWRI1608 and AWRI1626 in a mixed culture, strains were transformed with genes conferring resistance to either G418 (geneticin) or ClonNat (nourseothricin). Plasmids pMK-T-TDH1pr-KanMX and pMK-T-TDH1pr-NatMX were used to amplify transformation cassettes. In both plasmids the gene conferring antibiotic resistance is under the control of the strong *B. bruxellensis* promoter *TDH1*. *B. bruxellensis* strains were transformed by electroporation following the protocol suggested by (Miklenić et al., 2013). Briefly, strains were grown over night in liquid YPD medium (10 g.L<sup>-1</sup> yeast extract (Difco Laboratories, Detroit M1), 10 g.L<sup>-1</sup> bactopectone (Difco Laboratories, Detroit M1), and 20 g.L<sup>-1</sup> D-glucose (Sigma-Aldrich)). Then, 1 mL of culture was inoculated in 200 mL of fresh medium and incubated on orbital shaker (180 rpm, 28 °C) until a concentration of 5.10<sup>7</sup> cells.mL<sup>-1</sup> was achieved. Cells were then centrifuged at (3000 rpm, 4 min, room temperature), supernatant was carefully decanted and cells were washed with 50 mL deionised water (this step was repeated three times). Then, cells were re-suspended in 20 mL of a freshly made solution containing 35mM of dithiothreitol) and 100 mM lithium acetate, and incubated for 45 minutes at 28 °C with gentle shaking (140 rpm). After, cells were centrifuged (3000 rpm, 4 minutes, 4 °C) and pellet was washed with ice-cold sterile deionised water. From this step on, until the end of the electroporation procedure, the cells were maintained on ice. The washing procedure was then repeated two more times but with 20 mL of ice-cold 1M sorbitol solution. After, the cells were re-suspended in 1M sorbitol (ice-cold), so the total volume of suspension was 500 µL, 50 µL aliquots were distributed in micro-centrifuge tubes (1.5 mL, Eppendorf). A volume of 1 µL containing the DNA cassettes was then introduced into the samples, mixed thoroughly with a micropipette and incubated on ice for 5 minutes. Further, the samples were placed in separate 0.2 cm-gap electroporation cuvettes (Bio-Rad) previously kept on ice, and pulsed (1.8 kV, 5 ms) with Bio-Rad Gene Pulser Electroporator (600 Ω, 25 µF). Immediately

after the pulse, 1 mL of 1M sorbitol:YPD (1:1) ice-cold solution was added, and the samples were incubated at room temperature for 20 minutes without shaking. After the incubation step, the cells were placed in 10 mL sterile tubes and 1 mL of YPD was added. The samples were incubated overnight at 28 °C, 140 rpm and spread on YPD-G418 plates (G418, 200 mg.L<sup>-1</sup>) or YPD-Cln plates (Cln, 50 mg.L<sup>-1</sup>) depending on the transformation; each sample was inoculated on 2 plates. Plates were then incubated at 30 °C and transformants were visible after 5-10 days.

### 3.2.3. Transformation validation

To validate the successful transformation of the three *B. bruxellensis* strains, 8 random transformants for each strain were re-streaked on solid YPD medium containing the antibiotic to which the transformant was expected to be resistant (G418, 200 mg.L<sup>-1</sup> for KanMX transformants and ClonNat, 50 mg.L<sup>-1</sup> for NatMX transformants) (see Figure 3.1). Isolates were then tested by TYPEBrett PCR (method patent number PCT/FR2016/052701) to confirm the identity of the species and transformants' genetic group (Figure 3.1, Step 1.). Isolates were then streaked on plates containing the reciprocal antibiotic (e.g. G418 for isolates transformed with the NatMX cassette and ClonNat for isolates transformed with the KanMX cassette) (Figure 3.1, Step 2.); transformants which did not grow on reciprocal antibiotics were retained for further validation. DNA insertion in *B. bruxellensis* strains during transformation is non-homologous and random (Miklenić et al., 2013), and therefore phenotypical neutrality of the obtained transformants was evaluated. This was done by following cell growth in 96-well plates (Greiner, Frickenhausen, Germany) containing YNB medium (YNB, 6.7 g.l<sup>-1</sup>, D-Glucose, 2.5 g.l<sup>-1</sup>, and D-Fructose, 2.5 g.l<sup>-1</sup>, pH was adjusted to 3.5 with HCl 1M and the medium filtered with 0.22 µm pore filter) (Figure 3.1, Step 3.). Briefly, strains were first grown in YNB medium for 4 days and then, inoculated at OD<sub>600</sub> 0.1 in a final volume of 200 µL medium. Plates were covered with Breathe-Easy membranes (Diversified Biotech, Dedham, MA) and incubated at 28 °C. Inoculations were performed in triplicate and using the wild type

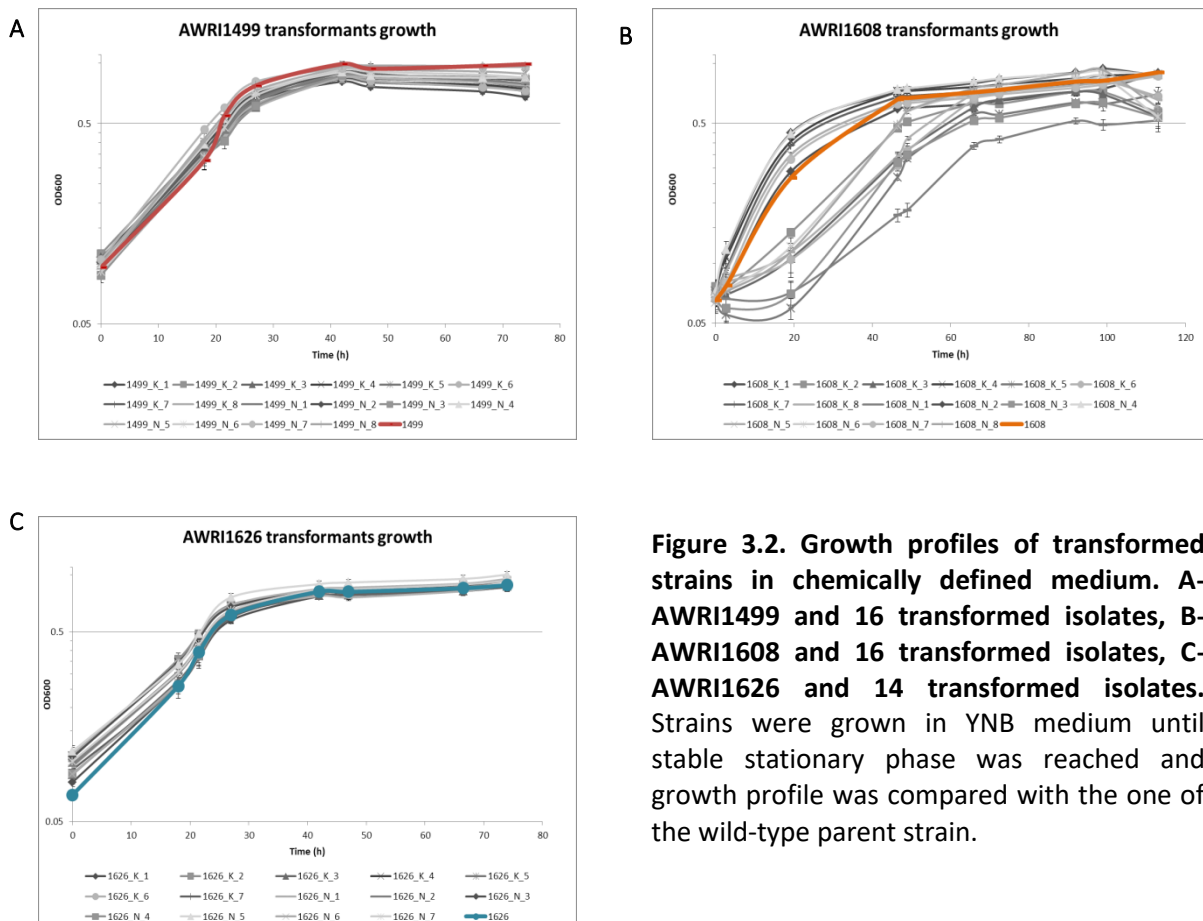
(WT) strains as a reference. Cell growth for each well was measured for 4 days with a microplate reader spectrophotometer (Spectramax M2, Molecular Devices). Transformants which showed similar growth curves to the corresponding wild-type strains were conserved in glycerol stock at -80 °C (Figure 3.1., Step 4.) and named according to strain, antibiotic cassette and isolate number, thus transformant name 1499\_K\_3, 1499 stands for wild type strain AWRI1499, K for the KanMX cassette inserted and 3 for the number of isolate from the eight that were chosen for transformation validation.



**Figure 3.1. Transformation validation steps.** After transformation, the culture containing the transformants is spread on YPD plates supplemented with antibiotic. Eight colonies are randomly picked and re-streaked on fresh plates. After 5-10 days of incubation, the transformants' species and genetic group are confirmed by PCR analysis (Step 1.). If the result corresponds to the expected one, biomass is re-streaked on another antibiotic to assess putative cross-resistance which is undesirable (Step 2.). If there is no growth, transformants' growth is assessed in YNB medium and compared to the wild type (WT) (Step 3.). If the transformants cover those parameters, they are conserved in glycerol stock at -80 °C for further use.

### 3.2.4. Competition experiments – media and growth conditions

Only transformants that had same growth profile as the wild type strains (Figure 3.2) were considered phenotypically neutral and were used for the rest of the experiment.



**Figure 3.2. Growth profiles of transformed strains in chemically defined medium. A- AWRI1499 and 16 transformed isolates, B- AWRI1608 and 16 transformed isolates, C- AWRI1626 and 14 transformed isolates.** Strains were grown in YNB medium until stable stationary phase was reached and growth profile was compared with the one of the wild-type parent strain.

Transformants used for competition experiments were grown in YNB medium until they reached stationary phase (4-5 days) (Figure 3.3, Step 1). Then competing pairs (combinations listed in Table 3.2) were inoculated in equivalent proportions in order to obtain an OD<sub>600</sub> 0.1 (*i.e.* 0.05 OD<sub>600</sub> for each strain) (Figure 3.3, Step 2.). Competition experiments were performed in synthetic media as described in Avramova et al., submitted, containing YNB, 6.7 g.L<sup>-1</sup>, D-Glucose, 2.5 g.L<sup>-1</sup>, D-Fructose, 2.5 g.L<sup>-1</sup>, ethanol, 5%, pH adjusted to 3.5 with HCl 1M and filtered with 0.22 μm pore filter. Potassium metabisulphite K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> was added at different concentrations from freshly made stock solution at 10 g.L<sup>-1</sup>. The final concentration was adjusted to obtain 0, 0.2, 0.4, and 0.6 mg.L<sup>-1</sup> molecular SO<sub>2</sub> (mSO<sub>2</sub>). Free SO<sub>2</sub> was measured by the aspiration/titration method (<http://www.moundtop.com/so2/SO2-Aspiration-Procedure2.pdf>) and molecular mSO<sub>2</sub> was estimated considering final temperature, pH and



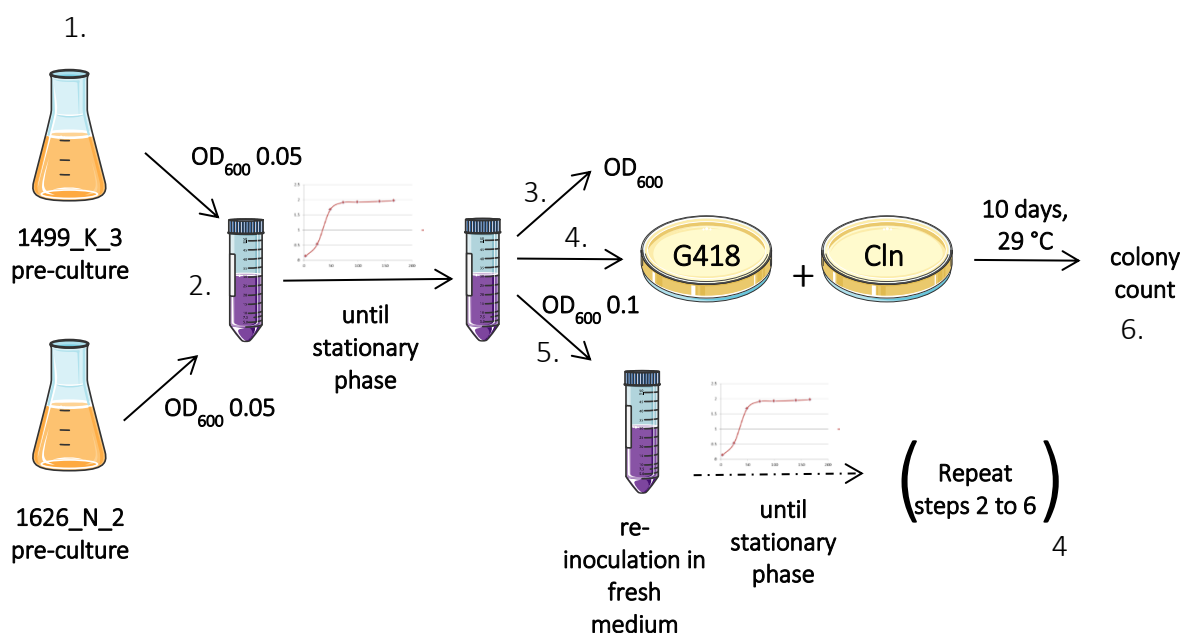
ethanol of the medium (calculations available at <https://www.vignevin-sudouest.com/services-professionnels/formulaires-calcul/so2-actif.php>). Competition experiments were performed in 50 mL centrifuge tubes (Grenier Bio-One) with 25 mL of medium at 28 °C and 140 rpm rotation shaking. Cultures were grown until an approximate OD<sub>600</sub> of 1.5-2 (roughly 4 generations) (Figure 3.3, Step 3.). At this point, samples were taken and spread on YPD plates containing antibiotics as indicated below (Figure 3.3, Step 4.), simultaneously cells were re-inoculated in fresh medium at OD<sub>600</sub> 0.1 (Figure 3.3, Step 5). These transfers were repeated until 20 generations of the total population were attained (or total of 4 transfer steps).

**Table 3.2. Competition couples**

Set 1	Set 2
<b>AWRI1626 vs AWRI1499</b>	<b>AWRI1608 vs AWRI1499</b>
1626_N_2 vs 1499_K_3	1608_N_4 vs 1499_K_3
1626_K_3 vs 1499_N_2	1608_K_1 vs 1499_N_2
1626_N_6 vs 1499_K_4	1608_N_5 vs 1499_K_4
1626_K_4 vs 1499_N_4	1608_K_4 vs 1499_N_4
1626_N_7 vs 1499_K_7	1608_N_7 vs 1499_K_7
1626_K_6 vs 1499_N_5	1608_K_7 vs 1499_N_5

DNA insertion in *B. bruxellensis* strains during transformation is non-homologous and random (Miklenić et al., 2013), and therefore controls were included to evaluate the possible impact of the transformation on the growth of the strains and their tolerance to SO<sub>2</sub>. To assess the possible impact of the introduced DNA cassette strains containing either antibiotic marker were evaluated, for instance strain A-KanMX vs strain B-NatMX and strain A-NatMX vs strain B-KanMX). Additionally, controls strain A-KanMX vs strain A-NatMX and strain B-KanMX vs strain B-NatMX were also included for all competing pairs at every mSO<sub>2</sub> concentrations (Supplementary Figure S-3.2 in Appendix). To avoid the potential bias introduced by using strains resulting from random insertion, different transformants (different transformed clones) were used as ‘biological triplicates’. To test the repeatability of the competition experiments

the same AWRI1499 transformants were used in both competition sets, AWRI1499 vs AWRI1608 and AWRI1499 vs AWRI1626.



**Figure 3.3. Transfer cycles.** Pre-cultures are grown for each transformant from a competition couple until stationary phase (1.). Further, both strains are inoculated in ratio 1:1 and total OD<sub>600</sub> 0.1 in fresh media (with different concentrations of SO<sub>2</sub>) (2.). The mixed culture is grown until stationary phase. At that point, sample is taken for OD<sub>600</sub> measure (3.); spreading on agar plates containing antibiotics (4.) which are later incubated and colonies are counter after 10 days; and sample is used for re-inoculation in fresh medium with the same concentration of SO<sub>2</sub> (5.). The cycles are repeated until approximate 20 generations, corresponding to 4 cycles.

### 3.2.5. Colony enumeration

Strain enumeration in mixed culture was assessed by plating on agar plates containing either G418, 200 mg.L<sup>-1</sup> or ClonNat, 50 mg.L<sup>-1</sup>. At each transfer step, samples were spread on agar plates (Cln and G418 separately) at two concentrations (no dilution and dilution 10<sup>-3</sup>; dilutions were done in PBS solution containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH adjusted to 7.4) using an automated spiral plater (Whitly). The spiral plater is dispensing a constant sample volume from the centre to the periphery of the plate, thus creating gradual decrease in cell concentration. Thus, the enumeration detection range covered 5 orders of magnitude (from 10<sup>2</sup> to 10<sup>7</sup>). Briefly, samples were thoroughly mixed with vortex machine

and 50  $\mu\text{L}$  were spread with spiral plater on plates containing 25 mL of medium. Plates were then incubated at 28 °C for 10 days and at least two enumerations per sample were performed using Protocol 3 Colony Counter (Synbiosis) using the “Two sector” option of the included software at maximum sensitivity and option “Split touching colonies”. The counts were manually adjusted to eliminate false positives and add colonies that were not taken into account by the software. Final colony counts were used to obtain  $\log_2$  ratios for each competing pair,  $\log_2(\text{AWRI1499}/\text{AWRI1626})$  and  $\log_2(\text{AWRI1499}/\text{AWRI1608})$ .  $\log_2$  ratios were then used in a heat map using heatmap.2 plot in gplots R package (<http://www.r-project.org/>).

### **3.2.6. Wild type control**

In order to test if the introduction of DNA affected strain growth and competition ability, wild-type strains were subjected to competition experiments. Thus, AWRI1499 and AWRI1608 strains were inoculated following the procedure indicated above. Fermentations were performed in triplicate for each  $\text{mSO}_2$  concentration (0, 0.2, 0.4, and 0.6  $\text{mg}\cdot\text{L}^{-1}$   $\text{mSO}_2$ ). Based on results obtained with transformed strains, samples from 0, 8 and 12 generations were chosen for colony enumeration. For each sample 30 random colonies were picked and lysed in 30  $\mu\text{L}$  of 20mM NaOH and then heated for 10 minutes at 99°C with iCycler thermal cycler (Biorad, Hercules, CA, USA). Then, TYPEBrett PCR (patent number PCT/FR2016/052701) was used to identify the genetic group of each colony.

### **3.3. Results**

Briefly, transformants were validated by testing their phenotypical neutrality in means of growth behaviour and cross antibiotic resistance. Validated transformants were used in pairwise competition – diploid AWRI1626 *vs* triploid AWRI1499, and triploid AWRI1608 *vs* triploid AWRI1608. The transformants are results from random DNA insertion of antibiotic resistance cassette *via* non-homologous recombination. Thus, controls comprising strains with

same genetic background but different antibiotic resistance markers were included in the experiment.

### **3.3.1. Validation of transformants**

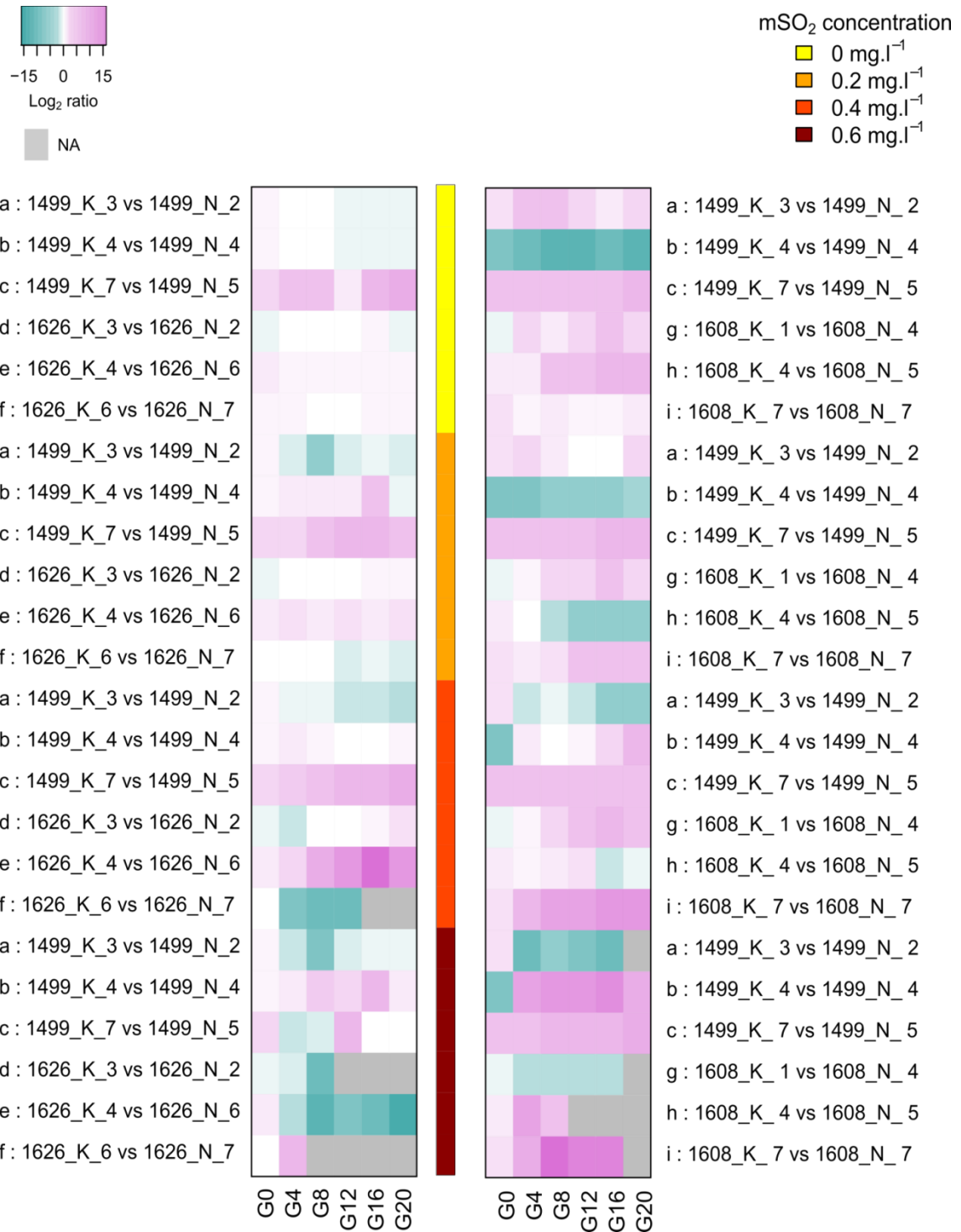
A total of 48 transformant colonies (corresponding to 2 antibiotic resistance marker cassettes per genetic background and 8 colonies per combination ‘genetic background and antibiotic resistance marker’) were validated confirming yeast species, genetic group identity, as well as antibiotic resistance (Table 3.3). This ensured that no contaminations occurred during transformation (as transformations of several strains were performed simultaneously) and that the DNA cassette insertion did not affect regions used for species and genetic group identification. Only one strain however, showed resistance to the reciprocal antibiotic (Table 3.3). Most transformants showed a similar growth profile to the corresponding wild-type strain (Figure 3.2), although some differences were observed for 6 of the AWRI1608 transformants. Therefore, AWRI1608 KanMX 2, 5, 6 and AWRI1608NatMX 1, 3, and 8 (Table 3.3) were not included in the competition experiments.

**Table 3.3. Transformation validation - validated (+) and non- validated (-) parameters**

Transformant	TYPEBrett	Antibiotic resistance	Growth on reciprocal antibiotic	Growth YNB
1499_K_1	+	+	+	+
1499_K_2	+	+	+	+
1499_K_3	+	+	+	+
1499_K_4	+	+	+	+
1499_K_5	+	+	+	+
1499_K_6	+	+	+	+
1499_K_7	+	+	+	+
1499_K_8	+	+	+	+
1499_N_1	+	+	+	+
1499_N_2	+	+	+	+
1499_N_3	+	+	+	+
1499_N_4	+	+	+	+
1499_N_5	+	+	+	+
1499_N_6	+	+	+	+
1499_N_7	+	+	+	+
1499_N_8	+	+	+	+
1626_K_1	+	+	+	+
1626_K_2	+	+	+	+
1626_K_3	+	+	+	+
1626_K_4	+	+	+	+
1626_K_5	+	+	+	+
1626_K_6	+	+	+	+
1626_K_7	+	+	+	+
1626_K_8	+	+	+	+
1626_N_1	+	+	+	+
1626_N_2	+	+	+	+
1626_N_3	+	+	+	+
1626_N_4	+	+	+	+
1626_N_5	+	+	+	+
1626_N_6	+	+	+	+
1626_N_7	+	+	+	+
1626_N_8	+	+	-	+
1608_K_1	+	+	+	+
1608_K_2	+	+	+	-
1608_K_3	+	+	+	+
1608_K_4	+	+	+	+
1608_K_5	+	+	+	-
1608_K_6	+	+	+	-
1608_K_7	+	+	+	+
1608_K_8	+	+	+	+
1608_N_1	+	+	+	-
1608_N_2	+	+	+	+
1608_N_3	+	+	+	-
1608_N_4	+	+	+	+
1608_N_5	+	+	+	+
1608_N_6	+	+	+	+
1608_N_7	+	+	+	+
1608_N_8	+	+	+	-

### **3.3.2. Competition experiments**

As controls, transformants with the same genetic background but different selective markers were subjected to competition experiments under different mSO<sub>2</sub> concentrations. Figure 3.4 shows the log<sub>2</sub> of the ratio between KanMX over NatMX transformants. No patterns were observed regarding antibiotic cassettes or mSO<sub>2</sub> concentration between competing pairs. This suggested that the dominance observed for some individual isolates was random and not related to the nature of the DNA cassette. In fact, differences in log<sub>2</sub> ratios for repeated AWRI1499 competing pairs were related to the initial ratio at inoculation time (G0).

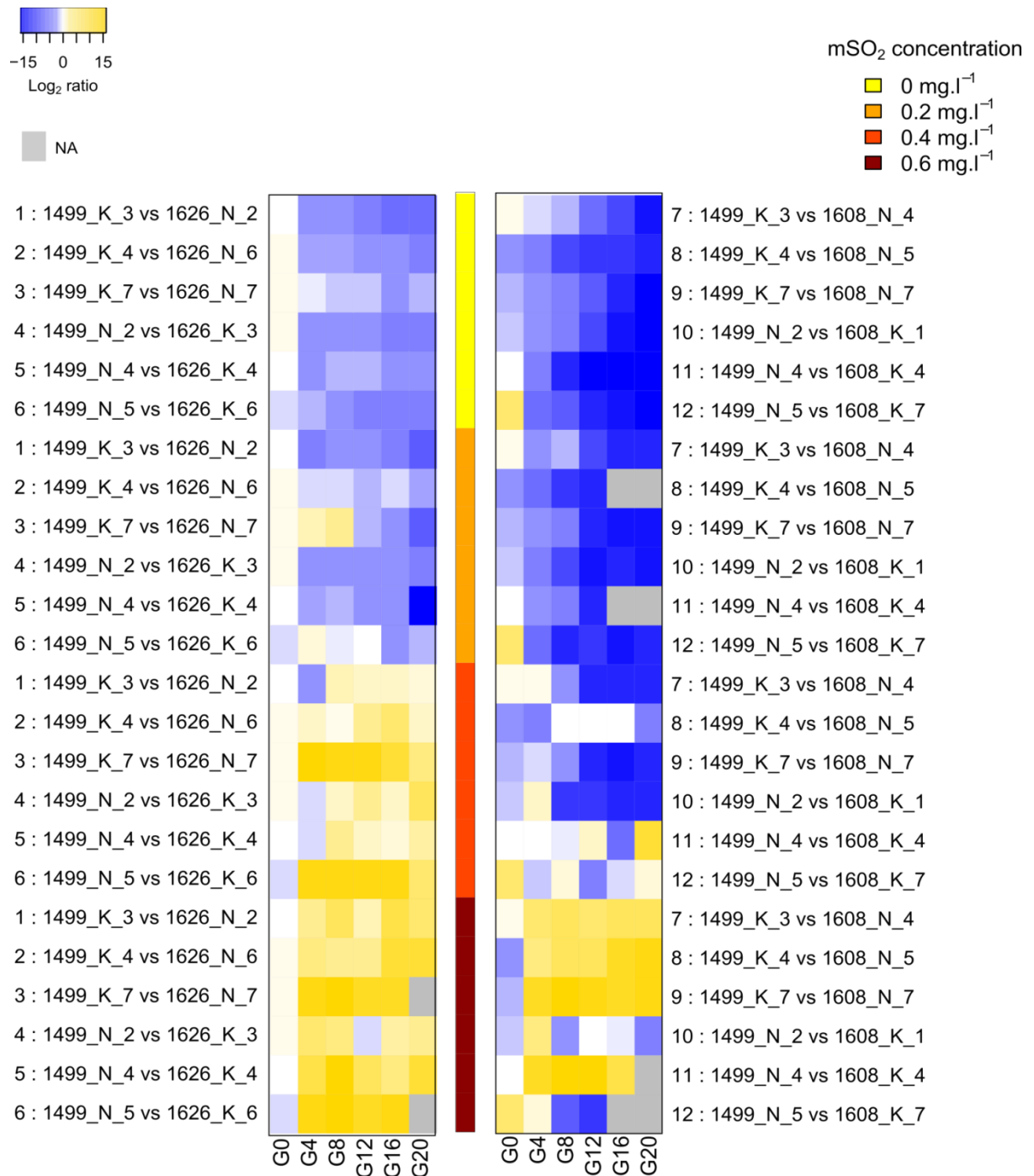


**Figure 3.4. Competition controls.** Transformants with the same genetic background but different antibiotic resistance cassettes were put in competition as controls of the competition experiments between strains with different genetic backgrounds. Each square represents the  $\text{log}_2$  of the ratio between strains (KanMX/NatMX).

Transformants for the three strains, AWRI1499, AWRI1608 and AWRI1626 were assessed by pairwise competition experiments (AWRI1499 vs AWRI1626, and AWRI1499 vs AWRI1608).

For AWRI1499 *vs* AWRI1626, two growth patterns were observed (Figure 3.5). At low mSO<sub>2</sub> concentrations (0 and 0.2 mg.L<sup>-1</sup>), AWRI1626 transformants outcompeted AWRI1499 transformants and this phenomenon is maintained and/or intensified over time depending on the competing pair. Although AWRI1626 transformants dominated over AWRI1499 transformants, AWRI1499 isolates showed moderate population numbers (in the order of 10<sup>5</sup>-10<sup>6</sup> UFC.mL<sup>-1</sup>, raw count numbers are shown in Supplementary Table S-3.1 in Appendix). On the contrary, at high mSO<sub>2</sub> concentrations (0.4 and 0.6 mg.L<sup>-1</sup>), AWRI1499 transformants outcompeted AWRI1626 isolates and this dominance seemed more intense for 0.6 mg.L<sup>-1</sup> of mSO<sub>2</sub> than for 0.4 mg.L<sup>-1</sup>. No differences were observed between the pairs 1499\_K *vs* 1626\_N and 1499\_N *vs* 1626\_K, suggesting that there is no effect of the type of cassette on the competition behaviour of the transformants.



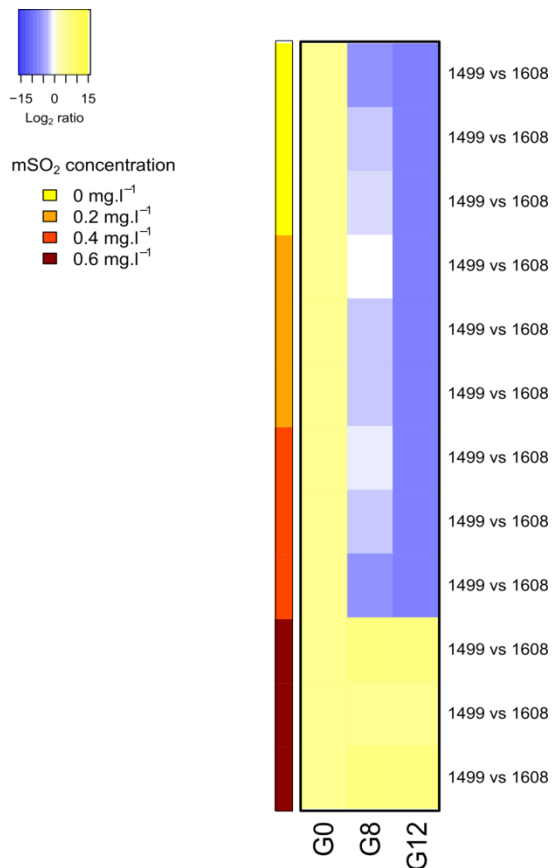


**Figure 3.5. Competition between transformants with different genetic backgrounds.** Transformants with different genetic background were put in competition. Each square represents the  $\log_2$  of the ratio between strains (1499/1626) on the left and (1499/1608) on the right.

In competitions experiments between AWRI1499 and AWRI1608, transformants of this latter strain outcompeted AWRI1499 isolates at 0, 0.2, and 0.4  $\text{mg.L}^{-1}$   $\text{mSO}_2$ , whereas at 0.6  $\text{mg.L}^{-1}$  AWRI1499 transformants outcompeted AWRI1608 isolates. For these strains, the proportions

of AWRI1499 and AWRI1608 in the initial inoculum were not always equal (see column G0 on Figure 3.6), but this did not affect the subsequent behaviour of the strains during the experiment, as pairs with higher initial inoculum of AWRI1608 than AWRI1499 behaved in the same way as competing pairs where at inoculation there were higher numbers of AWRI1499 or equal proportions between strains (e. g. couples 1 and 2 at 0 mSO<sub>2</sub>). Interestingly, at low mSO<sub>2</sub> concentrations AWRI1499 transformants were not detected by colony counting (<400 UFC.mL<sup>-1</sup>). This finding differs from the results obtained in competition experiments between AWRI1499 and AWRI1626 where both strains coexisted up to G20 stage (Supplementary Table S-3.1). At 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub>, two out of the six competing pairs (pairs 10 and 12, Figure 3.5) showed a dominance of the AWRI1608 transformant over the AWRI1499 isolate. This indicated that although transformants were carefully selected, the random nature of the transformation process can still affect the ability of certain isolates to compete.

Competition experiments performed with the wild-type strains AWRI1499 and AWRI1608 (Figure 3.6) validated the results obtained with the transformants and confirmed the reliability of the proposed experimental design. Wild-type competition experiments were particularly useful to confirm the dominance of AWRI1499 over AWRI1608 at 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub>.



**Figure 3.6. Wild-type controls of the competition experiments.** WT strains AWRI1499 and AWRI1608 were put in competition as controls.

### 3.4. Discussion

Previous studies highlighted contrasted tolerance to sulfur dioxide among *Brettanomyces bruxellensis* strains, which was correlated with differences in the strains' genetic background (C. Curtin et al., 2012b). It was further hypothesised that strains from AWRI1499-like group were specifically adapted to medium with SO<sub>2</sub> – a characteristic in relation with human activity (Avramova et al., submitted). However, to confirm the latter hypothesis, the relative selective advantage of these strains should be evaluated through competition experiments in presence or absence of SO<sub>2</sub>. It was previously demonstrated that, antibiotic resistance markers were suited for competition trials due to their negligible effect on transformants' growth rate when compared to wild type (Baganz et al., 1997). Different cassette types were developed (Goldstein and McCusker, 1999), allowing the broad application of this method for yeast relative fitness evaluation in industrial and clinical context. However, genetic transformation is still not ordinary part of laboratory work with *B. bruxellensis*. Despite the growing industrial

importance of this non-conventional microorganism, there is no protocol for its directed genetic transformation. Protocols for undirected genetic transformation of *B. bruxellensis* strains with both auxotrophic (Schifferdecker et al., 2016) and antibiotic resistance markers (Miklenić et al., 2015, 2013) were made available for the scientific community, but are still not broadly applied. Here, we used an optimised protocol for transformation with antibiotic resistance markers method based on non-homologous end joining for three previously sequenced *B. bruxellensis* strains (Borneman et al., 2014; Curtin et al., 2012; Piškur et al., 2012). We report the first successful genetic transformation of triploid *B. bruxellensis* strains to our knowledge – AWRI1499 and AWRI1608. The transformation protocol used in this study led to the construction of phenotypically neutral strains in means of cross-resistance and growth behaviour and opens the way for further genetic studies on this non-conventional yeast.

Previous studies highlighted major genetic and phenotypic differences between the strains AWRI1499, AWRI1608 and CBS 2499 (=AWRI1626) underscoring differences in ploidy level and genetic configuration. Here, we use those three strains as representatives of the major genetic clusters of the global *B. bruxellensis* population (Avramova et al., submitted), characterised by different ploidy level (diploid and triploid) and sulphite sensitivity (tolerant and sensitive). Pairwise competitions showed that, at low SO<sub>2</sub> concentrations, the diploid AWRI1626 strain has a relative selective advantage compared to the allotriploid AWRI1499 until a switching point (>0.2 mg.L<sup>-1</sup> mSO<sub>2</sub>), when AWRI1499 takes over AWRI1626. Similar situation was observed for AWRI1608 vs AWRI1499 (both triploid) but with a switching point at 0.4 mg.L<sup>-1</sup> mSO<sub>2</sub>. Strikingly, in the case of *B. bruxellensis*, all highly SO<sub>2</sub> tolerant wine strains are shown to be triploid (see Avramova et al., submitted). Stable polyploid state is thought to be maintained only if it confers selective advantage to the cell (Wertheimer et al., 2016), despite the possible slowing down of growth rate and cell metabolism (Otto, 2007). Polyploidy was shown to confer adaptive capacity to yeast cells through higher rate of

beneficial mutations (Selmecki et al., 2015), changes in cell physiology (Hegreness et al., 2006), or compensation of deleterious mutations. Other cases of adaptation to industrial environments through polyploidisation have already been described, such as the case of autotetraploid *S. cerevisiae* strains used in in baking for which the tetraploid state is assumed to confer fitness in the presence of high osmotic pressure (Albertin et al., 2009). The allopolyploid state of the strains AWRI1499 and AWRI1608 originate from independent hybridisation events between a diploid parent strain and unknown donors of haploid additional genome. *Brettanomyces bruxellensis* being supposedly an asexual organism (Avramova et al., submitted; Curtin and Pretorius, 2014), allopolyploidy is a plausible evolution strategy for expanding environmental tolerance. Similar cases are reported within *Saccharomyces* genus where triploid hybrids exist and often combine beneficial traits from two (or more) parents (Blein-Nicolas et al., 2015; Libkind et al., 2011; Marsit and Dequin, 2015; Masneuf et al., 1998; Querol and Bond, 2009). Thus, the question if the evolutionary success of allotriploid strains (AWRI1499-like and AWRI1608-like) would be related to characteristics other than SO<sub>2</sub> tolerance remains open and could be explored with the experimental approach presented here.

The contrasted relative fitness of the strain AWRI1499 at different SO<sub>2</sub> concentration confirms the previously stated hypothesis that AWRI1499-like strains could have a relative selective advantage in the presence of SO<sub>2</sub> (Borneman et al., 2014; Curtin et al., 2012). Similar situation of selective advantage related to SO<sub>2</sub> tolerance was reported when studying competition between *S. cerevisiae* strains (Pérez-Torrado et al., 2017; Zimmer et al., 2014). Indeed, it was pointed out that sulphite tolerance, particularly *SSUI* overexpression (Pérez-Torrado et al., 2017) and shorter lag phase in presence of SO<sub>2</sub> (Zimmer et al., 2014), take part in the factors leading to ecological advantage of dominant *S. cerevisiae* strains. Taking into account that AWRI1499 is specifically adapted to high concentrations of SO<sub>2</sub>, it can be assumed that, the

spoilage prevention practice of SO<sub>2</sub> addition, also leads to unwitting selection for a SO<sub>2</sub> tolerant genotype. This hypothesis is further confirmed by previous ecology studies on the species, reporting a high proportion of AWRI1499-like isolates which presented 92% of Australian isolates (C. Curtin et al., 2012b; Chris D. Curtin et al., 2007). This was also recently confirmed for the Bordeaux region, where AWRI1499-like strains represented 50% out of 731 isolates together with diploid CBS 2499-like isolates (Cibrario et al, in preparation). Moreover, an emergence of the AWRI1499-like group over the last 25 years (after the year 1990) was suggested (Cibrario et al, in preparation), which also strikingly correlates with the period when *B. bruxellensis* was associated for the first time with the presence of ethyl phenols in wine (Heresztyn, 1986; Chatonnet, 1992). This scientific discovery possibly drew an increased attention on this spoilage microorganism in the wine industry and was followed by rise of SO<sub>2</sub> use that acted as selection pressure on *B. bruxellensis* wine population. In *S. cerevisiae*, the translocations responsible for adaptation to sulphite (Pérez-Ortín et al., 2002; Zimmer et al., 2014), were demonstrated to be prevalent among wine strains (Zimmer et al., 2014). It was suggested that these strains may have been selected by human activity for their rapid colonisation of medium containing SO<sub>2</sub> (Zimmer et al., 2014). In *B. bruxellensis*, the existence of a major allotriploid group tolerant to sulphites is another example of human related selection process of wine microorganisms through SO<sub>2</sub> addition. However, for this species the molecular mechanisms behind SO<sub>2</sub> tolerance remain to be revealed.

Human-conducted fermentations provide complex environments where multiple genera, species, and strains of microorganisms coexist in the presence of various stress factors. Microbial interactions therefore take place and are important elements influencing wine quality (Fleet, 2003). Even if the interactions between wine yeast have been broadly studied at the interspecies level, the relations between strains of the same species have often been neglected (Fleet, 2003). In ecology, interactions are generally classified as cooperation (a behaviour

which provides a benefit to another individual, and which is selected for because of its beneficial effect on the recipient (West et al., 2007), and competition (the negative effects which one organism has upon another by consuming, or controlling access to, a resource that is limited in availability). More specifically, competition can be symmetrical (where the two populations coexist) and asymmetrical (where one population excludes the other)(Shearer, 1995). The competition trial performed here, highlighted coexistence of both AWRI1499 and AWRI1626 strains at 0, 0.2, and 0.4 mg.L<sup>-1</sup> mSO<sub>2</sub> (even if there was always dominance of one population over the other). This suggests populations of both genetic groups would be able to share the same niche space. The coexistence of AWRI1499 and AWRI1626 strains demonstrated in this experiment also supports the idea of a diploid-triploid complex suggested previously, where diploid and triploid *B. bruxellensis* populations coexist (see Avramova et al., submitted). It is interesting to notice that in the case of AWRI1499 (allotriploid) and AWRI1608 (divergent allotriploid), the relation is different, as AWRI1608 completely displaces AWRI1499 and at G20 AWRI1499 population is under the detection limit (<400 UFC.mL<sup>-1</sup>, Supplementary Table S-3.1 in Appendix). These results suggest that, at low SO<sub>2</sub> concentration, an actual competition with exclusion (Hibbing et al., 2010) occurs between the triploid strains AWRI1499 and AWRI1608. Thus, the comparison between the two competition couples demonstrates different interaction profiles and highlights the complexity of intraspecies relations for *B. bruxellensis*.

The industrially relevant yeast *B. bruxellensis* is an exceptional model of adaptation to diverse human-related environments *via* genome plasticity and acquisition of stable polyploid populations. The proposed competition protocol will allow taking into account the interactions between *B. bruxellensis* strains when studying this yeast's striking adaptation capacity.

## **Chapter 4. Molecular diagnosis of *Brettanomyces bruxellensis*' sulfur dioxide sensitivity through genotype specific method**

Marta Avramova<sup>1,2</sup>, Amélie Vallet-Courbin<sup>3</sup>, Julie Maupeu<sup>3</sup>, Isabelle Masneuf-Pomarède<sup>1,4</sup>, Warren Albertin<sup>1,5</sup>

<sup>1</sup>Univ. Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRA, Bordeaux INP, 33140 Villenave d'Ornon, France <sup>2</sup>School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia <sup>3</sup>Microflora-ADERA, ISVV, Unité de recherche Œnologie EA 4577, 33140 Villenave d'Ornon, France <sup>4</sup>Bordeaux Sciences Agro, 33170 Gradignan, France <sup>5</sup>ENSCBP, Bordeaux INP, 33600 Pessac, France

Status: in preparation





## 4.1. Introduction

Yeast metabolism is one of the multiple factors shaping wine aromatic and flavour properties (Fleet, 2003). Through various processes, winemakers manage the transformation of must into wine, aiming to obtain high quality product according to their wants and the expectations of their customers. However, wine chemical and microbiological properties being in constant evolution throughout the winemaking process, there are always parameters that are difficult to control. One example of such phenomenon is spoilage by *Brettanomyces bruxellensis* – yeast related to production of off-aromas with descriptors “barnyard”, “horse sweat”, “medicinal” (Heresztyn, 1986; Chatonnet, 1992). The most common method to prevent and/or control *B. bruxellensis* spoilage is the addition of sulfur dioxide into must and wine. Sulphites are used in winemaking at least since the 18<sup>th</sup> century and are introduced by both burning of sulphur tablets in barrels and in liquid form (mainly potassium bisulphite solution addition to must and wine)(Pascal Ribéreau-Gayon et al., 2017). Sulphur dioxide is broadly used in winemaking not only for its antiseptic action, but also for its antioxidant, and antioxidasic properties (Ribéreau-Gayon et al., 2017). Thus, SO<sub>2</sub> addition is the preferred choice when it comes to *B. bruxellensis* spoilage prevention. Unfortunately, over the last years, *B. bruxellensis* was reported to be tolerant to commonly used doses of SO<sub>2</sub>. Furthermore, its survival in presence of SO<sub>2</sub> was shown to be variable among isolates (Agnolucci et al., 2013; Barata et al., 2008; Curtin et al., 2012a). This variability makes the prediction of *B. bruxellensis* spoilage potential and the choice of adequate antimicrobial agent a challenge for winemakers. Recently, it was shown that *B. bruxellensis* SO<sub>2</sub> sensitivity correlates with genotype defined by AFLP (Curtin et al., 2012a) and microsatellite markers (Avramova et al., submitted). Among the six main clusters of *B. bruxellensis* population (Avramova et al., submitted), the AWRI1499-like genetic cluster was highlighted to comprise isolates with high SO<sub>2</sub> tolerance (Avramova et al., submitted). Furthermore, the strain AWRI1499 was demonstrated to have a selective advantage in presence

of SO<sub>2</sub> when compared to other wine strains, thus suggesting a specific adaptation of isolates from AWRI1499-like genetic cluster to this antimicrobial and higher wine spoilage potential (Avramova *et al.*, in preparation). The aims of this study were i) to extend the screening of SO<sub>2</sub> sensitivity to 106 additional isolates, ii) to confirm the correlation between genetic clusters and SO<sub>2</sub> sensitivity to a larger collection representative of the global *B. bruxellensis* population and iii) to validate the applicability of a method for *B. bruxellensis* SO<sub>2</sub> tolerance prediction through genetic markers analysis by using microsatellites.

## 4.2. Material and methods

### 4.2.1. Strains

In this study, 106 strains from different geographical and industrial fermentation origins were used based on their microsatellite profile as defined in the previous chapter. Those strains were evaluated for their tolerance to SO<sub>2</sub> using the same protocol as in Avramova *et al.*, submitted which made possible the combination of both datasets together to give a total of 145 strains (Table 4.1).

**Table 4.1. Strains used for sulfur dioxide tolerance assay**

Strain	Substrate	Country	Vintage	Genetic group	Phenotype analysis reference
13EN11C5	Wine	France	2013	CBS 2499-like	Avramova <i>et al.</i> , submitted
13E5A6	Wine	France	2013	CBS 2499-like	This study
12AVB1	Wine	France	1912	CBS 2499-like	This study
26AVB2	Wine	France	1926	CBS 2499-like	This study
59AVB3	Wine	France	1959	CBS 2499-like	This study
19b/19	Wine	Germany	NA	CBS 2499-like	This study
AWRI 2915	Wine	Australia	2014	CBS 2499-like	This study
AWRI1615	Wine	Australia	2003	CBS 2499-like	This study
B001-14 T28 7	Wine	France	2014	CBS 2499-like	This study
B002-14 T14 7	Wine	France	2014	CBS 2499-like	This study
CBS 2499	Wine	France	1990	CBS 2499-like	Avramova <i>et al.</i> , submitted
DEN6_12_10	Wine	Denmark	2012	CBS 2499-like	This study
DEN6_13_2	Wine	Denmark	2012	CBS 2499-like	This study
DEN612_9	Wine	Denmark	2012	CBS 2499-like	Avramova <i>et al.</i> , submitted
GB64	Wine	Italy	2014	CBS 2499-like	This study
GB70	Wine	Italy	2014	CBS 2499-like	This study
ISA2150	Wine	Portugal	2002	CBS 2499-like	This study
KOM14106	Kombucha	France	NA	CBS 2499-like	Avramova <i>et al.</i> , submitted
L02/E2 AZ	Wine	France	NA	CBS 2499-like	Avramova <i>et al.</i> , submitted

L0469	Wine	France	2004	CBS 2499-like	Avramova <i>et al.</i> , submitted
L0611	Wine	France	1938	CBS 2499-like	Avramova <i>et al.</i> , submitted
L0612	Wine	France	1941	CBS 2499-like	This study
L0614	Wine	France	1994	CBS 2499-like	This study
L0615	Wine	France	1994	CBS 2499-like	This study
L0616	Wine	France	1995	CBS 2499-like	This study
L0618	Wine	France	1949	CBS 2499-like	This study
L0619	Wine	France	1993	CBS 2499-like	This study
L14160	Kombucha	NA	2011	CBS 2499-like	Avramova <i>et al.</i> , submitted
L14163	Wine	New Zealand	2001	CBS 2499-like	Avramova <i>et al.</i> , submitted
L14168	Wine	South Africa	2004	CBS 2499-like	Avramova <i>et al.</i> , submitted
L14186	Cider	USA	2004	CBS 2499-like	Avramova <i>et al.</i> , submitted
Mauve-1991	Wine	France	1991	CBS 2499-like	This study
MRC78	Wine	Brazil	2003 - 2011	CBS 2499-like	This study
SJ12-2	Wine	France	2012	CBS 2499-like	This study
SJ12-5	Wine	France	2012	CBS 2499-like	This study
SJ12-6	Wine	France	2012	CBS 2499-like	This study
VP1528	Wine	Italy	2013	CBS 2499-like	This study
YJS5440	Wine	South Africa	2003	CBS 2499-like	This study
YJS5447	Wine	South Africa	2005	CBS 2499-like	This study
YJS5453	NA	Spain	NA	CBS 2499-like	This study
YJS5458	NA	South Africa	2004	CBS 2499-like	This study
YJS5461	NA	Spain	NA	CBS 2499-like	This study
CBS 3025	Beer	United Kingdom	1990	L14165-like	Avramova <i>et al.</i> , submitted
GB28	Wine	Italy	2014	L14165-like	This study
GB34	Wine	Italy	2014	L14165-like	This study
GB45	Beer	Belgium	NA	L14165-like	This study
GB48	Beer	Belgium	NA	L14165-like	This study
ISA1327	Wine	Portugal	1991	L14165-like	This study
KOM1449	Kombucha	France	NA	L14165-like	Avramova <i>et al.</i> , submitted
KOM1460	Kombucha	France	NA	L14165-like	This study
L0463	Wine	France	2004	L14165-like	This study
L14184	Beer	USA	2013	L14165-like	This study
MRC177	Wine	Brazil	2003 - 2011	L14165-like	This study
VP1502	Wine	Italy	2013	L14165-like	This study
YJS5310	NA	Netherlands	NA	L14165-like	This study
YJS5334	Wine	Italy	2006	L14165-like	This study
YJS5344	NA	Belgium	NA	L14165-like	This study
YJS5368	Wine	Italy	NA	L14165-like	This study
YJS5407	NA	Belgium	NA	L14165-like	This study
YJS5413	Wine	Italy	NA	L14165-like	This study
2OT13_01	Wine	France	2013	AWRI1608-like	This study
2OT13_09	Wine	France	2013	AWRI1608-like	This study
2OT13_10	Wine	France	2013	AWRI1608-like	This study
2OT14_02	Wine	France	2014	AWRI1608-like	This study
2OT14_05	Wine	France	2014	AWRI1608-like	This study
AWRI 1606	Wine	Australia	2002	AWRI1608-like	This study
AWRI1608	Wine	Australia	2001	AWRI1608-like	Avramova <i>et al.</i> , submitted
AWRI1677	Wine	Australia	NA	AWRI1608-like	Avramova <i>et al.</i> , submitted
CDR222	Wine	France	2003	AWRI1608-like	Avramova <i>et al.</i> , submitted
DENN12_8	Wine	Denmark	2012	AWRI1608-like	This study
DENN12_9	Wine	Denmark	2012	AWRI1608-like	This study

GB23	Wine	Italy	2013	AWRI1608-like	This study
GB27	Wine	Italy	2014	AWRI1608-like	This study
GB66	Wine	Italy	2014	AWRI1608-like	This study
GSP1504	Beer	Italy	2015	AWRI1608-like	Avramova <i>et al.</i> , submitted
GSP1513	Beer	Italy	2015	AWRI1608-like	This study
GSP1516	Beer	Italy	2015	AWRI1608-like	Avramova <i>et al.</i> , submitted
GSP1518	Beer	Italy	2015	AWRI1608-like	Avramova <i>et al.</i> , submitted
GSP1520	Beer	Italy	2015	AWRI1608-like	
ISA1700	Wine	Portugal	1995	AWRI1608-like	Avramova <i>et al.</i> , submitted
L0422	Wine	France	2003	AWRI1608-like	Avramova <i>et al.</i> , submitted
L14173	Wine	USA	2001	AWRI1608-like	This study
L14183	Wine	Uruguay	2005	AWRI1608-like	Avramova <i>et al.</i> , submitted
L14195	Beer	Belgium	1931	AWRI1608-like	This study
LAN1505	Wine	France	NA	AWRI1608-like	This study
MLC_296_2014_15	Wine	France	2012	AWRI1608-like	This study
MLC_296_2014_6	Wine	France	2012	AWRI1608-like	This study
YJS5396	NA	Belgium	NA	AWRI1608-like	This study
YJS5400	Wine	Italy	NA	AWRI1608-like	Avramova <i>et al.</i> , submitted
YJS5454	NA	Chile	NA	AWRI1608-like	This study
12ES26B8	Wine	France	2012	AWRI1499-like	This study
13EG55B1	Wine	France	2013	AWRI1499-like	This study
13EG55B2	Wine	France	2013	AWRI1499-like	This study
13EG55B3	Wine	France	2013	AWRI1499-like	This study
13EN11C11	Wine	France	2013	AWRI1499-like	Avramova <i>et al.</i> , submitted
13E2A8	Wine	France	2013	AWRI1499-like	This study
AWRI 1605	Wine	Australia	NA	AWRI1499-like	This study
AWRI 1651	Wine	Australia	2002	AWRI1499-like	This study
AWRI1499	Wine	Australia	2002	AWRI1499-like	Avramova <i>et al.</i> , submitted
AWRI1649	Wine	Australia	2001	AWRI1499-like	This study
CJ12-6	Wine	France	2012	AWRI1499-like	This study
CJ13-4	Wine	France	2013	AWRI1499-like	This study
GB06	Wine	Italy	2013	AWRI1499-like	This study
GB08	Wine	Italy	2013	AWRI1499-like	This study
GSP1509	Beer	Italy	2015	AWRI1499-like	This study
ISA2404	Wine	Portugal	2010	AWRI1499-like	Avramova <i>et al.</i> , submitted
KOM14120	Kombucha	France	NA	AWRI1499-like	This study
KOM1455	Kombucha	France	NA	AWRI1499-like	This study
L0417	Wine	France	2003	AWRI1499-like	Avramova <i>et al.</i> , submitted
L0424	Wine	France	2003	AWRI1499-like	Avramova <i>et al.</i> , submitted
L0516	Wine	France	1990	AWRI1499-like	Avramova <i>et al.</i> , submitted
L06/034 AZ	Wine	France	NA	AWRI1499-like	This study
L14156	Wine	Chile	2001	AWRI1499-like	This study
L14175	Wine	USA	2001	AWRI1499-like	This study
L14190	Wine	Argentina	2002	AWRI1499-like	Avramova <i>et al.</i> , submitted
LB15109p	Wine	France	2014	AWRI1499-like	Avramova <i>et al.</i> , submitted
Medoc-12-05	Wine	France	2012	AWRI1499-like	This study
Medoc-12-07	Wine	France	2012	AWRI1499-like	This study
MLC_296_2014_1	Wine	France	2012	AWRI1499-like	This study
MLC_296_2014_2	Wine	France	2012	AWRI1499-like	This study
MLC_296_2014_9	Wine	France	2012	AWRI1499-like	This study
VP1545	Wine	Italy	2013	AWRI1499-like	Avramova <i>et al.</i> , submitted
CBS 5513	Beer	South Africa	1990	CBS 5512-like	This study
CBS 6055	Beer	USA	1998	CBS 5512-like	This study
JP258V2013-C7	ethanol	Brazil	NA	CBS 5512-like	Avramova <i>et al.</i> , submitted
JP354V2014-C8	ethanol	Brazil	NA	CBS 5512-like	Avramova <i>et al.</i> , submitted
L14169	Fruit wine	Thailand	2002	CBS 5512-like	This study

UWOPS 92- 244.4	Tequila	Mexico	1992	CBS 5512-like	Avramova <i>et al.</i> , submitted
UWOPS 92- 255.4	Tequila	Mexico	1992	CBS 5512-like	Avramova <i>et al.</i> , submitted
UWOPS 92- 262.3	Tequila	Mexico	1992	CBS 5512-like	Avramova <i>et al.</i> , submitted
UWOPS 92- 297.4	Tequila	Mexico	1992	CBS 5512-like	This study
UWOPS 92- 300.5	Tequila	Mexico	1992	CBS 5512-like	This study
UWOPS 94- 263.2	Tequila	Mexico	1994	CBS 5512-like	This study
B001-14 T28 1	Wine	France	2014	L308-like	Avramova <i>et al.</i> , submitted
B001S-14 T49 3	Wine	France	2014	L308-like	Avramova <i>et al.</i> , submitted
CB3	Wine	South Africa	NA	L308-like	This study
Gamay 329 CM 6	Wine	France	2014	L308-like	This study
GB52	Wine	Italy	2014	L308-like	This study
GB54	Wine	Italy	2014	L308-like	This study
GB59	Wine	Italy	2014	L308-like	This study
L0308	Wine	France	1994	L308-like	This study
Merlot_329_CM_1	Wine	France	2014	L308-like	This study
VP1503	Wine	Italy	2013	L308-like	This study
VP1506	Wine	Italy	2013	L308-like	This study
YJS5382	NA	Chile	NA	L308-like	This study

#### 4.2.2. Sulphite tolerance assessment

The assay was performed in liquid medium containing 6.7 g.L<sup>-1</sup> of YNB (Difco™ Yeast Nitrogen Base, Beckton, Dickinson and Company), 2.5 g.L<sup>-1</sup> D-glucose, 2.5 g.L<sup>-1</sup> D-Fructose, 5% (v/v) ethanol and increasing concentrations of potassium metabisulphite (PMB, K<sub>2</sub>S<sub>2</sub>O<sub>5</sub>) (Thermo Fischer Scientific) in order to obtain 0, 0.2, 0.4 and 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub> final concentrations. For the calculation of mSO<sub>2</sub> it was considered that K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> corresponds to about 50% of total SO<sub>2</sub> (therefore a solution of 10 g.L<sup>-1</sup> K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> corresponds to approximately 5 g.L<sup>-1</sup> total SO<sub>2</sub>). In order to deduce the final mSO<sub>2</sub> concentration, the free SO<sub>2</sub> concentration was assessed by aspiration/titration method. Then, the mSO<sub>2</sub> was estimated by taking into account the final pH, temperature and alcohol content of the medium (resource available at <http://www.vignevin-sudouest.com/services-professionnels/formulaires-calcul/so2-actif.php>).

Final pH was adjusted to 3.5 with phosphoric acid (1M H<sub>3</sub>PO<sub>4</sub>) and the four media (corresponding to the 4 different concentrations of SO<sub>2</sub>) were filtered separately with 0.22 µm pore filter (Millipore).

Small-scale fermentations were performed in sterile 4 ml spectrophotometer cuvettes containing a sterile magnet stirrer (Dutscher, France). The cells were grown on YPD agar and inoculated into the YNB-based medium without SO<sub>2</sub>. After 96 h of pre-culture (the point at which all strains reached stationary phase), the cells were inoculated at OD<sub>600</sub> 0.1 in a final volume of 3 ml. The inoculated medium was then covered with 300 µL of sterile silicone oil (Sigma-Aldrich) to avoid oxidation of the medium which could favour the free SO<sub>2</sub> consumption. Then, the cuvette was capped with a plastic cap (Dutscher) and sealed with parafilm. A sterile needle was added by piercing the cap to allow CO<sub>2</sub> release. The “nano-fermenters” were then placed in a spectrophotometer cuvettes container box and on a 15 multi-positions magnetic stirrer plate at 25 °C (the final temperature in the “nano-fermenters” was therefore 29 °C due to the stirrer heating). Optical density (OD<sub>600</sub>) was measured every 24 hours during at least 300h to follow cell population growth until stationary phase was reached.

For each growth curve, the following three parameters were calculated: maximal OD was the maximal OD reached at 600 nm, the lag phase (in hours) was the time between inoculation and the beginning of cell growth (5% maximal OD increase), and finally, the maximal growth rate was calculated (maximal number of division per hour based on the OD measurement divided by time). A non-parametric Kruskal-Wallis test was used at  $\alpha=5\%$  to identify the means that were significantly different.

## **4.3. Results**

### **4.3.1. Growth behaviour in presence of SO<sub>2</sub>**

A total of more than 2050 small-scale fermentations were performed corresponding to each strain tested at increasing concentrations of mSO<sub>2</sub> (0, 0.2, 0.4 and 0.6 mg.L<sup>-1</sup>) at least in triplicate. The 145 strains had different response to sulphur dioxide concentrations in means of lag phase, growth rate, and maximum OD. According to those growth parameters' variation,

(Supplementary Table S-4.1 in Appendix), three growth curve types were defined for the different concentrations of SO<sub>2</sub>: i) sensitive strains with significantly longer lag phase and slower growth rate and/or lower maximum OD in presence of increasing concentrations of SO<sub>2</sub>, ii) tolerant strains for which lag phase was longer when SO<sub>2</sub> was present but the others parameters remained unchanged, and iii) resistant strains for which all parameters are not significantly impacted whatever the concentrations of SO<sub>2</sub> considered (examples of each growth profile are shown on Figure 4.1 with the respective values for different growth parameters).

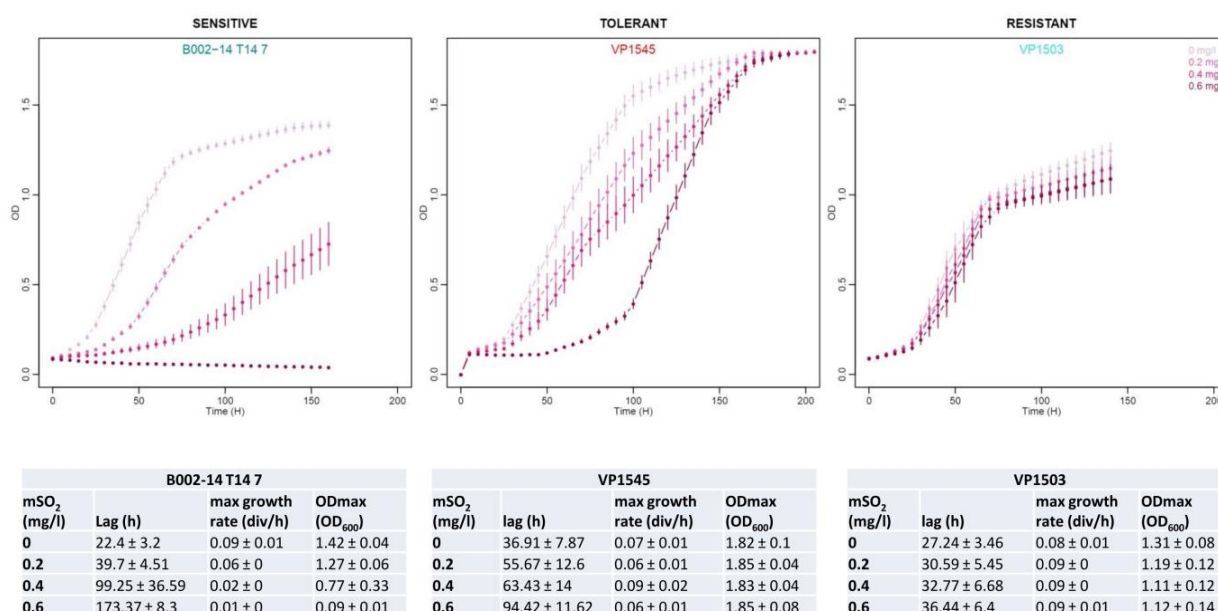


Figure 4.1. Examples of three different growth profiles at four mSO<sub>2</sub> concentrations.

#### 4.3.2. SO<sub>2</sub> tolerance/resistance and relation to genetic group

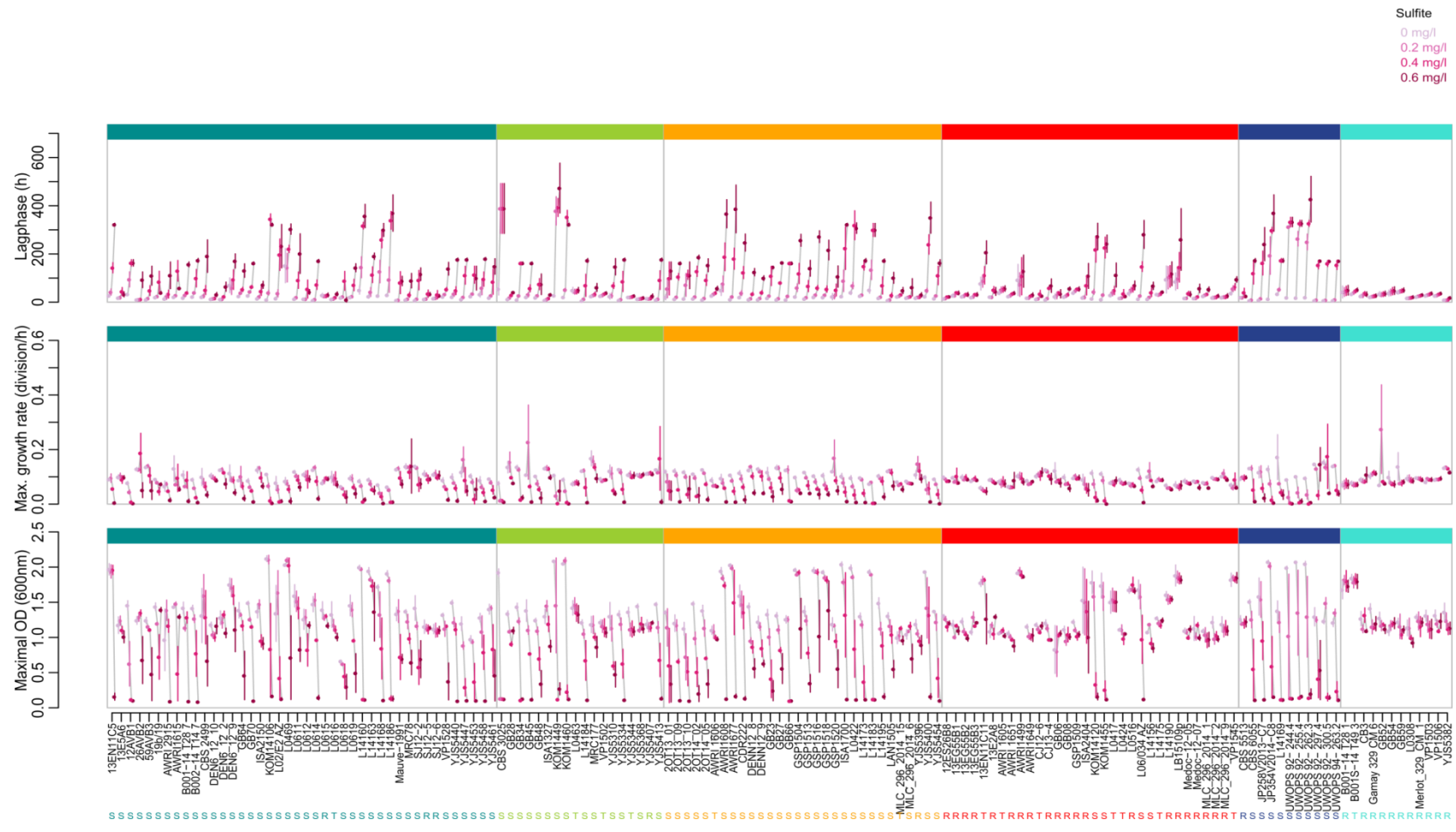
Differences in growth parameters were presented per genetic group (as defined by microsatellite analysis) (Figure 4.2, Supplementary Figure S-4.1 in Appendix, Table 4.2). It can be noted that strains that belong to the same genetic cluster have similar response to SO<sub>2</sub> in means of growth profile. Also, 48 out of 52 tolerant or resistant strains (Table 4.2) are isolated from wine (Table 4.1). All tolerant and resistant strains were isolated after 1990, and from



worldwide. The groups AWRI1499-like, and L0308-like presented 91% (40 out of 44 isolates) tolerant/resistant phenotypes, whereas the groups CBS 2499-like, L14165-like, AWRI1608-like and CBS 5512-like – 88% (89 out of 101 isolates) sensitive strains. For wine strains, the proportions were 95% and 87% respectively.

**Table 4.2. Number of isolates by genetic group and phenotype**

<b>Genetic group</b>	<b>Sensitive</b>	<b>Tolerant</b>	<b>Resistant</b>	<b>Total</b>
<b>CBS 2499-like</b>	38	1	3	42
<b>L14165-like</b>	14	3	1	18
<b>AWRI1608-like</b>	27	2	1	30
<b>AWRI1499-like</b>	4	7	21	32
<b>CBS 5512-like</b>	10	0	1	11
<b>L308-like</b>	0	1	11	12
<b>Total</b>	93	14	38	145



**Figure 4.2. Growth parameters of 145 *B. bruxellensis* isolates grown at different SO<sub>2</sub> concentrations.** Growth parameters lag phase (h), maximum growth rate (division per hour), and maximum OD are presented for each isolates. Isolates are grouped by genetic group as defined by Avramova et al., submitted: in order CBS 2499-like group (cyan), L14165-like (lawn green), AWRI1608-like (orange), AWRI1499-like (red), dark blue (CBS 3025-like), light blue (L0308-like). Vertical traits present standard deviations.

#### 4.4. Discussion

Sulphur dioxide is usually used by winemakers as preventive or curative treatment for *Brettanomyces bruxellensis* contamination. Concentrations of 0.2 to 0.5 mg.L<sup>-1</sup> molecular SO<sub>2</sub> are typically reported to inhibit growth in wine (Barata et al., 2008; Conterno et al., 2006). However, *B. bruxellensis* was considered to be rather sulphite tolerant (Agnolucci et al., 2013; A. Barata et al., 2008; C. Curtin et al., 2012b). Moreover, the tolerance to sulphite is strain dependant (Agnolucci et al., 2013; Avramova et al., submitted; Barata et al., 2008; Curtin et al., 2012a; Vigentini et al., 2008b) and sulphite efficiency is population level dependant (Longin et al., 2016a). Previous studies highlighted genotype dependant tolerance to sulfur dioxide for *B. bruxellensis* among Australian isolates (C. Curtin et al., 2012a), that was recently confirmed with 39 isolates representative of the global *B. bruxellensis* population (Avramova *et al.*, submitted). Taking into account the high intra-species genetic diversity of *B. bruxellensis*, 106 additional isolates from various origins were included to the previous phenotypic test to confirm the link between genotype and SO<sub>2</sub> tolerance at larger and finer scale. The final aim of this study was to explore the potential use of genetic markers to assess the genetic group and then, to deduce SO<sub>2</sub> tolerance of *B. bruxellensis* strains.

In our previous study, it was noticed that representatives of the triploid L0308-like group exhibited a peculiar profile characterised by unmodified growth parameters at all tested SO<sub>2</sub> concentrations. However, these observations were based on only two isolates with similar origin (Avramova *et al.*, submitted). To complement these results, we analysed 9 additional L0308-like strains from different origins and confirmed their “resistant” phenotype. Here, a resistant phenotype corresponds to behaviour, for which there were no significant differences for all studied growth parameters at increasing SO<sub>2</sub> concentration. On the other hand, “tolerant” strains were those for which lag phase was modified when SO<sub>2</sub> was increasing. Those two terms are used in clinical microbiology, where they serve to describe microbial pathogenicity

(Anderson, 2005; Brauner et al., 2016). Often, tolerance is related to the capacity of the organism to survive under inhibition by an agent, whereas resistance is linked to the capacity to actively proliferate in presence of antibiotic, and is measured as minimum inhibitory concentration or fitness (Anderson, 2005). The peculiarity of SO<sub>2</sub> application, however, is that the active antimicrobial fraction (mSO<sub>2</sub>) of this agent is variable (depending on environmental parameters such as temperature, alcohol content and pH) and decreases over time due to free SO<sub>2</sub> combination. Furthermore, *B. bruxellensis* is able to enter a VBNC (viable but not cultivable) state after sulphites addition (Agnolucci et al., 2010; Capozzi et al., 2016; Du Toit et al., 2005b; Longin et al., 2016a; Serpaggi et al., 2012), followed by growth recovery with sulphites decrease over time. In winemaking, sulphite levels are often re-adjusted at different time intervals, thus creating seasonality in SO<sub>2</sub> administration during the winemaking process. In these conditions, the actual survival of *B. bruxellensis* in wine could be related to i) survival and growth besides initial “hit” with SO<sub>2</sub>, that could be related to resistant-type mechanism and ii) survival at the initial SO<sub>2</sub> “hit” and until a stage when mSO<sub>2</sub> concentration is lower in the medium, followed by growth recovery that could be described as tolerance mechanism. Indeed, resistant and tolerant phenotypes are often interconnected and related to different types of metabolism and cell structure differences. In clinical microbiology, it is suggested that tolerant and resistant strains would be treated differently (resistant being treated with higher doses and shorter treatment, whereas tolerant strains would be treated with lower doses but extended treatment duration (Brauner et al., 2016). The detection of both resistant and tolerant growth profiles in the present dataset suggests that *B. bruxellensis* strains have developed not one, but multiple strategies to cope with SO<sub>2</sub> present in wine.

Here, the majority of tolerant or resistant strains were isolated from wine (46 out of 52). This suggests a strong link between SO<sub>2</sub> exposure and recent emergence of tolerant/resistant phenotypes that was discussed in previous studies (Previous chapters, Cibrario et al., in

preparation, personal communication, Curtin *et al.*, 2012). This hypothesis is also phenotypically supported as all resistant and tolerant strains were isolated from wines from recent vintages (after 1990) (Table 4.1). Furthermore, the strain AWRI1499 (a representative of allotriploid AWRI1499-like group) was shown to have a specific selective advantage in presence of SO<sub>2</sub> when compared to two other wine strains representatives of diploid CBS 2499-like and triploid AWRI1608-like groups (Avramova *et al.*, in preparation). This data taken together highlights the role of SO<sub>2</sub>, and therefore human activity, in shaping *B. bruxellensis* population structure, which was discussed in the previous chapters.

Sulphur dioxide resistance is broadly studied in *S. cerevisiae* and the main mechanisms related to this phenotype is efflux through Ssu1p active pump (Nardi *et al.*, 2010; Park and Bakalinsky, 2000; Pérez-Ortín *et al.*, 2002). It was demonstrated that *SSUI-R* allele which is involved in SO<sub>2</sub> resistance, is the product of reciprocal translocation between chromosomes VII and XVI, thus highlighting the importance of gross chromosomal rearrangements in the adaptive evolution of *S. cerevisiae* (Pérez-Ortín *et al.*, 2002). Later, another translocation involved in SO<sub>2</sub> tolerance (XV-t-XVI) was shown to shorten lag phase in presence of SO<sub>2</sub>, thus conferring relative selective advantage compared to nt-XVI strains (Zimmer *et al.*, 2014). Following those studies, it was suggested that those translocations were empirically selected by humans (Pérez-Ortín *et al.*, 2002; Zimmer *et al.*, 2014). The lack of effect of SO<sub>2</sub> on lag phase observed for the resistant *B. bruxellensis* strains could be related to similar mechanisms. Indeed, allele specific expression of efflux pump *BbSSUI* was detected by comparative transcriptomics (Curtin *et al.*, ISSY2015 abstract). The mechanisms related to the resistant phenotype in *B. bruxellensis* remain to be elucidated. As for the tolerant strains, the longer lag phase would reflect the time needed for the adaptation through complex mechanisms or the survival until a lower mSO<sub>2</sub> concentration is attained in the medium. Using staining with propidium iodide detection by flow cytometer analysis (Longin *et al.*, 2016a), Longin *et al.* showed that sulphite induces

increased yeast cell permeability, which probably leads to cell death. The ability of cells to restore functional cell permeability could constitute another sulphite adaptation mechanism for *B. bruxellensis*. The molecule of SO<sub>2</sub> having various effects on the cell structure, metabolism and genome (Divol et al., 2012), those mechanisms can be numerous including synthesis of binding molecules (like acetaldehyde), specific membrane structure, *etc.* (Divol et al., 2012).

The sensitivity/survival phenotype in presence of SO<sub>2</sub> correlates with genotypic profiles defined by microsatellite analysis of 39 strains (Avramova *et al.*, submitted) and this was validated here with the additional set of 106 strains. The groups CBS 2499-like (diploid), L14165-like (diploid), AWRI1608-like (triploid), and CBS 5512-like (triploid) are all susceptible to SO<sub>2</sub> presence in synthetic medium. On the contrary, AWRI1499-like (triploid) and L0308-like (triploid) survived in presence of high concentrations of SO<sub>2</sub>. This behaviour was confirmed by independent study performed in wine medium, where the strain L0417 (AWRI1499-like) was demonstrated to be more tolerant than L02E2 (CBS 2499-like). The use of microsatellites as selection markers was previously proposed for *S. cerevisiae* wine strains (Franco-Duarte et al., 2014, 2009). In the latter work, 30 different phenotype analyses were performed, and SO<sub>2</sub> tolerance was highlighted to be one of the factors that correlate the most with microsatellite patterns. In the winemaking context, SO<sub>2</sub> tolerance is a positive trait for the selection of *S. cerevisiae*, whereas it is the opposite for *B. bruxellensis* strains, for which it is directly related to spoilage potential. Defining SO<sub>2</sub> tolerance through genetic markers can therefore be used as an efficient tool to adapt antimicrobial treatment in the winery. Similar methods are used for resistance prediction for pathogenic fungi (Irinnyi et al., 2015; Park and Perlin, 2005). Namely, in the case of *C. albicans*, PCR-based methods were proposed for the detection of mutations related to fluconazole resistance (Park and Perlin, 2005). This method allows the adoption of alternative techniques to cope with this microorganism. Contrary to fluconazole which targets lanosterol 14-demethylase (the product of *ERG1*)(White et al.,

2002), SO<sub>2</sub> has a very broad range of activities on the cell on structural, genetic and metabolic level (Divol et al., 2012), and detection method of specific mutation responsible for resistance would be a challenge. Therefore, the strong correlation between genotype and SO<sub>2</sub> tolerance presents a reliable alternative for the prediction of this phenotype through microsatellite analysis. Indeed, resistant/tolerant genotypes were predicted at 95% (of 44 strains) based on the genotype profile. For comparison, this percentage was 91% for *C. albicans* (based on 32 isolates) when using targeted PCR (Park and Perlin, 2005). Combined with the fact that clonal populations of *B. bruxellensis* strains were isolated over a long period of time in the same winery (Albertin et al., 2014b), the use of microsatellite markers is also applicable as a prediction method based on spoilage populations from previous vintages. Here, we suggest the use of microsatellite markers as reliable genetic markers for predicting spoilage potential in means of SO<sub>2</sub> tolerance for *B. bruxellensis* populations. This method could allow application of adequate antimicrobial techniques according to the survival mechanism in presence of SO<sub>2</sub> of the contaminating *B. bruxellensis* population, and therefore assure efficient spoilage prevention with minimal intervention.

## Chapter 5. Discussion and perspectives

*Brettanomyces bruxellensis* is a yeast species that was known for more than a century and is associated to a peculiar aromatic profile in various fermented beverages. First described in beer, *B. bruxellensis* was revealed as an essential contributor to the organoleptic qualities of some specialty beer-types. In wine however, this same yeast is undesirable due to the production of ethyl phenols related to unpleasant aromas. Spoilage by this microorganism leads to consumers' rejection and subsequent economic losses for winemakers. Strains of this species were isolated from other fermented products like kombucha, cider, olives, *etc.* In particular, *B. bruxellensis* is of great industrial interest in the context of bioethanol production plants where it was initially considered a contamination organism but could be potentially used for the molasses fermentation. Thus, *B. bruxellensis* develops in multiple industrial fermentation environments where its presence (in some cases essential, whereas in others – undesirable) rarely remains unnoticed. It was mainly the wine spoilage potential of *B. bruxellensis* that drew great attention on this species on behalf of the scientific community over the last twenty years. Subsequently, high diversity was underscored for *B. bruxellensis* – both at the phenotypic and genetic levels. However, those studies remained marginal and didn't lead to a holistic picture of the species. To address this problem, we performed a population genetics study on a large number of *B. bruxellensis* strains through microsatellite markers analysis. This genotyping method has the advantage to give indication on the genetic relations between strains and the ploidy level of a given organism. Indeed, ploidy is an essential parameter to take into account when studying *B. bruxellensis*, as strains with different ploidy level were highlighted following whole genome sequencing. The results presented here confirm on a large scale the assumption that *B. bruxellensis* population is composed of strains with different ploidy level, as 57.8% of the isolates were shown to have more than 2 alleles for at least one locus. The studied



population is structured according to ploidy level, substrate of isolation and geographical origin of the strains, underlying the relative importance of each factor.

By the use of microsatellite analysis, we were able to confirm the triploid state of AWRI1499-like and AWRI1608-like strains, and we suggest the presence of other triploid groups, namely L0308-like wine group and L14165-like tequila/bioethanol group. Those results lead to the assumption that multiple polyploidisation events occurred among *B. bruxellensis* population and that polyploidy could present an adaptation mechanism to various environment types. Another interesting observation concerning the ploidy level of the species was that among the numerous and various isolates analysed, there was high heterozygosity level and no putative haploids, supporting the general view that *B. bruxellensis* could be an asexual organism. In perspective, the developed microsatellite genotyping method and the knowledge of the overall population structure of the species will allow to take into account the genetic diversity of *B. bruxellensis* when evaluating its phenotypic properties by choosing relevant representatives of the population for the experiments. Multiple morphological and physiological aspects on cell and population level remain to be explored. Namely, other parameters like pseudohyphal growth or tolerance to antimicrobial agents could be studied in the context of winemaking and other industrial fermentation environments. Subsequently, this would lead to better knowledge of the adaptation mechanisms of this yeast, as well as the factors that impact its survival, growth, and spoilage potential. Furthermore, the putative role of the triploid state of beer and bioethanol strains for the adaptation to their respective fermentation environments could be explored.

Intriguingly, the triploid AWRI1499-like strains presented the majority of Australian *B. bruxellensis* isolates from wine. Thus, a possible link between the polyploid state of those strains and the adaptation to winemaking environment was suggested. Here, we demonstrated that isolates from the AWRI1499-like genetic group: i) were highly disseminated in different

countries, ii) represented a high proportion of the overall wine isolates (>40%), and iii) were almost exclusively comprised of wine isolates. Thus, a strong support for the hypothesis of link between genotype and isolation environment was provided. This observation was extended to other niches than wine, as we found a correlation between genotypic profile and related industrial fermentation environment for beer, bioethanol/tequila, and kombucha. To further study the specific adaptation of the species to various fermentation environments, it would be interesting to optimise and apply the competition protocol developed for this study and explore other parameters than SO<sub>2</sub> tolerance. In particular, it would be of high interest to investigate the selective advantage of strains with different genetic background in other variable conditions relevant to winemaking – ethanol concentration, pH, temperature, *etc.* Also, to confirm the correlation between genotype (and associated ploidy level) and substrate of isolation, it would be interesting to perform competition experiments between strains isolated from different beverages and in media corresponding to the physicochemical characteristics of the latter (e.g. beer, bioethanol). Thus, it could be tested if strains from the different genetic groups have indeed a specific advantage in the media that they were isolated from.

Wine presents peculiar conditions for the development of microorganisms and one of the specificities related to winemaking is the use of sulfur dioxide. Indeed, it was previously demonstrated that isolates from the group AWRI1499 were highly tolerant to SO<sub>2</sub> and it was suggested that this was an important piece of the puzzle of the adaptation of this genetic group to wine. By this work, we highlight that AWRI1499-like triploid strains are tolerant to sulfur dioxide, whereas other genotypes were sensitive to this antimicrobial agent. The specific adaptation to SO<sub>2</sub> of the strain AWRI1499 was further tested and confirmed in this work. For this aim, *B. bruxellensis* isolates were transformed and put in pairwise competition at different SO<sub>2</sub> concentrations. Genetic transformation of triploid *B. bruxellensis* strains was successfully performed for the first time (to our knowledge). The results demonstrated the selective

advantage of AWRI1499 in presence of SO<sub>2</sub> thus supporting the hypothesis that this phenotypic characteristic can be a major component for the adaptation of this genetic group to winemaking and subsequently its high dissemination worldwide. It would be of interest to further verify those results in wine.

In fermentation environments different species and strains of microorganisms co-exist in the same space. Thus, the interactions that occur between them have impact on the final product and are interesting subject for future research. In particular, interspecies interaction between *S. cerevisiae* and *B. bruxellensis* can be interesting especially in the case of bioethanol production where both species were detected together. Also, the nature of intraspecific interactions for *B. bruxellensis* could be explored with the proposed competition protocol in winemaking and brewery context.

This strong link between genotype and sulphites tolerance presents an opportunity to diagnose SO<sub>2</sub> sensitivity for *B. bruxellensis*. Variable SO<sub>2</sub> tolerance of *B. bruxellensis* is a major concern for winemakers who aim to use optimal doses of this antimicrobial to attain a compromise between its beneficial and detrimental effects on wine quality. With the aim to design molecular markers that can be used in practice to deduce SO<sub>2</sub> sensitivity of spoilage-associated *B. bruxellensis* population, we have explored the SO<sub>2</sub> tolerance of a total of 145 isolates from various origins and substrates. Intriguingly, it was confirmed that only wine strains exhibited SO<sub>2</sub> tolerance. Furthermore, different degrees of SO<sub>2</sub> tolerance/resistance were highlighted, some phenotypes were characterised by unmodified growth even at 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub>. These observations suggest that there are probably not only one, but multiple strategies for coping with this antimicrobial for *B. bruxellensis*. To complement these results, it would be interesting to extend the study by performing the experiments in wine and follow the cell viability and physiological state of the cells by methods others than the spectrophotometry (e.g. flow cytometry). Microsatellite markers can therefore present a tool for molecular diagnosis of SO<sub>2</sub>

sensitivity in *B. bruxellensis*. In the future, the combination of population genetics and in-depth genome sequencing, would allow the elucidation of the molecular mechanisms underlying SO<sub>2</sub> tolerance in *B. bruxellensis*. Different strategies to cope with this toxic compound have been highlighted for the species *S. cerevisiae*. In particular, active efflux of SO<sub>2</sub> is related to translocations for the latter species. From an evolutionary point of view, it would be intriguing to verify if *B. bruxellensis* has acquired SO<sub>2</sub> tolerance through other genetic mechanisms, like polyploidy in particular and genomic plasticity in general. By the combination of high-quality genome sequencing and directed genetic transformation, candidate genes could be designated and tested for their contribution to SO<sub>2</sub> tolerance. Better understanding of the mechanism of SO<sub>2</sub> tolerance in *B. bruxellensis* will possibly give indications for the better management of this key issue in the winemaking context.

From a fundamental point of view, we provide a holistic picture of the population genetics of the species *B. bruxellensis*. Structuring according to substrate of isolation is highlighted, suggesting an influence of human activity on *B. bruxellensis* population structure. Furthermore, a coexistence of diploid and triploid populations is underscored and we propose that *B. bruxellensis* could be described as a diploid-triploid complex. These characteristics make of *B. bruxellensis* a non-conventional model microorganism for the study of polyploid populations and the impact of polyploidy on the adaptation capacities of an organism – a concept of industrial and clinical importance. Comparative genomics would allow the construction of more complete picture of the genome dynamics of the species. In particular, comparison between strains from different industrial environments could highlight genomic features associated with adaptation to specific niche-types which would lead to a better understanding of the evolution of the species and its putative relation with human activity. In perspective, a fortuitous isolation of *Brettanomyces* wild strains would allow the comparison between industrial and natural isolates. Thus, the relation between human activity and *B. bruxellensis*

evolution could be explored and verified. The availability of high-quality sequences could lead to the “discovery” of the mysterious donors of the additional genomes of allopolyploid *B. bruxellensis* strains.

Overall this work presents industrial interest for the winemaking community, as it demonstrates the dissemination of highly SO<sub>2</sub> tolerant strains worldwide. In perspective it would be of interest to explore growth behaviour and viability of “tolerant” and “resistant” *B. bruxellensis* strains at a finer scale. Phenotypic experiments combined with deeper knowledge of the genetics of the species would be useful for revealing the mechanisms behind those different phenotypes. The striking correlation between SO<sub>2</sub> tolerance and specific genotypic configuration presents a great tool for the evaluation of SO<sub>2</sub> sensitivity of resident *B. bruxellensis* strains. In the future, a finer diagnosis of *B. bruxellensis* spoilage population in means of genetic group, related SO<sub>2</sub> survival behaviour, and population level, would contribute to the use of optimal SO<sub>2</sub> concentrations at adequate time intervals, thus leading to more efficient SO<sub>2</sub> management.

## Chapter 6. Conclusions

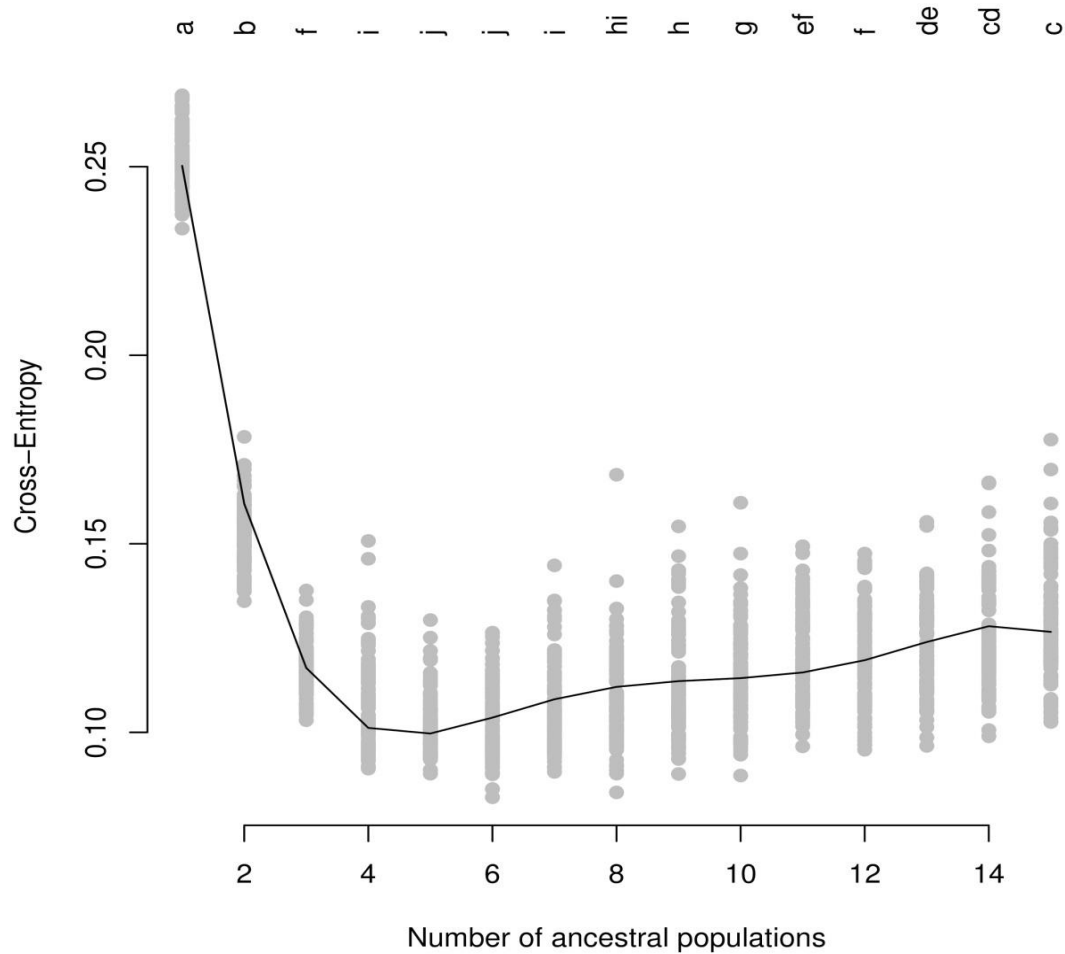
The present work led to several key findings:

- i) Polyploidy is a highly represented state among *B. bruxellensis* species revealing a diploid-triploid complex structured according to substrate of isolation and geographic origin.
- ii) A strong link between genotype and sulphite sensitivity was demonstrated for a large collection of isolates representative of the global *B. bruxellensis* population.
- iii) Wine triploid AWRI1499-like strains are tolerant to SO<sub>2</sub> concentrations as high as 0.6 mg.L<sup>-1</sup> mSO<sub>2</sub> and are highly disseminated across wine producing regions worldwide.
- iv) Competition experiments confirmed that tolerance to SO<sub>2</sub> confers a selective advantage compared to sensitive strains in medium containing SO<sub>2</sub>; this indicates specific adaptation of SO<sub>2</sub> tolerant *B. bruxellensis* populations through a human-related selection process.
- v) Three *B. bruxellensis* strains with different ploidy levels and genetic backgrounds were successfully transformed.
- vi) Microsatellite analysis can be used for molecular diagnosis of SO<sub>2</sub> sensitivity of *B. bruxellensis* strains.



## Appendix

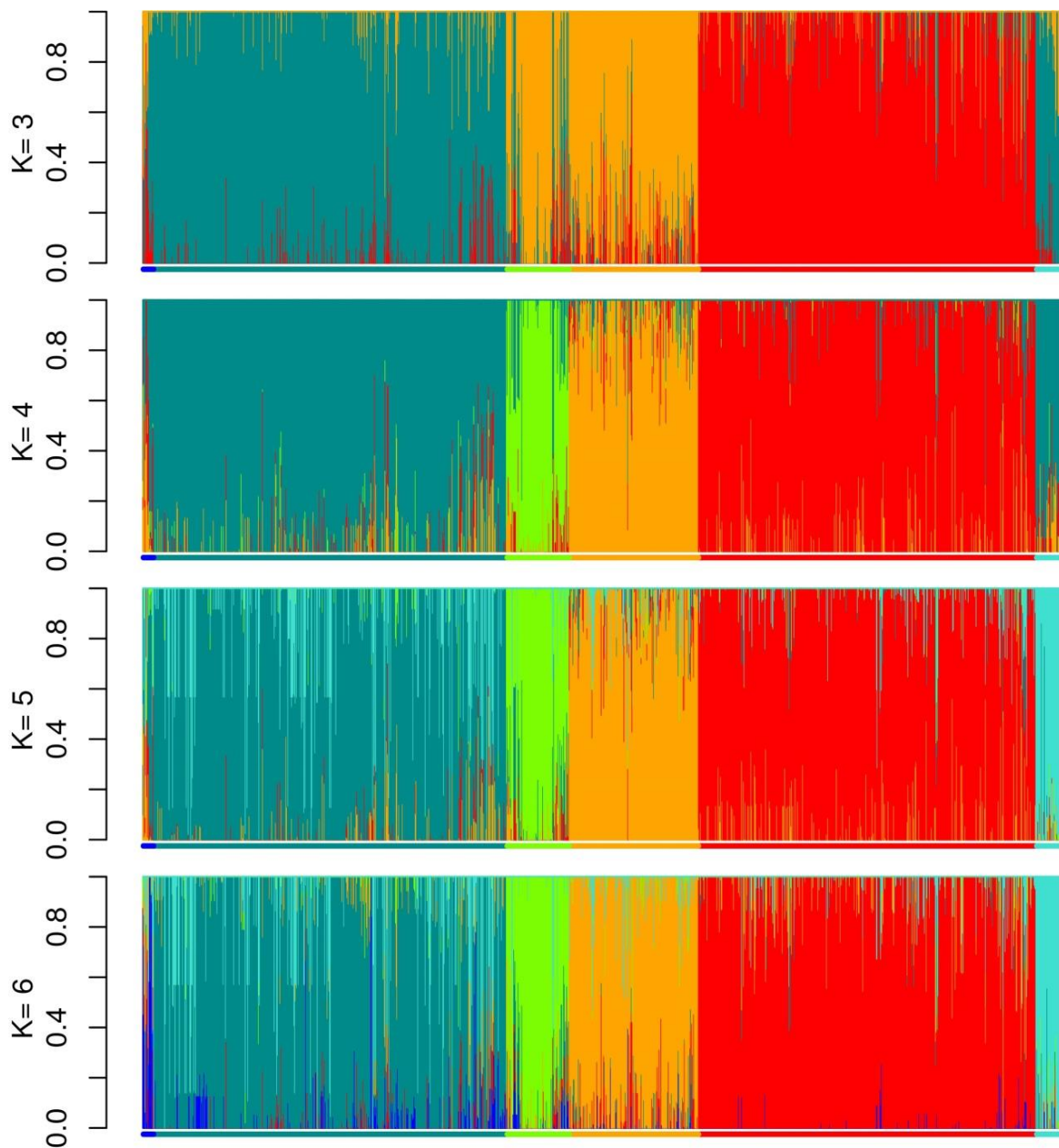
Supplementary Figure S-2.1.



**Supplementary Figure S-2.1. Entropy analysis for estimating the number of ancestral populations that explains the genotypic data in the best way.** Different letters correspond to the significance groups according to Kruskal-Wallis ( $\alpha=5\%$ ).

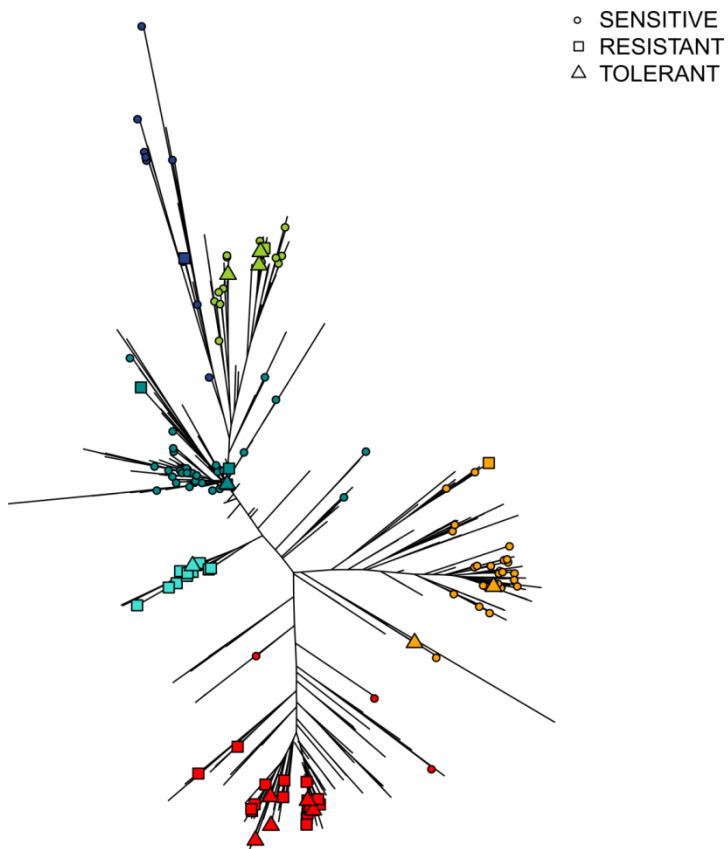


## Supplementary Figure S-2.2



**Supplementary Figure S-2.2. STRUCTURE plots for K= 3, 4, 5, and 6.** Each bar represents a strain and the colour of the bar represents the estimated ancestry proportion of each of the K clusters. The same colour code is kept as in previous figures. K=5 is the number of ancestral population with lowest entropy.

## Supplementary Figure S-4.1.



**Supplementary Figure S-4.1.** Position of isolates used in this study on dendrogram representing *B. bruxellensis* population structure. Dendrogram from Avramova et al., submitted; the colours correspond to different genetic clusters as defined in the latter.

**Supplementary Table S2.1. Microsatellite loci for *B. bruxellensis* genotyping.**

Microsatellite Motif name	Primers <sup>a</sup>	Fluorescent dyes	Tm, °C	Contig# (position), AWRI1499	Scaffold#, Number of strain recorded CBS2499 alleles (min-max size) <sup>b</sup>	Allele size (number of repeats), AWRI1499 <sup>b</sup>	Coding sequence	Ho (observed heterozygosity)	
B224	CTT/GCT F: TGCAAGTTCTGCAGCGTT; R: ACCAACAACAGCAAAGACACG	FAM		#224 (397513)	6	5 (103-127)	103(1); 106(640); 109(1499); 112(27); 127(554)	NA	0.459
B174	GAT F: TGGTGCTTAGAGCAGATGATG R: GCAACTGTTCCAATGAATTCC	FAM	53	#174 (2420882)	1	9 (184-214)	184(2); 187(8); 190(1); 193(91); 196(1497); 199(1); 202(2); 205(559); 214(1)	Riboflavin aldehyde-forming enzyme [EIF47072.1]	0.447
D9b	ATG F: TAATGAGAGAAGATGGAAAG R: TTGCACAACCTGTTTTTCTA	FAM	53	#190 (32569)	9	11 (308-443)	308(768); 323(5); 329(1); 332(1); 335(3); 338(46); 341(627); 383(1160); 389(178); 440(7); 443(1)	NA	0.899
B101	GAA/GAT F: CACGCAAAGAAGATGAGGA R: TGCCATTCTTATCCAAGTTG	HEX	56	#101 (118087)	3	8 (134-161)	134(7); 137(20); 140(960); 143(1088); 146(727); 149(1); 152(1); 161(7)	NA	0.858
B135c	CAA/AAC F: ACATGCAAGACGTGCTCAAAG R: TGATCTCTTCTGCAGCA F: GAAAACGAGTTCGGAAGACT	HEX	53	#135 (21220)	2	15 (247-289)	285(10); 288(2); 297(39); 312(8); 315(1); 318(1); 321(31); 324(3); 327(644); 333(26); 336(579); 339(24); 342(502); 345(2); 348(45); 354(4); 357(561); 363(201)	NA	0.865
B122c	ATC	HEX		#122 (18840)	7	11 (329-443)	329(7); 335(4); 338(1243); 341(6); 344(1); 353(529); 356(1); 377(1); 413(2); 428(90); 443(41)	Putative histone acetyltransferase saga complex component [EIF47840.1]	0.453
D2	TGA/TGG R: AAAAATTGAACGTGGTGATG F: GCCATCATTGTGACTGTCGTT R: CATTGCTTTCCAACCTCTCA	AT550	56	#104 (12956)	2	5 (94-118)	94(8); 103(5); 106(889); 109(758); 118(180)	NA	0.247
B301	TTG/CTG F: GTATGCTTGCGGGACTTGATT R: GCGACTTCAACAGCAGCTTAA	AT550		#301 (326230)	7	9 (139-181)	139(5); 142(3); 145(1493); 148(133); 151(542); 154(565); 175(208); 178(1); 181(39)	NA	0.615
D10b	TGC/TGT F: CTCTGCATTGCTTACTTAGAC R: TTCCATTATGATGGCAGGG	AT550	53	#96 (16790)	10	12 (219-282)	219(4); 234(1); 237(201); 246(5); 249(8); 252(1193); 255(602); 261(8); 264(13); 273(564); 276(7); 282(1)	NA	0.806
B273	TTA/ATT F: CTGCAAGAAGATGAATTGGAA R: ACCTTTGGATTGGCCCTTT F: TTAGTGGTTATCCGGAGGAG	AT565	53	#273 (521465)	6	3 (153-159)	153(1452); 156(794); 159(13)	NA	0.544
B22	ATG R: TATCCTCGTCAGCTTCTGCTT F: AGGAGGCTCCCGAAATGT	AT565	56	#22 (241906)	8	18 (189-273)	189(41); 195(1); 201(216); 204(8); 207(786); 210(1); 213(18); 216(802); 219(1); 222(4); 225(776); 228(1); 231(22); 243(1); 252(1); 261(7); 264(7); 273(524)	NA	0.625
D1	ACA R: GTGCAAATGGGGTGGAGA	AT565		#258 (60314)	1	18 (285-363)	285(10); 288(2); 297(39); 312(8); 315(1); 318(1); 321(31); 324(3); 327(644); 333(26); 336(579); 339(24); 342(502); 345(2); 348(45); 354(4); 357(561); 363(201)	NA	0.792

<sup>a</sup> Forward primers were tailed on 5'-end with M13 sequence (CACGACGTTGTAAACGAC)

<sup>b</sup> Allele size in bp

**Supplementary Table S-2.2. Sufite tolerance assay – growth parameters for 39 strains.** Mean values of the growth parameters lag phase, maximal growth rate and maximal OD per strain.

Strain	Lagphase (h) - 0 mg/l	Lagphase (h) - 0.2 mg/l	Lagphase (h) - 0.4 mg/l	Lagphase (h) - 0.6 mg/l	Max. growth rate 0 mg/l	Max. growth rate 0.2 mg/l	Max. growth rate 0.4 mg/l	Max. growth rate 0.6 mg/l	Maximal OD 0 mg/l	Maximal OD 0.2 mg/l	Maximal OD (600nm) - 0.4 mg/l	Maximal OD 0.6 mg/l
AWRI1608	31.3 +/- 16.08	58.6 +/- 29.24	169 +/- 53.74	NaN +/- NA	0.1 +/- 0.022	0.06 +/- 0.01	0.05 +/- 0.005	0 +/- 0	1.9 +/- 0.05	1.8 +/- 0.04	1.7 +/- 0.04	0.1 +/- 0.01
AWRI1677	0.9 +/- 0.88	7 +/- 2.34	133 +/- 48.3	490.5 +/- NA	0.12 +/- 0.013	0.13 +/- 0.004	0.04 +/- 0.016	0 +/- 0.001	2 +/- 0.02	2 +/- 0.04	1.5 +/- 0.46	0.2 +/- 0.03
CDR222	9.8 +/- 4.92	27.7 +/- 10.93	117.8 +/- 46.84	NaN +/- NA	0.12 +/- 0.01	0.08 +/- 0.028	0.04 +/- 0.002	0 +/- 0	1.9 +/- 0	1.9 +/- 0.02	1.9 +/- 0	0.1 +/- 0
GSP1504	11.6 +/- 1.66	41.8 +/- 10.12	99.2 +/- 36.57	235.5 +/- 21.64	0.11 +/- 0.011	0.05 +/- 0.01	0.05 +/- 0.006	0.04 +/- 0.008	2 +/- 0.02	1.9 +/- 0.02	1.9 +/- 0.03	1.1 +/- 0.43
GSP1516	11.1 +/- 3.95	18.2 +/- 5.32	46.1 +/- 11.38	233.2 +/- 40.84	0.13 +/- 0.017	0.1 +/- 0.017	0.06 +/- 0.007	0.03 +/- 0.019	2 +/- 0.04	1.9 +/- 0.02	1.9 +/- 0.04	1 +/- 0.51
GSP1518	10 +/- 0.41	17.9 +/- 1.33	39.6 +/- 12.79	182.3 +/- 43.67	0.13 +/- 0.006	0.08 +/- 0.002	0.05 +/- 0.008	0.03 +/- 0.012	1.9 +/- 0.02	1.9 +/- 0.03	1.8 +/- 0.05	1.4 +/- 0.42
ISA1700	5.5 +/- 0.5	59.5 +/- 39.33	142.2 +/- 96.55	NaN +/- NA	0.14 +/- 0.008	0.07 +/- 0.025	0.04 +/- 0.022	0 +/- 0	2 +/- 0.04	1.8 +/- 0.04	1.3 +/- 0.6	0.1 +/- 0
L0422	17.2 +/- 5.14	40.7 +/- 10.97	255.8 +/- 40.02	NaN +/- NA	0.11 +/- 0.018	0.07 +/- 0.017	0.02 +/- 0.013	0 +/- 0	2 +/- 0.04	1.9 +/- 0.03	0.8 +/- 0.41	0.1 +/- 0
L14183	25.1 +/- 8.01	98.1 +/- 36.59	NaN +/- NA	NaN +/- NA	0.09 +/- 0.02	0.07 +/- 0.02	0 +/- 0	0 +/- 0	1.9 +/- 0.03	1.9 +/- 0.01	0.1 +/- 0.01	0.1 +/- 0
YJS5400	7.4 +/- 2.43	19.8 +/- 2.01	176.2 +/- 34.43	339 +/- NA	0.14 +/- 0.007	0.07 +/- 0.025	0.03 +/- 0.011	0 +/- 0.001	1.9 +/- 0.01	1.4 +/- 0.43	1.3 +/- 0.4	0.2 +/- 0.04
JP258V2013-C7	4.8 +/- 2.17	11.7 +/- 2.62	278.5 +/- 12.66	NaN +/- NA	0.16 +/- 0.02	0.11 +/- 0.01	0.04 +/- 0.022	0 +/- 0	2.1 +/- 0.03	2 +/- 0.01	1.2 +/- 0.55	0.1 +/- 0
JP354V2014-C8	5.8 +/- 2.22	179.6 +/- 79.14	291 +/- NA	NaN +/- NA	0.14 +/- 0.016	0.08 +/- 0.019	0.01 +/- 0.014	0 +/- 0	2.1 +/- 0.02	2 +/- 0.05	0.6 +/- 0.43	0.2 +/- 0.01
UWOPS 92- 244.4	1.2 +/- 0.75	270 +/- NA	NaN +/- NA	NaN +/- NA	0.15 +/- 0.002	0.02 +/- 0.018	0 +/- 0	0 +/- 0	2 +/- 0.02	1 +/- 0.88	0.1 +/- 0	0.1 +/- 0
UWOPS 92- 255.4	1.8 +/- 1.01	203.2 +/- 22.66	NaN +/- NA	NaN +/- NA	0.14 +/- 0.011	0.03 +/- 0.016	0 +/- 0	0 +/- 0	2.1 +/- 0.01	1.4 +/- 0.61	0.1 +/- 0.01	0.2 +/- 0.02
UWOPS 92- 262.3	2.3 +/- 0.6	182.8 +/- 1.02	NaN +/- NA	329 +/- NA	0.14 +/- 0.004	0.02 +/- 0.012	0 +/- 0	0 +/- 0.002	2 +/- 0.04	1.3 +/- 0.57	0.1 +/- 0.01	0.2 +/- 0.07
13EN11C5	8.8 +/- 3.59	19.5 +/- 8.08	106.3 +/- 21.58	123.5 +/- NA	0.14 +/- 0.009	0.11 +/- 0.031	0.04 +/- 0.003	0 +/- 0.002	2 +/- 0.08	1.9 +/- 0.1	2 +/- 0.07	0.2 +/- 0.05
CBS 2499	19.5 +/- 2.12	23.9 +/- 2.62	59.9 +/- 19.3	272.2 +/- 96.7	0.11 +/- 0.002	0.09 +/- 0.011	0.09 +/- 0.007	0.02 +/- 0.013	1.9 +/- 0.07	1.9 +/- 0.01	1.9 +/- 0.05	1 +/- 0.49
DEN6_12_9	88.6 +/- 38.44	69 +/- 34.2	100.7 +/- 61.52	145.2 +/- 40.85	0.09 +/- 0.023	0.08 +/- 0.018	0.08 +/- 0.015	0.05 +/- 0.012	1.9 +/- 0.06	2 +/- 0.04	1.9 +/- 0.04	1.5 +/- 0.36
KOM14106	0 +/- 0	17.8 +/- 6.1	352 +/- NA	NaN +/- NA	0.11 +/- 0.006	0.09 +/- 0.017	0.01 +/- 0.011	0 +/- 0	2.1 +/- 0.04	2.1 +/- 0.06	0.8 +/- 0.65	0.2 +/- 0.01
L02/E2 AZ	12.8 +/- 7.26	40.1 +/- 18.46	362.5 +/- NA	NaN +/- NA	0.12 +/- 0.01	0.09 +/- 0.025	0.01 +/- 0.006	0 +/- 0	2 +/- 0.01	2 +/- 0.05	0.6 +/- 0.45	0.2 +/- 0.02
L0469	121.2 +/- 62.1	112 +/- 53.46	184.7 +/- 17.18	227.5 +/- NA	0.08 +/- 0.032	0.07 +/- 0.034	0.04 +/- 0.005	0.01 +/- 0.01	2 +/- 0.02	2.1 +/- 0.03	2 +/- 0.1	0.7 +/- 0.6
L0611	9.3 +/- 1.59	18.7 +/- 0.93	174 +/- 3.62	360 +/- NA	0.12 +/- 0.015	0.08 +/- 0.008	0.06 +/- 0.009	0.01 +/- 0.015	2 +/- 0.01	1.9 +/- 0.01	1.9 +/- 0.02	0.5 +/- 0.33
L14160	31.3 +/- 13.37	123.7 +/- 40.47	NaN +/- NA	NaN +/- NA	0.09 +/- 0.014	0.06 +/- 0.007	0 +/- 0	0 +/- 0	2 +/- 0.03	2 +/- 0.02	0.1 +/- 0	0.1 +/- 0
L14163	9.9 +/- 4.13	22.5 +/- 10.75	73.6 +/- 28.24	125.1 +/- 15.11	0.12 +/- 0.011	0.1 +/- 0.016	0.06 +/- 0.011	0.04 +/- 0.014	1.9 +/- 0.04	1.8 +/- 0.03	1.8 +/- 0.07	1.5 +/- 0.34
L14168	11.8 +/- 1.38	93.8 +/- 39.63	197.5 +/- 28.99	NaN +/- NA	0.1 +/- 0.003	0.03 +/- 0.007	0.02 +/- 0.011	0 +/- 0	1.7 +/- 0.03	1.3 +/- 0.32	0.8 +/- 0.44	0.1 +/- 0
L14186	17.6 +/- 3.86	79 +/- 52.86	0 +/- NA	0 +/- NA	0.07 +/- 0.009	0.06 +/- 0.008	0 +/- 0	0 +/- 0	1.9 +/- 0.04	1.8 +/- 0.04	0.1 +/- 0.04	0.1 +/- 0.04
CBS 3025	43.2 +/- 27.39	NaN +/- NA	NaN +/- NA	NaN +/- NA	0.09 +/- 0.016	0 +/- 0	0 +/- 0	0 +/- 0	1.9 +/- 0.05	0.1 +/- 0	0.1 +/- 0.01	0.1 +/- 0.01
KOM1449	9.7 +/- 1.3	364.2 +/- 74.51	NaN +/- NA	336.2 +/- 98.18	0.14 +/- 0.003	0.02 +/- 0.01	0 +/- 0	0 +/- 0.001	2.1 +/- 0.01	1.5 +/- 0.68	0.1 +/- 0	0.3 +/- 0.09
B001-14 T28 1	34 +/- 23.38	32 +/- 15.16	36 +/- 16.9	40 +/- 18.5	0.08 +/- 0.023	0.08 +/- 0.026	0.08 +/- 0.028	0.08 +/- 0.021	1.7 +/- 0.13	1.8 +/- 0.07	1.8 +/- 0.15	1.7 +/- 0.09
B001S-14 T49 3	20 +/- 3.41	25.2 +/- 3.23	31 +/- 3.97	35.8 +/- 4.58	0.08 +/- 0.006	0.07 +/- 0.007	0.07 +/- 0.008	0.06 +/- 0.008	1.8 +/- 0.06	1.8 +/- 0.08	1.8 +/- 0.08	1.8 +/- 0.1
13EN11C11	52.9 +/- 24.31	60.8 +/- 21.78	84.9 +/- 33.7	116.5 +/- 40.43	0.07 +/- 0.015	0.06 +/- 0.014	0.06 +/- 0.012	0.03 +/- 0.012	1.8 +/- 0.05	1.8 +/- 0.06	1.8 +/- 0.02	1.3 +/- 0.36
AWRI1499	75 +/- 55.67	56.5 +/- 39.77	91.5 +/- 69.88	110.3 +/- 60.23	0.07 +/- 0.014	0.09 +/- 0.022	0.08 +/- 0.014	0.07 +/- 0.01	1.9 +/- 0.03	2 +/- 0.05	1.9 +/- 0.05	1.9 +/- 0.02
ISA2404	15 +/- 4.27	17.5 +/- 5.07	76.7 +/- 39.07	95.5 +/- 49.4	0.12 +/- 0.015	0.1 +/- 0.011	0.07 +/- 0.011	0.05 +/- 0.026	2 +/- 0.04	2 +/- 0.02	1.8 +/- 0.21	1.3 +/- 0.58
L0417	19.1 +/- 6.5	28.8 +/- 10.16	45.5 +/- 20.17	130 +/- 62.96	0.09 +/- 0.018	0.07 +/- 0.017	0.07 +/- 0.018	0.05 +/- 0.016	1.8 +/- 0.1	1.8 +/- 0.15	1.8 +/- 0.09	1.8 +/- 0.09
L0424	40.2 +/- 22.83	55.6 +/- 30.3	81.4 +/- 47.48	176.8 +/- 64.95	0.08 +/- 0.013	0.06 +/- 0.008	0.05 +/- 0.006	0.04 +/- 0.008	1.8 +/- 0.06	1.8 +/- 0.09	1.8 +/- 0.07	1.7 +/- 0.07
L0516	26.2 +/- 11.46	36.8 +/- 11.45	41.7 +/- 13.26	55.3 +/- 18.46	0.07 +/- 0.011	0.06 +/- 0.01	0.06 +/- 0.01	0.06 +/- 0.007	1.7 +/- 0.05	1.7 +/- 0.02	1.8 +/- 0.11	1.7 +/- 0.04
L14190	75.3 +/- 55.39	80.8 +/- 45.6	86.3 +/- 43.47	96.7 +/- 49.46	0.06 +/- 0.008	0.05 +/- 0.004	0.05 +/- 0.006	0.05 +/- 0.003	1.6 +/- 0.05	1.5 +/- 0.07	1.5 +/- 0.06	1.5 +/- 0.04
LB15109pp	58.8 +/- 30	71 +/- 35.99	118.1 +/- 58.11	162.9 +/- 64.17	0.08 +/- 0.029	0.08 +/- 0.023	0.06 +/- 0.018	0.06 +/- 0.021	1.9 +/- 0.15	1.9 +/- 0.08	1.8 +/- 0.08	1.8 +/- 0.05
VP1545	18.4 +/- 5.06	28.9 +/- 8.88	34.8 +/- 10.8	57.6 +/- 8.14	0.1 +/- 0.015	0.08 +/- 0.021	0.08 +/- 0.019	0.05 +/- 0.005	1.8 +/- 0.1	1.9 +/- 0.04	1.8 +/- 0.04	1.8 +/- 0.08

**Supplementary Table S-3.1. Raw counting numbers (competition experiments AWRI1499 vs AWRI1626)**

SO2	Couple	Transformant	RAW					
			0	4	8	12	16	20
0	1499 vs 1626	1499_K_3	1.94E+06	3.20E+06	8.55E+06	3.10E+06	1.60E+06	1.24E+06
0	1499 vs 1626	1626_N_2	1.72E+06	3.58E+07	8.06E+07	6.75E+07	7.17E+07	1.06E+08
0	1499 vs 1626	1499_N_2	2.00E+06	4.73E+06	1.15E+07	5.49E+06	1.50E+06	1.88E+06
0	1499 vs 1626	1626_K_3	1.52E+06	2.88E+07	8.06E+07	6.83E+07	5.36E+07	4.85E+07
0	1499 vs 1626	1499_K_4	2.32E+06	5.36E+06	1.61E+07	4.48E+06	4.72E+06	3.68E+06
0	1499 vs 1626	1626_N_6	1.62E+06	2.22E+07	6.88E+07	3.98E+07	5.58E+07	7.12E+07
0	1499 vs 1626	1499_N_4	1.82E+06	3.40E+06	1.95E+07	1.29E+07	6.23E+06	4.40E+06
0	1499 vs 1626	1626_K_4	1.58E+06	2.73E+07	6.94E+07	4.89E+07	4.45E+07	4.56E+07
0	1499 vs 1626	1499_K_7	1.03E+05	2.61E+07	2.34E+07	1.81E+07	7.07E+06	9.45E+06
0	1499 vs 1626	1626_N_7	8.61E+04	3.50E+07	6.40E+07	5.07E+07	7.91E+07	3.18E+07
0	1499 vs 1626	1499_N_5	3.65E+04	9.52E+06	8.20E+06	3.06E+06	4.02E+06	3.44E+06
0	1499 vs 1626	1626_K_6	7.78E+04	3.32E+07	4.96E+07	4.27E+07	9.07E+07	6.46E+07
0.2	1499 vs 1626	1499_K_3	1.94E+06	1.58E+06	1.90E+06	5.60E+05	1.62E+06	2.18E+04
0.2	1499 vs 1626	1626_N_2	1.72E+06	2.06E+07	9.26E+06	3.00E+06	2.25E+07	6.53E+06
0.2	1499 vs 1626	1499_N_2	2.00E+06	2.40E+05	1.04E+07	4.57E+06	1.32E+06	1.20E+06
0.2	1499 vs 1626	1626_K_3	1.52E+06	2.40E+06	4.74E+07	4.89E+07	1.06E+07	3.09E+07
0.2	1499 vs 1626	1499_K_4	2.32E+06	3.20E+06	3.47E+07	8.00E+05	1.14E+06	1.17E+07
0.2	1499 vs 1626	1626_N_6	1.62E+06	5.68E+06	5.84E+07	2.96E+06	2.43E+06	4.78E+07
0.2	1499 vs 1626	1499_N_4	1.82E+06	9.40E+05	1.69E+07	4.32E+06	7.24E+06	3.99E+02
0.2	1499 vs 1626	1626_K_4	1.58E+06	4.02E+06	5.88E+07	2.96E+07	4.23E+07	2.51E+07
0.2	1499 vs 1626	1499_K_7	1.03E+05	1.72E+06	1.45E+07	4.20E+05	5.07E+06	1.30E+04
0.2	1499 vs 1626	1626_N_7	8.61E+04	5.80E+05	1.24E+06	1.64E+06	3.47E+07	3.24E+06
0.2	1499 vs 1626	1499_N_5	3.65E+04	2.18E+07	9.00E+05	3.07E+07	1.22E+06	6.13E+06
0.2	1499 vs 1626	1626_K_6	7.78E+04	1.30E+07	1.34E+06	3.46E+07	6.98E+06	2.35E+07
0.4	1499 vs 1626	1499_K_3	1.94E+06	3.60E+05	4.60E+07	6.80E+05	5.33E+07	1.58E+06
0.4	1499 vs 1626	1626_N_2	1.72E+06	1.56E+06	1.31E+07	2.89E+05	1.96E+07	9.60E+05
0.4	1499 vs 1626	1499_N_2	2.00E+06	8.40E+05	3.69E+07	7.00E+05	1.66E+06	6.27E+06
0.4	1499 vs 1626	1626_K_3	1.52E+06	1.36E+06	1.37E+07	1.57E+05	6.60E+05	2.06E+04
0.4	1499 vs 1626	1499_K_4	2.32E+06	1.42E+06	4.09E+07	2.26E+06	2.04E+06	4.64E+06
0.4	1499 vs 1626	1626_N_6	1.62E+06	6.00E+05	3.03E+07	1.82E+05	3.97E+04	1.78E+06
0.4	1499 vs 1626	1499_N_4	1.82E+06	6.20E+05	2.06E+07	1.31E+05	1.66E+06	1.04E+07
0.4	1499 vs 1626	1626_K_4	1.58E+06	1.12E+06	4.16E+06	4.53E+04	7.80E+05	2.48E+06
0.4	1499 vs 1626	1499_K_7	1.03E+05	1.70E+07	6.13E+06	1.14E+07	5.80E+06	3.28E+06
0.4	1499 vs 1626	1626_N_7	8.61E+04	6.00E+02	4.20E+02	1.34E+03	3.20E+03	1.52E+05
0.4	1499 vs 1626	1499_N_5	3.65E+04	1.09E+07	4.67E+06	7.33E+06	5.87E+06	9.80E+05
0.4	1499 vs 1626	1626_K_6	7.78E+04	5.80E+02	3.99E+02	6.40E+02	6.00E+02	1.21E+04
0.6	1499 vs 1626	1499_K_3	1.94E+06	4.56E+06	5.33E+06	1.73E+05	2.29E+07	2.18E+06
0.6	1499 vs 1626	1626_N_2	1.72E+06	9.00E+05	2.73E+04	5.23E+04	5.26E+04	2.23E+04
0.6	1499 vs 1626	1499_N_2	2.00E+06	4.16E+06	4.08E+06	1.12E+05	1.14E+06	7.80E+05
0.6	1499 vs 1626	1626_K_3	1.52E+06	8.00E+04	1.48E+05	1.88E+05	2.80E+05	1.76E+05
0.6	1499 vs 1626	1499_K_4	2.32E+06	4.49E+07	4.96E+06	7.02E+03	7.00E+05	3.80E+05
0.6	1499 vs 1626	1626_N_6	1.62E+06	2.44E+06	7.00E+05	8.80E+02	3.99E+02	3.99E+02
0.6	1499 vs 1626	1499_N_4	1.82E+06	3.84E+06	1.76E+07	1.64E+06	1.02E+04	6.20E+05
0.6	1499 vs 1626	1626_K_4	1.58E+06	5.10E+03	3.99E+02	7.06E+03	3.99E+02	3.99E+02
0.6	1499 vs 1626	1499_K_7	1.03E+05	7.07E+06	1.15E+07	2.24E+06	2.72E+06	NA
0.6	1499 vs 1626	1626_N_7	8.61E+04	3.99E+02	3.99E+02	3.99E+02	3.99E+02	NA
0.6	1499 vs 1626	1499_N_5	3.65E+04	3.42E+06	1.23E+07	3.04E+06	3.28E+06	NA
0.6	1499 vs 1626	1626_K_6	7.78E+04	3.99E+02	3.99E+02	3.99E+02	3.99E+02	NA

**Supplementary Table S-3.1. (continued) AWRI1499 vs AWRI1608**

RAW								
SO2	Couple	Doublings	0	4	8	12	16	20
	01499 vs 1608	1499_K_3	4.60E+05	7.48E+06	2.38E+06	8.52E+04	1.65E+04	1.60E+03
	01499 vs 1608	1608_N_4	3.80E+05	1.44E+07	8.13E+06	5.08E+06	1.46E+07	1.25E+07
	01499 vs 1608	1499_N_2	5.66E+04	1.32E+06	3.60E+05	2.21E+04	1.40E+03	3.00E+02
	01499 vs 1608	1608_K_1	1.56E+05	1.39E+07	1.23E+07	8.31E+06	1.18E+07	8.64E+06
	01499 vs 1608	1499_K_4	2.99E+04	7.69E+04	1.93E+04	1.94E+04	2.50E+04	1.16E+03
	01499 vs 1608	1608_N_5	2.02E+05	2.62E+06	8.15E+06	2.00E+07	4.53E+07	7.40E+06
	01499 vs 1608	1499_N_4	7.20E+05	2.14E+05	1.66E+03	6.60E+02	3.99E+02	3.99E+02
	01499 vs 1608	1608_K_4	6.60E+05	2.72E+06	6.40E+06	4.93E+07	2.05E+07	9.47E+06
	01499 vs 1608	1499_K_7	6.17E+04	2.78E+06	4.60E+05	4.27E+04	2.85E+03	8.00E+02
	01499 vs 1608	1608_N_7	2.10E+05	2.10E+07	1.01E+07	1.03E+07	1.20E+07	2.27E+07
	01499 vs 1608	1499_N_5	1.10E+06	2.37E+05	3.54E+04	2.36E+03	3.99E+02	3.99E+02
	01499 vs 1608	1608_K_7	1.14E+04	1.91E+07	1.02E+07	7.17E+06	8.32E+06	9.52E+06
	0.21499 vs 1608	1499_K_3	4.60E+05	1.97E+05	4.80E+05	1.42E+04	5.00E+02	3.99E+02
	0.21499 vs 1608	1608_N_4	3.80E+05	1.52E+06	1.80E+06	5.28E+06	2.76E+06	2.70E+06
	0.21499 vs 1608	1499_N_2	5.66E+04	1.04E+05	1.66E+03	3.99E+02	3.99E+02	3.99E+02
	0.21499 vs 1608	1608_K_1	1.56E+05	2.72E+06	1.00E+06	6.47E+06	2.30E+06	5.20E+06
	0.21499 vs 1608	1499_K_4	2.99E+04	5.66E+04	6.20E+02	3.99E+02	NA	NA
	0.21499 vs 1608	1608_N_5	2.02E+05	3.12E+06	9.80E+05	1.96E+06	NA	NA
	0.21499 vs 1608	1499_N_4	7.20E+05	6.60E+05	5.40E+04	7.80E+02	NA	NA
	0.21499 vs 1608	1608_K_4	6.60E+05	3.12E+06	8.80E+05	3.88E+06	NA	NA
	0.21499 vs 1608	1499_K_7	6.17E+04	2.15E+05	5.95E+04	5.00E+02	3.99E+02	3.99E+02
	0.21499 vs 1608	1608_N_7	2.10E+05	2.38E+06	7.60E+05	1.60E+06	4.04E+06	5.53E+06
	0.21499 vs 1608	1499_N_5	1.10E+06	4.34E+04	5.40E+02	3.99E+02	3.99E+02	3.99E+02
	0.21499 vs 1608	1608_K_7	1.14E+04	2.58E+06	1.44E+06	8.80E+05	5.03E+06	1.50E+06
	0.41499 vs 1608	1499_K_3	4.60E+05	4.20E+05	1.11E+05	3.99E+02	3.99E+02	3.99E+02
	0.41499 vs 1608	1608_N_4	3.80E+05	3.40E+05	5.00E+05	1.62E+06	2.00E+06	2.12E+06
	0.41499 vs 1608	1499_N_2	5.66E+04	2.42E+06	1.04E+03	4.67E+03	3.99E+02	3.99E+02
	0.41499 vs 1608	1608_K_1	1.56E+05	9.60E+05	1.12E+06	6.20E+06	1.32E+06	2.02E+06
	0.41499 vs 1608	1499_K_4	2.99E+04	1.53E+05	1.28E+06	3.56E+06	3.56E+06	7.00E+05
	0.41499 vs 1608	1608_N_5	2.02E+05	2.30E+06	1.31E+06	3.92E+06	3.92E+06	1.63E+07
	0.41499 vs 1608	1499_N_4	7.20E+05	1.48E+06	1.08E+07	1.15E+07	1.09E+05	7.00E+05
	0.41499 vs 1608	1608_K_4	6.60E+05	1.38E+06	1.31E+07	4.52E+06	5.06E+06	3.99E+02
	0.41499 vs 1608	1499_K_7	6.17E+04	1.14E+05	3.60E+05	3.99E+02	3.99E+02	3.99E+02
	0.41499 vs 1608	1608_N_7	2.10E+05	1.81E+05	2.22E+06	2.57E+06	3.96E+06	1.42E+06
	0.41499 vs 1608	1499_N_5	1.10E+06	3.60E+05	1.95E+07	6.61E+04	4.60E+05	6.03E+06
	0.41499 vs 1608	1608_K_7	1.14E+04	7.80E+05	1.01E+07	1.20E+06	8.60E+05	3.04E+06
	0.61499 vs 1608	1499_K_3	4.60E+05	5.29E+07	2.72E+06	8.71E+06	5.95E+07	3.60E+06
	0.61499 vs 1608	1608_N_4	3.80E+05	7.20E+05	1.33E+04	1.72E+05	4.80E+05	2.18E+04
	0.61499 vs 1608	1499_N_2	5.66E+04	1.50E+06	2.14E+05	2.28E+06	4.00E+06	1.93E+04
	0.61499 vs 1608	1608_K_1	1.56E+05	1.04E+05	1.10E+06	2.46E+06	5.19E+06	5.80E+05
	0.61499 vs 1608	1499_K_4	2.99E+04	5.07E+06	4.78E+07	3.83E+07	7.95E+07	7.03E+07
	0.61499 vs 1608	1608_N_5	2.02E+05	1.86E+05	1.98E+05	2.23E+05	1.93E+04	4.57E+03
	0.61499 vs 1608	1499_N_4	7.20E+05	6.28E+07	2.25E+07	2.16E+07	1.63E+05	NA
	0.61499 vs 1608	1608_K_4	6.60E+05	1.61E+04	3.99E+02	3.99E+02	3.99E+02	NA
	0.61499 vs 1608	1499_K_7	6.17E+04	2.10E+06	3.03E+07	5.17E+06	2.84E+06	5.28E+06
	0.61499 vs 1608	1608_N_7	2.10E+05	3.99E+02	3.99E+02	3.99E+02	3.99E+02	3.99E+02
	0.61499 vs 1608	1499_N_5	1.10E+06	3.92E+06	1.08E+03	3.99E+02	NA	NA
	0.61499 vs 1608	1608_K_7	1.14E+04	1.84E+06	2.20E+05	7.00E+05	NA	NA



## Written communications

### Why is the genetic diversity of *Brettanomyces bruxellensis* important for winemakers and is it related to sulfur dioxide tolerance?

M. Avramova<sup>1,6</sup>, A. Cibrario<sup>1</sup>, M. Coton<sup>2</sup>, E. Coton<sup>2</sup>, F. Salin<sup>3</sup>, W. Albertin<sup>1,4</sup>, C. Curtin<sup>6</sup>, I. Masneuf-Pomarède<sup>1,5</sup>

<sup>1</sup>University of Bordeaux, ISVV, Unité de recherche Œnologie INRA, Bordeaux INP, Villenave d'Ornon, France

<sup>2</sup>Université de Brest, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France

<sup>3</sup>INRA, UMR Biodiversité Gènes et Ecosystèmes, PlateForme Génomique, Cestas, France

<sup>4</sup>ENSCBP, Bordeaux INP, Pessac, France

<sup>5</sup>Bordeaux Sciences Agro, Gradignan, France

<sup>6</sup>The Australian Wine Research Institute, Urrbrae, SA, Australia

Corresponding author's email: [avramova.marta@gmail.com](mailto:avramova.marta@gmail.com)

### Abstract

The environmental conditions of wine are unfavourable for growth of many microorganisms; however, *Brettanomyces bruxellensis* is highly adapted to the winemaking process, which implies resistance to sulfur dioxide, high ethanol tolerance, growth on limited nitrogen sources and tolerance of low pH. This yeast's metabolism results in an alteration of the wine's flavour profile (unpleasant 'leathery' and/or 'mousy' characters), thus leading to economic losses. *B. bruxellensis* is also associated with other industrial fermentations such as beer, cider, kombucha (fermented tea), kefir and bioethanol. In these products, the desirability/undesirability of this yeast is unclear and still debated.

The industrial importance of *B. bruxellensis* has led to the study of its genome and population structure. Previous studies revealed a high genotypic diversity at intra-species level and that phenotypic characteristics are strain-dependent. Furthermore, a comparison of genome assemblies revealed the coexistence of diploid and triploid populations and high dissemination of a triploid population in wine fermentations in Australia. We have conducted a genotyping study of a large population of *B. bruxellensis* isolates from five continents and different substrates using microsatellite markers. The results suggest that *B. bruxellensis* species is structured according to ploidy level and substrate. The potential contribution of the triploid state to the adaptation to industrial fermentations and to the dissemination of *B. bruxellensis* is discussed. This work focuses on the ability of different strains of *B. bruxellensis* to survive in the presence of sulfur dioxide.

### Introduction

*Brettanomyces bruxellensis* (*B. bruxellensis*) is a spoilage yeast associated with major wine aroma defects which are present in about 25% of red wines (Gerbaux et al. 2000; Conterno et al. 2006). The 'Brett' character often leads to consumers' rejection and therefore economic loss. Even if numerous prevention and elimination methods are available for winemakers, the problem often persists from one year to another. Thus, controlling *B. bruxellensis* contamination risk is often a priority when vinification and wine ageing management decisions



are made. The importance of the *B. bruxellensis* issue is underscored by the fact that this species is detected worldwide and in substrates other than wine.

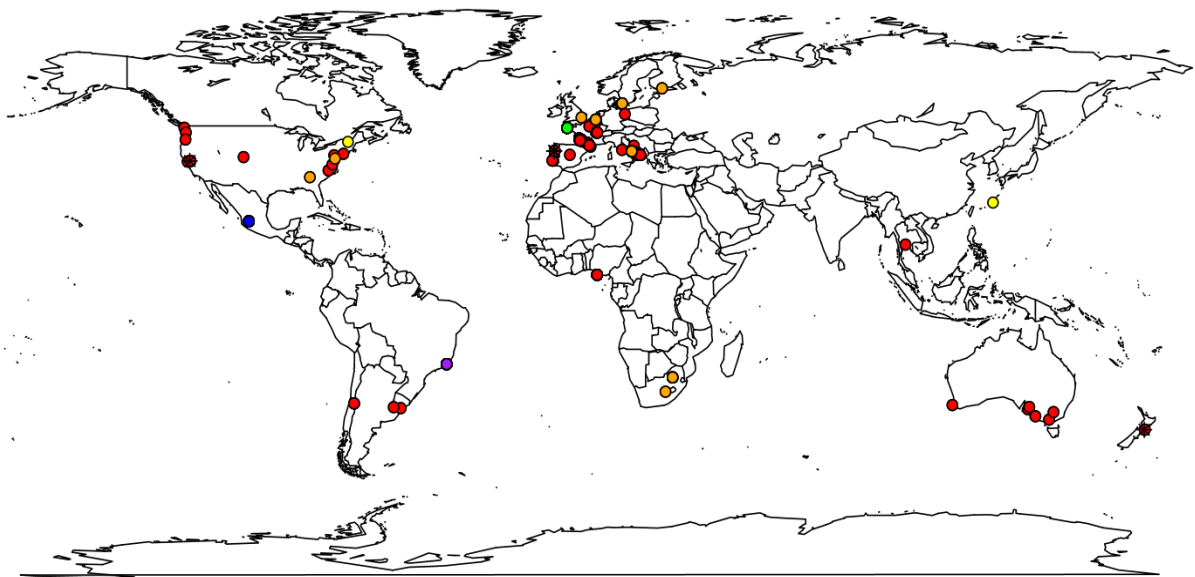
Sulfur dioxide (SO<sub>2</sub>) addition is the most common technique to prevent *B. bruxellensis* development during the winemaking process. However, high SO<sub>2</sub> levels are not always associated with premium wines styles and many wine consumers are seeking wines with lower SO<sub>2</sub> levels. Furthermore, it was recently shown that some *B. bruxellensis* strains are tolerant to SO<sub>2</sub>. The use of SO<sub>2</sub> as a prevention method against *B. bruxellensis* contamination might not prove a long-term strategy.

Here, we present our results concerning the intraspecific diversity of *B. bruxellensis* – on both genotypic and phenotypic level – and we focus particularly on *B. bruxellensis*' sensitivity to SO<sub>2</sub>.

### *Brettanomyces bruxellensis* and its genetic diversity

Numerous tools allow the detection and quantification of the species *B. bruxellensis*, although few were developed for genotypic analysis at intra-species level. Therefore, little evidence is available on the species' ecology and genetic diversity. Even though the population level can be quantified in a wine or must sample in a reliable way, it was, until now, relatively challenging to assess the nature of the strains present in the sample and their contamination and spoilage activity.

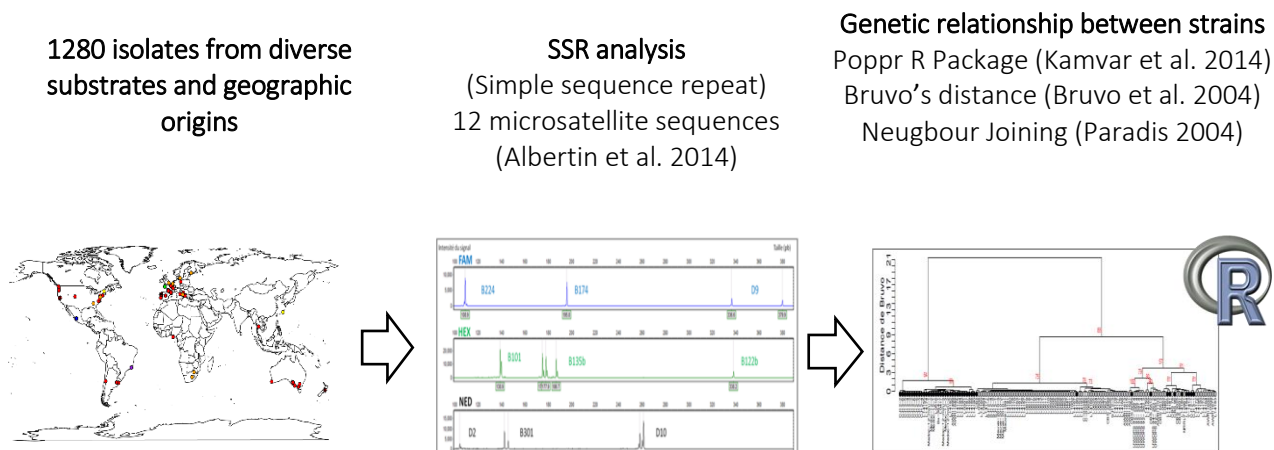
A collection of 1280 *B. bruxellensis* isolates from 29 countries was assembled and considered in this study (Figure 1).



**Figure 1.** Geographic origin of *B. bruxellensis* strains used in this study. The substrate of origin is indicated by the colour as follows: red – wine, grape and wine equipment; orange – beer; blue – tequila; green – kombucha; violet – bioethanol; yellow – others (cider and other fermented beverages).

Scientific teams from all over the world have focused their work on the genome sequencing of different strains of *B. bruxellensis*. Thanks to this recent knowledge, and particularly the sequences provided by the work of Curtin et al. (2012b), our team developed 12 genetic markers based on

microsatellite sequence repeats (Albertin et al. 2014) (a scheme representing the main steps of the method is provided in Figure 2). The strains used in the study were isolated from wine, sourced from other laboratories or were already present in Centre Ressources Biologiques (CRB) oenologie strain collection. DNA extraction was performed from a single fresh colony by treatment with 30  $\mu$ l of 20 mM NaOH and 99 °C heat for 10 minutes. Microsatellite analysis was done by amplifying Simple Sequence Repeat (or SSR) regions as described by Albertin et al. (2014). Amplicon sizes were measured by ABI3730 DNA analyser and GeneMarker® software. Raw data was treated on R software using Poppr Package (Kamvar et al. 2014).



**Figure 2.** Schematic of experimental approach

This method allows the establishment of the genetic links between strains, revealing significant genetic diversity within the species. In agreement with the first genome studies of the species (Curtin et al. 2012b; Borneman et al. 2014), we confirm the existence of triploid strains possessing every gene in three copies instead of the common two. These triploid strains possess an additional genome whose origin remains unknown at present. Remarkably, a high proportion of genotyped strains are triploid. The hypothesis is that the triploid state could confer specific traits to *B. bruxellensis* which would be advantageous for the adaptation to wine-type environment.

The 1280 *B. bruxellensis* isolates were genotyped by microsatellite analysis highlighting 617 genetic profiles clustered in three main genetic groups (A, B, and C). Group A consists of strains that are triploid and isolated from wine, group B consists of a second type of triploid strains isolated from beer and wine, and group C consists of mostly diploid strains isolated from wine and other substrates (kombucha, tequila, bioethanol, etc.). This significant genetic diversity may help to explain the considerable phenotypic variation of the strains shown in previous studies, particularly growth capacity, ethyl phenol production and/or SO<sub>2</sub> tolerance. Interestingly, different genetic groups have been shown to co-exist in the same winery or wine sample and from one sample to another taken from the same source.

### *Brettanomyces bruxellensis* and use of sulfur dioxide

The active form of SO<sub>2</sub> (i.e. molecular SO<sub>2</sub>) is the most common method to fight against *B. bruxellensis*. The efficiency of SO<sub>2</sub> depends on the dose applied, the pH, ethanol content and temperature of the medium. It is generally considered that 0.5 mg/l molecular SO<sub>2</sub> is sufficient to inhibit *B. bruxellensis*

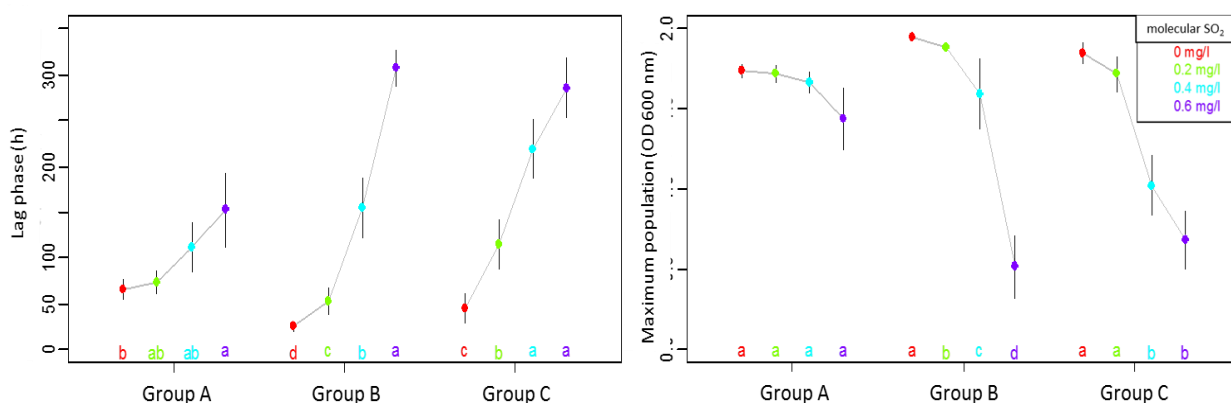
growth and that 0.7-0.8 mg/l of molecular SO<sub>2</sub> is a lethal dose (Chatonnet 2012), although the levels change with wine pH: 30 mg/l of free SO<sub>2</sub> at pH 3.6 equates to 60 mg/l at pH 3.9.

The existence of strains that are tolerant to SO<sub>2</sub> was recently highlighted. In Australia, a strategy to control *B. bruxellensis* based on the use of SO<sub>2</sub> was applied over the last ten years or more. *B. bruxellensis* isolates were collected from different wines treated with SO<sub>2</sub> and the studied population clustered in eight different genetic profiles as estimated by Amplified Fragment Length Polymorphism (Curtin 2007). A group comprising the majority among these strains (85 % of the isolates) was shown to be highly tolerant to SO<sub>2</sub> and cells could grow at 0.6 mg/l of molecular SO<sub>2</sub> (Curtin et al. 2012a). These findings suggest that using SO<sub>2</sub> to manage *B. bruxellensis* may apply a selective pressure to the population and ultimately lead to the emergence of highly resistant strains. Thus, it is important to understand any links between the genetic linkage of the strains and their resistance to SO<sub>2</sub>.

To spread these observations to other winemaking regions, we performed at laboratory scale a phenotypic test to evaluate the SO<sub>2</sub> tolerance of 33 strains representative of the genetic diversity of the species (strains varied in their geographical region, substrate of isolation and genetic group A, B or C as previously defined by microsatellite analysis). Strain growth was characterised in a synthetic laboratory medium in triplicate and under anaerobic conditions.

It was observed that growth in the presence of increasing concentrations of SO<sub>2</sub> is significantly different for each genetic group (Figure 3). For the strains from group A, the lag phase is slightly but significantly longer with an increasing SO<sub>2</sub> concentration. However, once the growth has started, the SO<sub>2</sub> concentration doesn't have any significant effect on the other growth parameters (i.e. growth rate, maximum population attained and time taken to attain the maximum population; only maximum population is shown) and this observation was valid even for the concentration of 0.6 mg/l of molecular SO<sub>2</sub>. Thus, these strains are considered tolerant to SO<sub>2</sub>: apart from the longer lag phase, they have a 'normal' growth from 0.2 to 0.6 mg/l molecular SO<sub>2</sub>. On the contrary, the growth of the strains belonging to strains B and C is strongly affected by the concentration of molecular SO<sub>2</sub> and this is valid for doses higher than 0.4 mg/l (and even 0.2 for several strains). These strains are considered sensitive to SO<sub>2</sub>.

Consequently, the adjustment of the molecular SO<sub>2</sub> even at 0.6 mg/l could be insufficient when SO<sub>2</sub> tolerant strains are present.



**Figure 3.** Growth parameters of different *B. bruxellensis* strains belonging to three major genetic groups (A - 8 strains, B - 8 strains and C - 17 strains) in media with increasing molecular SO<sub>2</sub> concentrations. Kruskal-Wallis test was performed for every parameter and every group of strains, different letters (a, b, c, d, ab) indicate significantly different mean values at 5% threshold.

## A tool for diagnosing *Brettanomyces bruxellensis*' SO<sub>2</sub> sensitivity

In our laboratory, we have developed and filed a patent for a simple molecular test which highlights the genetic group of a given *B. bruxellensis* isolate (A, B or C) and therefore predicts its SO<sub>2</sub> sensitivity. This test is based on simple Polymerase Chain Reaction (PCR) analysis on colonies isolated on selective solid medium. The analysis relies on the size of the amplification fragment produced at the end of the PCR, which varies with the genetic group of the strain. An example of a result after the test is performed on three different colonies is shown in Figure 4.



**Figure 4.** Result from the PCR to determine if a *B. bruxellensis* isolate belongs to the group A, B, or C. The 470 bp band is specific to the *B. bruxellensis* species (Ibeas at al. 1996), a 281 band is specific to strains from group A, a 356 band is specific to strains from group B and there is no band specific to group C.

Thus, a single analysis permits (i) to confirm that the isolate belongs to *B. bruxellensis* species and (ii) to predict its sensitivity to SO<sub>2</sub>. Of the 1280 isolates studied, 435 belong to group A, 206 to group B and 639 to group C. The group A isolates come from various wine regions in France (Bordeaux, Bourgogne, Jura, Languedoc, Côtes du Rhône) but also from Italy and Australia. No link was established between the genetic group and the geographical origin of the strains.

As a next step, we aim to develop a quantitative PCR-tool that would eliminate the cultivation step of the analysis and allow faster quantification.

## Conclusion

The study of *B. bruxellensis*' genetic diversity revealed an unexpected genomic complexity. Various *B. bruxellensis* groups exist which differ in terms of sulfite tolerance. In the collection of isolates studied by microsatellite analysis, 34% of the strains are potentially very tolerant to SO<sub>2</sub>, illustrating the fast adaptation capacity of the species. The phenomenon is widespread – the sulfite-tolerant isolates were detected not only in different French winemaking regions but also in other countries such as Australia.

In the actual context of chemical input reduction in the wine industry (particularly the use of SO<sub>2</sub>), it is now possible to assess the SO<sub>2</sub> sensitivity of *B. bruxellensis* contaminating a wine sample. This may help the winemaker to select a strategy to prevent and control spoilage, and avoid the use SO<sub>2</sub> when it is not likely to be effective – winemakers should utilise SO<sub>2</sub> addition only on wines that are not contaminated by tolerant strains.

## References

- Albertin, W.; Panfili, A.; Miot-Sertier, C.; Goulielmakis, A.; Delcamp, A.; Salin, F.; Lonvaud-Funel, A.; Curtin, C.; Masneuf-Pomarede, I. (2014) Development of microsatellite markers for the rapid and reliable genotyping of *Brettanomyces bruxellensis* at strain level. *Food Microbiol.* 42: 188-195.
- Borneman, A.R.; Zeppel, R.; Chambers, P.J.; Curtin, C.D. (2014) Insights into the *Dekkera bruxellensis* genomic landscape: comparative genomics reveals variations in ploidy and nutrient utilisation potential amongst wine isolates. *Plos Genet.* 10(2): e1004161.
- Bruvo, R.; Michelis, N.K.; D'Souza, T.G.; Schulenburg, H. (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Mol. Ecol.* 13: 2101-2106.
- Chatonnet, P. (2012) *Brettanomyces*: mythes et réalités. *Revue des œnologues.* 144: 42-48.
- Conterno, L.; Joseph, C.M.L.; Arvik, T.J.; Henick-Kling, T.; Bisson, L.F. (2006) Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. *Am. J. Enol. Vitic.* 57(2): 139-147.
- Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W., de Barros Lopes, M.A. (2007) Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Res.* 7(3):471-81.
- Curtin, C.D.; Kennedy, E.; Henschke, P.A. (2012a) Genotype-dependent sulphite tolerance of Australian *Dekkera* (*Brettanomyces*) *bruxellensis* wine isolates. *Lett. Appl. Microbiol.* 55(1): 56-61.
- Curtin, C.D.; Borneman, A.R.; Chambers, P.J., Pretorius, I.S. (2012b) De-novo assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast *Dekkera bruxellensis* AWRI1499. *Plos One* 7(3).
- Gerbaux, V.; Jeudy, S.; Monamy, C. (2000) Étude des phénols volatils dans les vins de Pinot noir en Bourgogne. *Bulletin de l'OIV* 73: 581-599.
- Ibeas, J.I.; Lozano, I.; Perdigones, F.; Jimenez, J. (1996) Detection of *Dekkera*-*Brettanomyces* strains in sherry by a nested PCR method. *Appl. Environ. Microbiol.* 62(3): 998-1003.
- Kamvar, Z.N.; Javier, F.T.; Grünwald, N.J. (2014) Poppr : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *Peer J*: 2:e281.
- Paradis, E.; Claude, J.; Strimmer, K. (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289-290.

## References

- Aa, E., Townsend, J.P., Adams, R.I., Nielsen, K.M., Taylor, J.W., 2006. Population structure and gene evolution in *Saccharomyces cerevisiae*. *FEMS Yeast Research* 6, 702–715. <https://doi.org/10.1111/j.1567-1364.2006.00059.x>
- Agnolucci, M., Cristani, C., Maggini, S., Rea, F., Cossu, A., Tirelli, A., Nuti, M., 2013. Impact of sulphur dioxide on the viability, culturability, and volatile phenol production of *Dekkera bruxellensis* in wine. *Ann Microbiol* 64, 653–659. <https://doi.org/10.1007/s13213-013-0698-6>
- Agnolucci, M., Rea, F., Sbrana, C., Cristani, C., Fracassetti, D., Tirelli, A., Nuti, M., 2010. Sulphur dioxide affects culturability and volatile phenol production by *Brettanomyces/Dekkera bruxellensis*. *International Journal of Food Microbiology* 143, 76–80. <https://doi.org/10.1016/j.ijfoodmicro.2010.07.022>
- Agnolucci, M., Vigentini, I., Capurso, G., Merico, A., Tirelli, A., Compagno, C., Foschino, R., Nuti, M., 2009. Genetic diversity and physiological traits of *Brettanomyces bruxellensis* strains isolated from Tuscan Sangiovese wines. *International Journal of Food Microbiology* 130, 238–244. <https://doi.org/10.1016/j.ijfoodmicro.2009.01.025>
- Albertin, W., Chasseriaud, L., Comte, G., Panfili, A., Delcamp, A., Salin, F., Marullo, P., Bely, M., 2014a. Winemaking and Bioprocesses Strongly Shaped the Genetic Diversity of the Ubiquitous Yeast *Torulaspora delbrueckii*. *PLoS ONE* 9, e94246. <https://doi.org/10.1371/journal.pone.0094246>
- Albertin, W., Marullo, P., 2012. Polyploidy in fungi: evolution after whole-genome duplication. *Proc. Biol. Sci.* 279, 2497–2509. <https://doi.org/10.1098/rspb.2012.0434>
- Albertin, W., Marullo, P., Aigle, M., Bourgeois, A., Bely, M., Dillmann, C., De Vienne, D., Sicard, D., 2009. Evidence for autotetraploidy associated with reproductive isolation in *Saccharomyces cerevisiae*: towards a new domesticated species. *Journal of Evolutionary Biology* 22, 2157–2170. <https://doi.org/10.1111/j.1420-9101.2009.01828.x>
- Albertin, W., Panfili, A., Miot-Sertier, C., Goulielmakis, A., Delcamp, A., Salin, F., Lonvaud-Funel, A., Curtin, C., Masneuf-Pomarede, I., 2014b. Development of microsatellite markers for the rapid and reliable genotyping of *Brettanomyces bruxellensis* at strain level. *Food Microbiology* 42, 188–195. <https://doi.org/10.1016/j.fm.2014.03.012>
- Albertin, W., Setati, M.E., Miot-Sertier, C., Mostert, T.T., Colonna-Ceccaldi, B., Coulon, J., Girard, P., Moine, V., Pillet, M., Salin, F., Bely, M., Divol, B., Masneuf-Pomarede, I., 2016. *Hanseniaspora uvarum* from Winemaking Environments Show Spatial and Temporal Genetic Clustering. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.01569>
- Almeida, P., Barbosa, R., Zalar, P., Imanishi, Y., Shimizu, K., Turchetti, B., Legras, J.-L., Serra, M., Dequin, S., Couloux, A., Guy, J., Bensasson, D., Gonçalves, P., Sampaio, J.P., 2015. A population genomics insight into the Mediterranean origins of wine yeast domestication. *Mol Ecol* 24, 5412–5427. <https://doi.org/10.1111/mec.13341>
- Almeida, P., Gonçalves, C., Teixeira, S., Libkind, D., Bontrager, M., Masneuf-Pomarede, I., Albertin, W., Durrens, P., Sherman, D., Marullo, P., Hittinger, C.T., Gonçalves, P., Sampaio, J.P., 2014. A Gondwanan Imprint on Global Diversity and Domestication of Wine and Cider Yeast *Saccharomyces uvarum*. *Nat Commun* 5, 4044. <https://doi.org/10.1038/ncomms5044>
- Anacleto, J., van Uden, N., 1982. Kinetics and activation energetics of death in *Saccharomyces cerevisiae* induced by sulfur dioxide. *Biotechnol. Bioeng.* 24, 2477–2486. <https://doi.org/10.1002/bit.260241112>

- Anderson, J.B., 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat Rev Micro* 3, 547–556. <https://doi.org/10.1038/nrmicro1179>
- Aranda, A., Jiménez-Martí, E., Orozco, H., Matallana, E., del Olmo, M., 2006. Sulfur and Adenine Metabolisms Are Linked, and Both Modulate Sulfite Resistance in Wine Yeast. *J. Agric. Food Chem.* 54, 5839–5846. <https://doi.org/10.1021/jf060851b>
- Arroyo-López, F.N., Orlić, S., Querol, A., Barrio, E., 2009. Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. *Int. J. Food Microbiol.* 131, 120–127. <https://doi.org/10.1016/j.ijfoodmicro.2009.01.035>
- Ayoub, M.-J., Legras, J.-L., Saliba, R., Gaillardin, C., 2006. Application of Multi Locus Sequence Typing to the analysis of the biodiversity of indigenous *Saccharomyces cerevisiae* wine yeasts from Lebanon. *Journal of Applied Microbiology* 100, 699–711. <https://doi.org/10.1111/j.1365-2672.2006.02817.x>
- Babcock, E.B. (Ernest B., 1877, B., Stebbins, G.L. (George L., 1906-, 1938. American species of *Crepis*.
- Baganz, F., Hayes, A., Marren, D., Gardner, D.C.J., Oliver, S.G., 1997. Suitability of replacement markers for functional analysis studies in *Saccharomyces cerevisiae*. *Yeast* 13, 1563–1573. [https://doi.org/10.1002/\(SICI\)1097-0061\(199712\)13:16<1563::AID-YEA240>3.0.CO;2-6](https://doi.org/10.1002/(SICI)1097-0061(199712)13:16<1563::AID-YEA240>3.0.CO;2-6)
- Baker, E., Wang, B., Bellora, N., Peris, D., Hulfachor, A.B., Koshalek, J.A., Adams, M., Libkind, D., Hittinger, C.T., 2015. The Genome Sequence of *Saccharomyces eubayanus* and the Domestication of Lager-Brewing Yeasts. *Mol Biol Evol* 32, 2818–2831. <https://doi.org/10.1093/molbev/msv168>
- Bakker, J., Bridle, P., Bellworthy, S.J., Garcia-Viguera, C., Reader, H.P., Watkins, S.J., 1998. Effect of sulphur dioxide and must extraction on colour, phenolic composition and sensory quality of red table wine. *J. Sci. Food Agric.* 78, 297–307. [https://doi.org/10.1002/\(SICI\)1097-0010\(199811\)78:3<297::AID-JSFA117>3.0.CO;2-G](https://doi.org/10.1002/(SICI)1097-0010(199811)78:3<297::AID-JSFA117>3.0.CO;2-G)
- Barata, A., Caldeira, J., Botelho, R., Pagliara, D., Malfeito-Ferreira, M., Loureiro, V., 2008. Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. *International Journal of Food Microbiology* 121, 201–207. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.020>
- Barata, A., Pagliara, D., Piccininno, T., Tarantino, F., Ciardulli, W., Malfeito-Ferreira, M., Loureiro, V., 2008. The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine. *FEMS Yeast Res.* 8, 1097–1102. <https://doi.org/10.1111/j.1567-1364.2008.00415.x>
- Barbin, P., Cheval, J.-L., Gilis, J.-F., Strehaiano, P., Taillandier, P., 2008. Diversity in spoilage yeast *Dekkera/Brettanomyces bruxellensis* isolated from French red wine. Assessment during fermentation of synthetic wine medium. *Journal of the Institute of Brewing* 114, 69–75.
- Barquet, M., Martín, V., Medina, K., Pérez, G., Carrau, F., Gaggero, C., 2012. Tandem repeat-tRNA (TRtRNA) PCR method for the molecular typing of non-*Saccharomyces* subspecies. *Appl Microbiol Biotechnol* 93, 807–814. <https://doi.org/10.1007/s00253-011-3714-4>
- Basílio, A.C.M., de Araújo, P.R.L., de Moraes, J.O.F., da Silva Filho, E.A., de Moraes, M.A., Simões, D.A., 2008. Detection and identification of wild yeast contaminants of the industrial fuel ethanol fermentation process. *Curr. Microbiol.* 56, 322–326. <https://doi.org/10.1007/s00284-007-9085-5>

- Beckner, M., Ivey, M.L., Phister, T.G., 2011. Microbial contamination of fuel ethanol fermentations. *Lett. Appl. Microbiol.* 53, 387–394. <https://doi.org/10.1111/j.1472-765X.2011.03124.x>
- Belloch, C., Orlic, S., Barrio, E., Querol, A., 2008. Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. *Int. J. Food Microbiol.* 122, 188–195. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.083>
- Benito, S., Palomero, F., Morata, A., Uthurry, C., Suárez-Lepe, J.A., 2009. Minimization of ethylphenol precursors in red wines via the formation of pyranoanthocyanins by selected yeasts. *International Journal of Food Microbiology* 132, 145–152. <https://doi.org/10.1016/j.ijfoodmicro.2009.04.015>
- Bing, J., Han, P.-J., Liu, W.-Q., Wang, Q.-M., Bai, F.-Y., 2014. Evidence for a Far East Asian origin of lager beer yeast. *Current Biology* 24, R380–R381. <https://doi.org/10.1016/j.cub.2014.04.031>
- Blein-Nicolas, M., Albertin, W., Da, S., Valot, B., Balliau, T., Masneuf-Pomarède, I., Bely, M., Marullo, P., Sicard, D., Dillmann, C., De, V., Zivy, M., 2015. A systems approach to elucidate heterosis of protein abundances in yeast. *Molecular and Cellular Proteomics* 14, 2056–2071. <https://doi.org/10.1074/mcp.M115.048058>
- Blomqvist, J., Eberhard, T., Schnürer, J., Passoth, V., 2010. Fermentation characteristics of *Dekkera bruxellensis* strains. *Appl Microbiol Biotechnol* 87, 1487–1497. <https://doi.org/10.1007/s00253-010-2619-y>
- Blomqvist, J., Passoth, V., 2015. *Dekkera bruxellensis*—spoilage yeast with biotechnological potential, and a model for yeast evolution, physiology and competitiveness. *FEMS Yeast Research* 15, fov021. <https://doi.org/10.1093/femsyr/fov021>
- Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. Brewhouse-Resident Microbiota Are Responsible for Multi-Stage Fermentation of American Coolship Ale. *PLOS ONE* 7, e35507. <https://doi.org/10.1371/journal.pone.0035507>
- Börlin, M., Venet, P., Claisse, O., Salin, F., Legras, J.-L., Masneuf-Pomarede, I., 2016. Cellar-Associated *Saccharomyces cerevisiae* Population Structure Revealed High-Level Diversity and Perennial Persistence at Sauternes Wine Estates. *Appl. Environ. Microbiol.* 82, 2909–2918. <https://doi.org/10.1128/AEM.03627-15>
- Borneman, A.R., Desany, B.A., Riches, D., Affourtit, J.P., Forgan, A.H., Pretorius, I.S., Egholm, M., Chambers, P.J., 2012. The genome sequence of the wine yeast VIN7 reveals an allotriploid hybrid genome with *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* origins. *FEMS Yeast Res.* 12, 88–96. <https://doi.org/10.1111/j.1567-1364.2011.00773.x>
- Borneman, A.R., Zeppel, R., Chambers, P.J., Curtin, C.D., 2014. Insights into the *Dekkera bruxellensis* Genomic Landscape: Comparative Genomics Reveals Variations in Ploidy and Nutrient Utilisation Potential amongst Wine Isolates. *PLoS Genetics* 10. <https://doi.org/10.1371/journal.pgen.1004161>
- Botstein, D., Chervitz, S.A., Cherry, M., 1997. Yeast as a Model Organism. *Science* 277, 1259–1260. <https://doi.org/10.1126/science.277.5330.1259>
- Bradbury, J.E., Richards, K.D., Niederer, H.A., Lee, S.A., Dunbar, P.R., Gardner, R.C., 2006. A homozygous diploid subset of commercial wine yeast strains. *Antonie Van Leeuwenhoek* 89, 27–37. <https://doi.org/10.1007/s10482-005-9006-1>
- Brauner, A., Fridman, O., Gefen, O., Balaban, N.Q., 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Micro* 14, 320–330. <https://doi.org/10.1038/nrmicro.2016.34>
- Bruvo, R., Michiels, N.K., D'souza, T.G., Schulenburg, H., 2004. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology* 13, 2101–2106. <https://doi.org/10.1111/j.1365-294X.2004.02209.x>



- Cadez, N., Raspor, P., Cock, D., W.a.m, A., Boekhout, T., Smith, M.T., 2002. Molecular identification and genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*. *FEMS Yeast Res* 1, 279–289. <https://doi.org/10.1111/j.1567-1364.2002.tb00046.x>
- Campolongo, S., Rantsiou, K., Giordano, M., Gerbi, V., Cocolin, L., 2010. Prevalence and Biodiversity of *Brettanomyces bruxellensis* in Wine from Northwestern Italy. *Am. J. Enol. Vitic.* 61, 486–491. <https://doi.org/10.5344/ajev.2010.10034>
- Capozzi, V., Di Toro, M.R., Grieco, F., Michelotti, V., Salma, M., Lamontanara, A., Russo, P., Orrù, L., Alexandre, H., Spano, G., 2016. Viable But Not Culturable (VBNC) state of *Brettanomyces bruxellensis* in wine: New insights on molecular basis of VBNC behaviour using a transcriptomic approach. *Food Microbiology* 59, 196–204. <https://doi.org/10.1016/j.fm.2016.06.007>
- Cappello, M.S., Poltronieri, P., Blaiotta, G., Zacheo, G., 2010. Molecular and physiological characteristics of a grape yeast strain containing atypical genetic material. *International Journal of Food Microbiology, The 16th CBL (Club des Bactéries Lactiques) Symposium, May 2009, Toulouse, France* 144, 72–80. <https://doi.org/10.1016/j.ijfoodmicro.2010.08.013>
- Capusoni, C., Arioli, S., Zambelli, P., Moktaduzzaman, M., Mora, D., Compagno, C., 2016. Effects of oxygen availability on acetic acid tolerance and intracellular pH in *Dekkera bruxellensis*. *Appl. Environ. Microbiol.* AEM.00515-16. <https://doi.org/10.1128/AEM.00515-16>
- Carmona, L., Varela, J., Godoy, L., Ganga, M.A., 2016. Comparative proteome analysis of *Brettanomyces bruxellensis* under hydroxycinnamic acid growth. *Electronic Journal of Biotechnology* 23, 37–43. <https://doi.org/10.1016/j.ejbt.2016.07.005>
- Carrasco-Sánchez, V., John, A., Marican, A., Santos, L.S., Laurie, V.F., 2015. Removal of 4-Ethylphenol and 4-Ethylguaiacol with Polyaniline-Based Compounds in Wine-Like Model Solutions and Red Wine. *Molecules* 20, 14312–14325. <https://doi.org/10.3390/molecules200814312>
- Chakravarti, A., 1999. Population genetics—making sense out of sequence. *Nat Genet* 21, 56–60. <https://doi.org/10.1038/4482>
- Chambers, P.J., Pretorius, I.S., 2010. Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Rep* 11, 914–920. <https://doi.org/10.1038/embor.2010.179>
- Chatonnet, P., Dubourdieu, D., Boidron, J.N., 1995a. The Influence of *Brettanomyces/Dekkera* sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines. *Am. J. Enol. Vitic.* 46, 463–468.
- Chatonnet, P., Dubourdieu, D., Boidron, J.N., 1995b. The Influence of *Brettanomyces/Dekkera* sp. Yeasts and Lactic Acid Bacteria on the Ethylphenol Content of Red Wines. *Am J Enol Vitic.* 46, 463–468.
- Chatonnet, P., Dubourdieu, D., Boidron, J.N., 1992. Incidence des conditions de fermentation et d'élevage des vins blancs secs en barriques sur leur composition en substances cédées par le bois de chêne. *Sciences des aliments* 12, 665–685.
- Chen, Z.J., 2010. Molecular mechanisms of polyploidy and hybrid vigor. *Trends in Plant Science* 15, 57–71. <https://doi.org/10.1016/j.tplants.2009.12.003>
- Clark, L.V., Schreier, A.D., 2017. Resolving microsatellite genotype ambiguity in populations of allopolyploid and diploidized autopolyploid organisms using negative correlations between allelic variables. *Mol Ecol Resour* 17, 1090–1103. <https://doi.org/10.1111/1755-0998.12639>

- Claussen, N.H., 1904. On a Method for the Application of Hansen's Pure Yeast System in the Manufacturing of Well-Conditioned English Stock Beers. *Journal of the Institute of Brewing* 10, 308–331. <https://doi.org/10.1002/j.2050-0416.1904.tb04656.x>
- Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R., Comi, G., 2004. Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalous* in spoiled wines. *Appl. Environ. Microbiol.* 70, 1347–1355.
- Comai, L., 2005. The advantages and disadvantages of being polyploid. *Nat Rev Genet* 6, 836–846. <https://doi.org/10.1038/nrg1711>
- Comitini, F., Ciani, M., 2011. *Kluyveromyces wickerhamii* killer toxin: purification and activity towards *Brettanomyces/Dekkera* yeasts in grape must. *FEMS Microbiol Lett* 316, 77–82. <https://doi.org/10.1111/j.1574-6968.2010.02194.x>
- Conant, G.C., Wolfe, K.H., 2007. Increased glycolytic flux as an outcome of whole-genome duplication in yeast. *Mol Syst Biol* 3. <https://doi.org/10.1038/msb4100170>
- Conterno, L., Joseph, C.M.L., Arvik, T.J., Henick-Kling, T., Bisson, L.F., 2006. Genetic and Physiological Characterization of *Brettanomyces bruxellensis* Strains Isolated from Wines. *Am. J. Enol. Vitic.* 57, 139–147.
- Coton, E., Coton, M., Levert, D., Casaregola, S., Sohier, D., 2006. Yeast ecology in French cider and black olive natural fermentations. *International Journal of Food Microbiology* 108, 130–135. <https://doi.org/10.1016/j.ijfoodmicro.2005.10.016>
- Coton, M., Pawtowski, A., Taminiou, B., Burgaud, G., Deniel, F., Coulloume-Labarthe, L., Fall, A., Daube, G., Coton, E., 2017. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiol. Ecol.* 93. <https://doi.org/10.1093/femsec/fix048>
- Crauwels, S., Assche, A.V., Jonge, R. de, Borneman, A.R., Verreth, C., Troels, P., Samblanx, G.D., Marchal, K., Peer, Y.V. de, Willems, K.A., Verstrepen, K.J., Curtin, C.D., Lievens, B., 2015. Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. *Appl Microbiol Biotechnol* 99, 9123–9134. <https://doi.org/10.1007/s00253-015-6769-9>
- Crauwels, S., Van Opstaele, F., Jaskula-Goiris, B., Steensels, J., Verreth, C., Bosmans, L., Paulussen, C., Herrera-Malaver, B., de Jonge, R., De Clippeleer, J., Marchal, K., De Samblanx, G., Willems, K.A., Verstrepen, K.J., Aerts, G., Lievens, B., 2017. Fermentation assays reveal differences in sugar and (off-) flavor metabolism across different *Brettanomyces bruxellensis* strains. *FEMS Yeast Res* 17. <https://doi.org/10.1093/femsyr/fow105>
- Crauwels, S., Zhu, B., Steensels, J., Busschaert, P., Samblanx, G.D., Marchal, K., Willems, K.A., Verstrepen, K.J., Lievens, B., 2014. Assessing Genetic Diversity among *Brettanomyces* Yeasts by DNA Fingerprinting and Whole-Genome Sequencing. *Appl. Environ. Microbiol.* 80, 4398–4413. <https://doi.org/10.1128/AEM.00601-14>
- Cubillos, F.A., Vásquez, C., Faugeron, S., Ganga, A., Martínez, C., 2009. Self-fertilization is the main sexual reproduction mechanism in native wine yeast populations. *FEMS Microbiol. Ecol.* 67, 162–170. <https://doi.org/10.1111/j.1574-6941.2008.00600.x>
- Curtin, C., Kennedy, E., Henschke, P. a., 2012a. Genotype-dependent sulphite tolerance of Australian *Dekkera* (*Brettanomyces*) *bruxellensis* wine isolates. *Letters in Applied Microbiology* 55, 56–61. <https://doi.org/10.1111/j.1472-765X.2012.03257.x>
- Curtin, C., Kennedy, E., Henschke, P.A., 2012b. Genotype-dependent sulphite tolerance of Australian *Dekkera* (*Brettanomyces*) *bruxellensis* wine isolates. *Lett. Appl. Microbiol.* 55, 56–61. <https://doi.org/10.1111/j.1472-765X.2012.03257.x>

- Curtin, C., Varela, C., Borneman, A., 2015. Harnessing improved understanding of *Brettanomyces bruxellensis* biology to mitigate the risk of wine spoilage. *Australian Journal of Grape and Wine Research* 21, 680–692. <https://doi.org/10.1111/ajgw.12200>
- Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W., De, B.L., 2007. Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Research* 7, 471–481. <https://doi.org/10.1111/j.1567-1364.2006.00183.x>
- Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W., Lopes, M.A. de B., 2007. Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Research* 7, 471–481. <https://doi.org/10.1111/j.1567-1364.2006.00183.x>
- Curtin, C.D., Borneman, A.R., Chambers, P.J., Pretorius, I.S., 2012. De-Novo Assembly and Analysis of the Heterozygous Triploid Genome of the Wine Spoilage Yeast *Dekkera bruxellensis* AWRI1499. *PLOS ONE* 7, e33840. <https://doi.org/10.1371/journal.pone.0033840>
- Curtin, C.D., Langhans, G., Henschke, P.A., Grbin, P.R., 2013. Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma. *Food Microbiol.* 36, 241–247. <https://doi.org/10.1016/j.fm.2013.06.008>
- Curtin, C.D., Pretorius, I.S., 2014. Genomic insights into the evolution of industrial yeast species *Brettanomyces bruxellensis*. *FEMS Yeast Res* 14, 997–1005. <https://doi.org/10.1111/1567-1364.12198>
- de Barros Lopes, M., Bellon, J.R., Shirley, N.J., Ganter, P.F., 2002. Evidence for multiple interspecific hybridization in *Saccharomyces sensu stricto* species. *FEMS Yeast Research* 1, 323–331. <https://doi.org/10.1111/j.1567-1364.2002.tb00051.x>
- de Souza Liberal, A.T., Basílio, A.C.M., do Monte Resende, A., Brasileiro, B.T.V., da Silva-Filho, E.A., de Moraes, J.O.F., Simões, D.A., de Moraes, M.A., 2007. Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. *J. Appl. Microbiol.* 102, 538–547. <https://doi.org/10.1111/j.1365-2672.2006.03082.x>
- Deak, T., Beuchat, L.R., 1995. Modified indirect conductimetric technique for detecting low populations of yeasts in beverage concentrates and carbonated beverages. *Food Microbiology* 12, 165–172. [https://doi.org/10.1016/S0740-0020\(95\)80093-X](https://doi.org/10.1016/S0740-0020(95)80093-X)
- Delfini, C., Gaia, P., Schellino, R., Strano, M., Pagliara, A., Ambrò, S., 2002. Fermentability of grape must after inhibition with dimethyl dicarbonate (DMDC). *J. Agric. Food Chem.* 50, 5605–5611.
- Delsart, C., Grimi, N., Boussetta, N., Miot Sertier, C., Ghidossi, R., Vorobiev, E., Mietton Peuchot, M., 2016. Impact of pulsed-electric field and high-voltage electrical discharges on red wine microbial stabilization and quality characteristics. *J. Appl. Microbiol.* 120, 152–164. <https://doi.org/10.1111/jam.12981>
- Denoth Lippuner, A., Julou, T., Barral, Y., 2014. Budding yeast as a model organism to study the effects of age. *FEMS Microbiol. Rev.* 38, 300–325. <https://doi.org/10.1111/1574-6976.12060>
- Dequin, S., Casaregola, S., 2011. The genomes of fermentative *Saccharomyces*. *C. R. Biol.* 334, 687–693. <https://doi.org/10.1016/j.crv.2011.05.019>
- Di Toro, M.R., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., Tufariello, M., Grieco, F., Spano, G., 2015. Intraspecific biodiversity and “spoilage potential” of *Brettanomyces bruxellensis* in Apulian wines. *LWT - Food Science and Technology* 60, 102–108. <https://doi.org/10.1016/j.lwt.2014.06.059>
- Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M., Loureiro, V., 2003. Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. *Food Microbiology* 20, 377–384. [https://doi.org/10.1016/S0740-0020\(03\)00023-6](https://doi.org/10.1016/S0740-0020(03)00023-6)

- Divol, B., Du, T., Duckitt, E., 2012. Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Applied Microbiology and Biotechnology* 95, 601–613. <https://doi.org/10.1007/s00253-012-4186-x>
- Du Toit, W. j., Pretorius, I. s., Lonvaud-Funel, A., 2005a. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal of Applied Microbiology* 98, 862–871. <https://doi.org/10.1111/j.1365-2672.2004.02549.x>
- Du Toit, W. j., Pretorius, I. s., Lonvaud-Funel, A., 2005b. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal of Applied Microbiology* 98, 862–871. <https://doi.org/10.1111/j.1365-2672.2004.02549.x>
- Duarte, F.L., Coimbra, L., Baleiras-Couto, M., 2017. Filter Media Comparison for the Removal of *Brettanomyces bruxellensis* from Wine. *Am J Enol Vitic. ajev.2017.17003*. <https://doi.org/10.5344/ajev.2017.17003>
- Dubourdieu, D., Sokol, A., Zucca, J., Thalouarn, P., Dattee, A., Aigle, M., 1987. Identification des souches de levures isolées de vins par l’analyse de leur ADN mitochondrial. *OENO One* 21, 267. <https://doi.org/10.20870/oeno-one.1987.21.4.1286>
- Dunn, B., Levine, R.P., Sherlock, G., 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics* 6, 53. <https://doi.org/10.1186/1471-2164-6-53>
- Dweck, H.K.M., Ebrahim, S.A.M., Farhan, A., Hansson, B.S., Stensmyr, M.C., 2015. Olfactory Proxy Detection of Dietary Antioxidants in *Drosophila*. *Current Biology* 25, 455–466. <https://doi.org/10.1016/j.cub.2014.11.062>
- Erny, C., Raoult, P., Alais, A., Butterlin, G., Delobel, P., Matei-Radoi, F., Casaregola, S., Legras, J.L., 2012. Ecological Success of a Group of *Saccharomyces cerevisiae*/*Saccharomyces kudriavzevii* Hybrids in the Northern European Wine-Making Environment. *Appl. Environ. Microbiol.* 78, 3256–3265. <https://doi.org/10.1128/AEM.06752-11>
- Esteve-Zarzoso, B., Peris-Torán, M.J., García-Maiquez, E., Uruburu, F., Querol, A., 2001. Yeast Population Dynamics during the Fermentation and Biological Aging of Sherry Wines. *Appl Environ Microbiol* 67, 2056–2061. <https://doi.org/10.1128/AEM.67.5.2056-2061.2001>
- Etievant, P.X. (Institut N. de la R.A., Issanchou, S.N., Marie, S., Ducruet, V., Flanzy, C., 1989. Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration and time of storage. *Sciences des Aliments (France)*.
- Fabrizio, V., Vigentini, I., Parisi, N., Picozzi, C., Compagno, C., Foschino, R., 2015. Heat inactivation of wine spoilage yeast *Dekkera bruxellensis* by hot water treatment. *Lett Appl Microbiol* 61, 186–191. <https://doi.org/10.1111/lam.12444>
- Fay, J.C., Benavides, J.A., 2005. Evidence for Domesticated and Wild Populations of *Saccharomyces cerevisiae*. *PLOS Genetics* 1, e5. <https://doi.org/10.1371/journal.pgen.0010005>
- Feldmesser, M., Kress, Y., Casadevall, A., 2001. Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. *Microbiology (Reading, Engl.)* 147, 2355–2365. <https://doi.org/10.1099/00221287-147-8-2355>
- Fleet, G.H., 2003. Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22.
- Fournier, T., Gounot, J.-S., Freel, K.C., Cruaud, C., Lemainque, A., Aury, J.-M., Wincker, P., Schacherer, J., Friedrich, A., 2017. High-quality de novo genome assembly of the *Dekkera bruxellensis* UMY321 yeast isolate using Nanopore MinION sequencing. *bioRxiv* 151167. <https://doi.org/10.1101/151167>

- Franco-Duarte, R., Mendes, I., Umek, L., Drumonde-Neves, J., Zupan, B., Schuller, D., 2014. Computational models reveal genotype–phenotype associations in *Saccharomyces cerevisiae*. *Yeast* 31, 265–277. <https://doi.org/10.1002/yea.3016>
- Franco-Duarte, R., Umek, L., Zupan, B., Schuller, D., 2009. Computational approaches for the genetic and phenotypic characterization of a *Saccharomyces cerevisiae* wine yeast collection. *Yeast* 26, 675–692. <https://doi.org/10.1002/yea.1728>
- Frawley, L.E., Orr-Weaver, T.L., 2015. Polyploidy. *Current Biology* 25, R353–R358. <https://doi.org/10.1016/j.cub.2015.03.037>
- Frichot, E., François, O., 2015. LEA: An R package for landscape and ecological association studies. *Methods Ecol Evol* 6, 925–929. <https://doi.org/10.1111/2041-210X.12382>
- Fugelsang, K.C., Zoecklein, B.W., 2003. Population Dynamics and Effects of *Brettanomyces bruxellensis* Strains on Pinot noir (*Vitis vinifera* L.) Wines. *Am J Enol Vitic.* 54, 294–300.
- Fujiwara, T., Bandi, M., Nitta, M., Ivanova, E.V., Bronson, R.T., Pellman, D., 2005. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. *Nature* 437, 1043–1047. <https://doi.org/10.1038/nature04217>
- Fundyga, R.E., Lott, T.J., Arnold, J., 2002. Population structure of *Candida albicans*, a member of the human flora, as determined by microsatellite loci. *Infection, Genetics and Evolution* 2, 57–68. [https://doi.org/10.1016/S1567-1348\(02\)00088-6](https://doi.org/10.1016/S1567-1348(02)00088-6)
- Galafassi, S., Merico, A., Pizza, F., Hellborg, L., Molinari, F., Piškur, J., Compagno, C., 2011. Dekkera/*Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. *J Ind Microbiol Biotechnol* 38, 1079–1088. <https://doi.org/10.1007/s10295-010-0885-4>
- Gallardo-Chacón, J.J., Karbowski, T., 2015. Sorption of 4-ethylphenol and 4-ethylguaiacol by suberin from cork. *Food Chem* 181, 222–226. <https://doi.org/10.1016/j.foodchem.2015.02.102>
- Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L., Teiling, C., Steffy, B., Taylor, M., Schwartz, A., Richardson, T., White, C., Baele, G., Maere, S., Verstrepen, K.J., 2016. Domestication and Divergence of *Saccharomyces cerevisiae* Beer Yeasts. *Cell* 166, 1397–1410.e16. <https://doi.org/10.1016/j.cell.2016.08.020>
- Ganga, M.A., Martínez, C., 2004. Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *J. Appl. Microbiol.* 96, 76–83.
- Gangl, H., Batusic, M., Tscheik, G., Tiefenbrunner, W., Hack, C., Lopandic, K., 2009. Exceptional fermentation characteristics of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. *N Biotechnol* 25, 244–251. <https://doi.org/10.1016/j.nbt.2008.10.001>
- Garcia, D., Gomez-Caballero, A., Guerreiro, A., Goicolea, M.A., Barrio, R.J., 2015. Molecularly imprinted polymers as a tool for the study of the 4-ethylphenol metabolic pathway in red wines. *J Chromatogr A* 1410, 164–172. <https://doi.org/10.1016/j.chroma.2015.07.103>
- Gayevskiy, V., Goddard, M.R., 2016. *Saccharomyces eubayanus* and *Saccharomyces arboricola* reside in North Island native New Zealand forests. *Environ Microbiol* 18, 1137–1147. <https://doi.org/10.1111/1462-2920.13107>
- Gerstein, A.C., Fu, M.S., Mukaremera, L., Li, Z., Ormerod, K.L., Fraser, J.A., Berman, J., Nielsen, K., 2015. Polyploid Titan Cells Produce Haploid and Aneuploid Progeny To Promote Stress Adaptation. *mBio* 6, e01340-15. <https://doi.org/10.1128/mBio.01340-15>
- Gilliland, R.B., 1961. *Brettanomyces*. I. Occurrence, Characteristics, and Effects on Beer Flavour. *Journal of the Institute of Brewing* 67, 257–261. <https://doi.org/10.1002/j.2050-0416.1961.tb01791.x>

- Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Lukasik, R., 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, Special Issue on Lignocellulosic Bioethanol: Current Status and Perspectives 101, 4775–4800. <https://doi.org/10.1016/j.biortech.2010.01.088>
- Goddard, M.R., Greig, D., 2015. *Saccharomyces cerevisiae*: a nomadic yeast with no niche? *FEMS Yeast Res.* 15. <https://doi.org/10.1093/femsyr/fov009>
- Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., Oliver, S.G., 1996. Life with 6000 Genes. *Science* 274, 546–567. <https://doi.org/10.1126/science.274.5287.546>
- Goldstein, A.L., McCusker, J.H., 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553. [https://doi.org/10.1002/\(SICI\)1097-0061\(199910\)15:14<1541::AID-YEA476>3.0.CO;2-K](https://doi.org/10.1002/(SICI)1097-0061(199910)15:14<1541::AID-YEA476>3.0.CO;2-K)
- González, S.S., Barrio, E., Gafner, J., Querol, A., 2006. Natural hybrids from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces kudriavzevii* in wine fermentations. *FEMS Yeast Res.* 6, 1221–1234. <https://doi.org/10.1111/j.1567-1364.2006.00126.x>
- González, S.S., Barrio, E., Querol, A., 2008. Molecular Characterization of New Natural Hybrids of *Saccharomyces cerevisiae* and *S. kudriavzevii* in Brewing. *Appl. Environ. Microbiol.* 74, 2314–2320. <https://doi.org/10.1128/AEM.01867-07>
- González, S.S., Gallo, L., Climent, M.D., Barrio, E., Querol, A., 2007. Enological characterization of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. *International Journal of Food Microbiology* 116, 11–18. <https://doi.org/10.1016/j.ijfoodmicro.2006.10.047>
- González-Arenzana, L., Sevenich, R., Rauh, C., López, R., Knorr, D., López-Alfaro, I., 2016. Inactivation of *Brettanomyces bruxellensis* by High Hydrostatic Pressure technology. *Food Control* 59, 188–195. <https://doi.org/10.1016/j.foodcont.2015.04.038>
- Grangeteau, C., Gerhards, D., Rousseaux, S., von Wallbrunn, C., Alexandre, H., Guilloux-Benatier, M., 2015. Diversity of yeast strains of the genus *Hanseniaspora* in the winery environment: What is their involvement in grape must fermentation? *Food Microbiology* 50, 70–77. <https://doi.org/10.1016/j.fm.2015.03.009>
- Grbin, P.R., Henschke, P.A., 2000. Mousy off-flavour production in grape juice and wine by *Dekkera* and *Brettanomyces* yeasts. *Australian Journal of Grape and Wine Research* 6, 255–262. <https://doi.org/10.1111/j.1755-0238.2000.tb00186.x>
- Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Léger, P., Lepais, O., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F., Petit, R.J., 2011. Current trends in microsatellite genotyping. *Mol Ecol Resour* 11, 591–611. <https://doi.org/10.1111/j.1755-0998.2011.03014.x>
- Guijo, S., Mauricio, J.C., Salmon, J.M., Ortega, J.M., 1997. Determination of the relative ploidy in different *Saccharomyces cerevisiae* strains used for fermentation and “flor” film ageing of dry sherry-type wines. *Yeast* 13, 101–117. [https://doi.org/10.1002/\(SICI\)1097-0061\(199702\)13:2<101::AID-YEA66>3.0.CO;2-H](https://doi.org/10.1002/(SICI)1097-0061(199702)13:2<101::AID-YEA66>3.0.CO;2-H)
- Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J., Querol, A., 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch Microbiol* 169, 387–392. <https://doi.org/10.1007/s002030050587>
- Hammes, W.P., Brandt, M.J., Francis, K.L., Rosenheim, J., Seitter, M.F.H., Vogelmann, S.A., 2005. Microbial ecology of cereal fermentations. *Trends in Food Science & Technology*, Second International Symposium on Sourdough - From Fundamentals to Applications 16, 4–11. <https://doi.org/10.1016/j.tifs.2004.02.010>

- Harris, V., Ford, C.M., Jiranek, V., Grbin, P.R., 2008. Dekkera and Brettanomyces growth and utilisation of hydroxycinnamic acids in synthetic media. *Appl Microbiol Biotechnol* 78, 997–1006. <https://doi.org/10.1007/s00253-007-1328-7>
- Hazen, K.C., 1995. New and emerging yeast pathogens. *Clin. Microbiol. Rev.* 8, 462–478.
- Hegreness, M., Shores, N., Hartl, D., Kishony, R., 2006. An Equivalence Principle for the Incorporation of Favorable Mutations in Asexual Populations. *Science* 311, 1615–1617. <https://doi.org/10.1126/science.1122469>
- Hellborg, L., Piškur, J., 2009. Complex Nature of the Genome in a Wine Spoilage Yeast, *Dekkera bruxellensis*. *Eukaryot Cell* 8, 1739–1749. <https://doi.org/10.1128/EC.00115-09>
- Heresztyn, T., 1986. Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast. *Arch. Microbiol.* 146, 96–98. <https://doi.org/10.1007/BF00690165>
- Hibbett, D.S., Taylor, J.W., 2013. Fungal systematics: is a new age of enlightenment at hand? *Nature Reviews Microbiology* 11, nrmicro2963. <https://doi.org/10.1038/nrmicro2963>
- Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* 8, 15–25. <https://doi.org/10.1038/nrmicro2259>
- Hickman, M.A., Zeng, G., Forche, A., Hirakawa, M.P., Abbey, D., Harrison, B.D., Wang, Y.-M., Su, C., Bennett, R.J., Wang, Y., Berman, J., 2013. The ‘obligate diploid’ *Candida albicans* forms mating-competent haploids. *Nature* 494, 55–59. <https://doi.org/10.1038/nature11865>
- Hinze, H., Holzer, H., 1986. Analysis of the energy metabolism after incubation of *Saccharomyces cerevisiae* with sulfite or nitrite. *Arch. Microbiol.* 145, 27–31.
- Höhna, S., May, M.R., Moore, B.R., 2016. TESS: an R package for efficiently simulating phylogenetic trees and performing Bayesian inference of lineage diversification rates. *Bioinformatics* 32, 789–791. <https://doi.org/10.1093/bioinformatics/btv651>
- Hong, K.-K., Nielsen, J., 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. *Cell. Mol. Life Sci.* 69, 2671–2690. <https://doi.org/10.1007/s00018-012-0945-1>
- Hou, J., Tyo, K.E.J., Liu, Z., Petranovic, D., Nielsen, J., 2012. Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 12, 491–510. <https://doi.org/10.1111/j.1567-1364.2012.00810.x>
- Hranilovic, A., Bely, M., Masneuf-Pomarede, I., Jiranek, V., Albertin, W., 2017. The evolution of *Lachancea thermotolerans* is driven by geographical determination, anthropisation and flux between different ecosystems. *PLOS ONE* 12, e0184652. <https://doi.org/10.1371/journal.pone.0184652>
- Ibeas, J.I., Lozano, I., Perdignes, F., Jimenez, J., 1996. Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. *Appl. Environ. Microbiol.* 62, 998–1003.
- Irinyi, L., Serena, C., Garcia-Hermoso, D., Arabatzis, M., Desnos-Ollivier, M., Vu, D., Cardinali, G., Arthur, I., Normand, A.-C., Giraldo, A., da Cunha, K.C., Sandoval-Denis, M., Hendrickx, M., Nishikaku, A.S., de Azevedo Melo, A.S., Merseguel, K.B., Khan, A., Parente Rocha, J.A., Sampaio, P., da Silva Briones, M.R., e Ferreira, R.C., de Medeiros Muniz, M., Castañón-Olivares, L.R., Estrada-Barcenas, D., Cassagne, C., Mary, C., Duan, S.Y., Kong, F., Sun, A.Y., Zeng, X., Zhao, Z., Gantois, N., Botterel, F., Robbertse, B., Schoch, C., Gams, W., Ellis, D., Halliday, C., Chen, S., Sorrell, T.C., Piarroux, R., Colombo, A.L., Pais, C., de Hoog, S., Zancopé-Oliveira, R.M., Taylor, M.L., Toriello, C., de Almeida Soares, C.M., Delhaes, L., Stubbe, D., Dromer, F., Ranque, S., Guarro, J., Cano-Lira, J.F., Robert, V., Velegriki, A., Meyer, W., 2015. International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA

- barcoding database--the quality controlled standard tool for routine identification of human and animal pathogenic fungi. *Med. Mycol.* 53, 313–337. <https://doi.org/10.1093/mmy/myv008>
- Ishchuk, O.P., Zeljko, T.V., Schifferdecker, A.J., Wisén, S.M., Hagström, Å.K., Rozpędowska, E., Andersen, M.R., Hellborg, L., Ling, Z., Sibirny, A.A., Piškur, J., 2016. Novel Centromeric Loci of the Wine and Beer Yeast *Dekkera bruxellensis* CEN1 and CEN2. *PLOS ONE* 11, e0161741. <https://doi.org/10.1371/journal.pone.0161741>
- Joan, D.-E., Neus, F.-M., José, C., Esther, V., Antonio, V., 2009. Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories* 8, 17.
- Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. <https://doi.org/10.1111/1567-1364.12111>
- Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24, 1403–1405. <https://doi.org/10.1093/bioinformatics/btn129>
- Jombart, T., Devillard, S., Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* 11, 94. <https://doi.org/10.1186/1471-2156-11-94>
- Jørgen, H., L, M.B., Claudia, M., Ana, B., H, M.U., Kiran, P., 2010. Improved vanillin production in baker's yeast through *in silico* design. *Microbial Cell Factories* 9, 84.
- Joseph, C.M.L., Gorton, L.W., Ebeler, S.E., Bisson, L.F., 2013. Production of Volatile Compounds by Wine Strains of *Brettanomyces bruxellensis* Grown in the Presence of Different Precursor Substrates. *Am J Enol Vitic.* 64, 231–240. <https://doi.org/10.5344/ajev.2013.12095>
- Kamvar, Z.N., Tabima, J.F., Grünwald, N.J., 2014. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2, e281. <https://doi.org/10.7717/peerj.281>
- Kluyver, A.J., Custers, M.T.J., n.d. Onderzoekingen over het gistgeslacht *Brettanomyces*.
- Kosse, D., Seiler, H., Amann, R., Ludwig, W., Scherer, S., 1997. Identification of yoghurt-spoiling yeasts with 18S rRNA-targeted oligonucleotide probes. *Systematic and applied microbiology*.
- Krumbholz, G.; Tauschanoff, W. 1933. *Mycotorula intermedia* n. sp., ein Beitrag zur Kenntnis der Gärungserreger im Wein.. *Zentralblatt für Bakteriologie und Parasitenkunde Abteilung 2.* 88:366-373 [WWW Document], n.d. URL <http://www.mycobank.org/BioloMICS.aspx?TableKey=14682616000000061&Rec=10013&Fields=All> (accessed 10.29.17).
- Kuo, H.-P., Wang, R., Lin, Y.-S., Lai, J.-T., Lo, Y.-C., Huang, S.-T., 2017. Pilot scale repeated fed-batch fermentation processes of the wine yeast *Dekkera bruxellensis* for mass production of resveratrol from *Polygonum cuspidatum*. *Bioresource Technology* 243, 986–993. <https://doi.org/10.1016/j.biortech.2017.07.053>
- Lachance, M.A., 1995. Yeast communities in a natural tequila fermentation. *Antonie Van Leeuwenhoek* 68, 151–160.
- Lappe-Oliveras, P., Moreno-Terrazas, R., Arrizón-Gaviño, J., Herrera-Suárez, T., García-Mendoza, A., Gschaedler-Mathis, A., 2008. Yeasts associated with the production of Mexican alcoholic nondistilled and distilled Agave beverages. *FEMS Yeast Res* 8, 1037–1052. <https://doi.org/10.1111/j.1567-1364.2008.00430.x>
- Larcher, R., Puecher, C., Rohregger, S., Malacarne, M., Nicolini, G., 2012. 4-Ethylphenol and 4-ethylguaiacol depletion in wine using esterified cellulose. *Food Chemistry, 6th International Conference on Water in Food* 132, 2126–2130. <https://doi.org/10.1016/j.foodchem.2011.12.012>



- Lattey, K. a., Bramley, B. r., Francis, I. l., 2010. Consumer acceptability, sensory properties and expert quality judgements of Australian Cabernet Sauvignon and Shiraz wines. *Australian Journal of Grape and Wine Research* 16, 189–202. <https://doi.org/10.1111/j.1755-0238.2009.00069.x>
- Laureys, D., De Vuyst, L., 2014. Microbial Species Diversity, Community Dynamics, and Metabolite Kinetics of Water Kefir Fermentation. *Appl Environ Microbiol* 80, 2564–2572. <https://doi.org/10.1128/AEM.03978-13>
- Le Jeune, C., Lollier, M., Demuyter, C., Erny, C., Legras, J.-L., Aigle, M., Masneuf-Pomarède, I., 2007. Characterization of natural hybrids of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* var. *uvarum*. *FEMS Yeast Res* 7, 540–549. <https://doi.org/10.1111/j.1567-1364.2007.00207.x>
- Legras, J.-L., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol Lett* 221, 249–255. [https://doi.org/10.1016/S0378-1097\(03\)00205-2](https://doi.org/10.1016/S0378-1097(03)00205-2)
- Legras, J.-L., Merdinoglu, D., Cornuet, J.-M., Karst, F., 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Molecular Ecology* 16, 2091–2102. <https://doi.org/10.1111/j.1365-294X.2007.03266.x>
- Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P., Sampaio, J.P., 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *PNAS* 108, 14539–14544. <https://doi.org/10.1073/pnas.1105430108>
- Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., Davey, R.P., Roberts, I.N., Burt, A., Koufopanou, V., Tsai, I.J., Bergman, C.M., Bensasson, D., O’Kelly, M.J.T., van Oudenaarden, A., Barton, D.B.H., Bailes, E., Nguyen, A.N., Jones, M., Quail, M.A., Goodhead, I., Sims, S., Smith, F., Blomberg, A., Durbin, R., Louis, E.J., 2009. Population genomics of domestic and wild yeasts. *Nature* 458, 337–341. <https://doi.org/10.1038/nature07743>
- Longin, C., Degueurce, C., Julliat, F., Guilloux-Benatier, M., Rousseaux, S., Alexandre, H., 2016a. Efficiency of population-dependent sulfite against *Brettanomyces bruxellensis* in red wine. *Food Research International* 89, 620–630. <https://doi.org/10.1016/j.foodres.2016.09.019>
- Longin, C., Julliat, F., Serpaggi, V., Maupeu, J., Bourbon, G., Rousseaux, S., Guilloux-Benatier, M., Alexandre, H., 2016b. Evaluation of three *Brettanomyces* qPCR commercial kits: results from an interlaboratory study. *OENO One* 50. <https://doi.org/10.20870/oenone.2016.50.4.1274>
- Lopandic, K., Gangl, H., Wallner, E., Tschek, G., Leitner, G., Querol, A., Borth, N., Breitenbach, M., Prillinger, H., Tiefenbrunner, W., 2007. Genetically different wine yeasts isolated from Austrian vine-growing regions influence wine aroma differently and contain putative hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. *FEMS Yeast Res.* 7, 953–965. <https://doi.org/10.1111/j.1567-1364.2007.00240.x>
- Lopes, C.A., Jofré, V., Sangorrín, M.P., 2009. Spoilage yeasts in Patagonian winemaking: molecular and physiological features of *Pichia guilliermondii* indigenous isolates. *Rev. Argent. Microbiol.* 41, 177–184.
- Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*, 23rd International Specialized Symposium on Yeasts (ISSY-23) 86, 23–50. [https://doi.org/10.1016/S0168-1605\(03\)00246-0](https://doi.org/10.1016/S0168-1605(03)00246-0)
- Luo, H., Schmid, F., Grbin, P.R., Jiranek, V., 2012. Viability of common wine spoilage organisms after exposure to high power ultrasonics. *Ultrasonics Sonochemistry* 19, 415–420. <https://doi.org/10.1016/j.ultsonch.2011.06.009>

- Lustrato, G., Vigentini, I., De Leonardis, A., Alfano, G., Tirelli, A., Foschino, R., Ranalli, G., 2010. Inactivation of wine spoilage yeasts *Dekkera bruxellensis* using low electric current treatment (LEC). *Journal of Applied Microbiology* 109, 594–604. <https://doi.org/10.1111/j.1365-2672.2010.04686.x>
- Mable, B.K., 2001. Ploidy evolution in the yeast *Saccharomyces cerevisiae*: a test of the nutrient limitation hypothesis. *Journal of Evolutionary Biology* 14, 157–170. <https://doi.org/10.1046/j.1420-9101.2001.00245.x>
- Macris, B.J., Markakis, P., 1974. Transport and toxicity of sulphur dioxide in *Saccharomyces cerevisiae* var *ellipsoideus*. *J. Sci. Food Agric.* 25, 21–29. <https://doi.org/10.1002/jsfa.2740250104>
- Magwene, P.M., 2014. Revisiting Mortimer's Genome Renewal Hypothesis: Heterozygosity, Homothallism, and the Potential for Adaptation in Yeast, in: Landry, C.R., Aubin-Horth, N. (Eds.), *Ecological Genomics, Advances in Experimental Medicine and Biology*. Springer Netherlands, pp. 37–48. [https://doi.org/10.1007/978-94-007-7347-9\\_3](https://doi.org/10.1007/978-94-007-7347-9_3)
- Manzano, M., Vizzini, P., Jia, K., Adam, P.-M., Ionescu, R.E., n.d. Development of Localized Surface Plasmon Resonance biosensors for the detection of *Brettanomyces bruxellensis* in wine. *Sensors and Actuators B: Chemical*. <https://doi.org/10.1016/j.snb.2015.09.099>
- Marsit, S., Dequin, S., 2015. Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. *FEMS Yeast Res.* 15. <https://doi.org/10.1093/femsyr/fov067>
- Martorell, P., Barata, A., Malfeito-Ferreira, M., Fernández-Espinar, M.T., Loureiro, V., Querol, A., 2006. Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources. *International Journal of Food Microbiology* 106, 79–84. <https://doi.org/10.1016/j.ijfoodmicro.2005.05.014>
- Masneuf, I., Hansen, J., Groth, C., Piskur, J., Dubourdiou, D., 1998. New Hybrids between *Saccharomyces Sensu Stricto* Yeast Species Found among Wine and Cider Production Strains. *Appl. Environ. Microbiol.* 64, 3887–3892.
- Masneuf-Pomarede, I., Bely, M., Marullo, P., Albertin, W., 2016. The Genetics of Non-conventional Wine Yeasts: Current Knowledge and Future Challenges. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.01563>
- Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W., 2015. The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Res.* 15, fov045. <https://doi.org/10.1093/femsyr/fov045>
- May, R.C., Stone, N.R.H., Wiesner, D.L., Bicanic, T., Nielsen, K., 2016. *Cryptococcus*: from environmental saprophyte to global pathogen. *Nat. Rev. Microbiol.* 14, 106–117. <https://doi.org/10.1038/nrmicro.2015.6>
- McDonald, B.A., 1997. The Population Genetics of Fungi: Tools and Techniques. *Phytopathology* 87, 448–453. <https://doi.org/10.1094/PHYTO.1997.87.4.448>
- McGovern, P.E., Glusker, D.L., Exner, L.J., Voigt, M.M., 1996. Neolithic resinated wine. *Nature* 381, 480–481. <https://doi.org/10.1038/381480a0>
- McGovern, P.E., Zhang, J., Tang, J., Zhang, Z., Hall, G.R., Moreau, R.A., Nuñez, A., Butrym, E.D., Richards, M.P., Wang, C., Cheng, G., Zhao, Z., Wang, C., 2004. Fermented beverages of pre- and proto-historic China. *PNAS* 101, 17593–17598. <https://doi.org/10.1073/pnas.0407921102>
- Mehlomakulu, N.N., Setati, M.E., Divol, B., 2015. Non-*Saccharomyces* killer toxins: Possible biocontrol agents against *Brettanomyces* in wine? *South African Journal of Enology and Viticulture* 36, 94–104.

- Mehlomakulu, N.N., Setati, M.E., Divol, B., 2014. Characterization of novel killer toxins secreted by wine-related non-Saccharomyces yeasts and their action on Brettanomyces spp. *International Journal of Food Microbiology* 188, 83–91. <https://doi.org/10.1016/j.ijfoodmicro.2014.07.015>
- Melaragno, J.E., Mehrotra, B., Coleman, A.W., 1993. Relationship between Endopolyploidy and Cell Size in Epidermal Tissue of Arabidopsis. *Plant Cell* 5, 1661–1668. <https://doi.org/10.1105/tpc.5.11.1661>
- Meroth, C.B., Hammes, W.P., Hertel, C., 2003. Identification and Population Dynamics of Yeasts in Sourdough Fermentation Processes by PCR-Denaturing Gradient Gel Electrophoresis. *Appl Environ Microbiol* 69, 7453–7461. <https://doi.org/10.1128/AEM.69.12.7453-7461.2003>
- Miklenić, M., Štafa, A., Bajić, A., Žunar, B., Lisnić, B., Svetec, I.-K., 2013. Genetic transformation of the yeast *Dekkera/Brettanomyces bruxellensis* with non-homologous DNA. *J. Microbiol. Biotechnol.* 23, 674–680.
- Miklenić, M., Žunar, B., Štafa, A., Svetec, I.-K., 2015. Improved electroporation procedure for genetic transformation of *Dekkera/Brettanomyces bruxellensis*. *FEMS Yeast Res.* 15. <https://doi.org/10.1093/femsyr/fov096>
- Miot-Sertier, C., Lonvaud-Funel, A., 2007. Development of a molecular method for the typing of *Brettanomyces bruxellensis* (*Dekkera bruxellensis*) at the strain level. *Journal of Applied Microbiology* 102, 555–562. <https://doi.org/10.1111/j.1365-2672.2006.03069.x>
- Morales, L., Dujon, B., 2012. Evolutionary Role of Interspecies Hybridization and Genetic Exchanges in Yeasts. *Microbiol. Mol. Biol. Rev.* 76, 721–739. <https://doi.org/10.1128/MMBR.00022-12>
- Morrissey, W.F., Davenport, B., Querol, A., Dobson, A.D.W., 2004. The role of indigenous yeasts in traditional Irish cider fermentations. *J. Appl. Microbiol.* 97, 647–655. <https://doi.org/10.1111/j.1365-2672.2004.02354.x>
- Mortimer, R., Polsinelli, M., 1999. On the origins of wine yeast. *Research in Microbiology* 150, 199–204. [https://doi.org/10.1016/S0923-2508\(99\)80036-9](https://doi.org/10.1016/S0923-2508(99)80036-9)
- Mortimer, R.K., 2000a. Evolution and Variation of the Yeast (*Saccharomyces*) Genome. *Genome Res.* 10, 403–409. <https://doi.org/10.1101/gr.10.4.403>
- Mortimer, R.K., 2000b. Evolution and Variation of the Yeast (*Saccharomyces*) Genome. *Genome Res.* 10, 403–409. <https://doi.org/10.1101/gr.10.4.403>
- Mortimer, R.K., Romano, P., Suzzi, G., Polsinelli, M., 1994. Genome renewal: A new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. *Yeast* 10, 1543–1552. <https://doi.org/10.1002/yea.320101203>
- Mulla, W., Zhu, J., Li, R., 2014. Yeast: a simple model system to study complex phenomena of aneuploidy. *FEMS Microbiol Rev* 38, 201–212. <https://doi.org/10.1111/1574-6976.12048>
- Muñoz, R., Gómez, A., Robles, V., Rodríguez, P., Cebollero, E., Tabera, L., Carrascosa, A.V., Gonzalez, R., 2009. Multilocus sequence typing of oenological *Saccharomyces cerevisiae* strains. *Food Microbiology* 26, 841–846. <https://doi.org/10.1016/j.fm.2009.05.009>
- Nardi, T., Corich, V., Giacomini, A., Blondin, B., 2010. A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. *Microbiology* 156, 1686–1696. <https://doi.org/10.1099/mic.0.036723-0>
- Naumov, G., 1996. Genetic identification of biological species in the *Saccharomyces sensu stricto* complex. *Journal of Industrial Microbiology & Biotechnology* 17, 295–302. <https://doi.org/10.1007/BF01574704>

- Naumov, G.I., Naumova, E.S., Masneuf, I., Aigle, M., Kondratieva, V.I., Dubourdieu, D., 2000. Natural Polyploidization of Some Cultured Yeast *Saccharomyces Sensu Stricto*: Auto- and Allotetraploidy. *Systematic and Applied Microbiology* 23, 442–449. [https://doi.org/10.1016/S0723-2020\(00\)80076-4](https://doi.org/10.1016/S0723-2020(00)80076-4)
- Nguyen, H.V., Lepingle, A., Gaillardin, C.A., 2000. Molecular typing demonstrates homogeneity of *Saccharomyces uvarum* strains and reveals the existence of hybrids between *S. uvarum* and *S. cerevisiae*, including the *S. bayanus* type strain CBS 380. *Syst. Appl. Microbiol.* 23, 71–85.
- Novo, M., Bigey, F., Beyne, E., Galeote, V., Gavory, F., Mallet, S., Cambon, B., Legras, J.-L., Wincker, P., Casaregola, S., Dequin, S., 2009. Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118. *PNAS* 106, 16333–16338. <https://doi.org/10.1073/pnas.0904673106>
- Odds, F.C., 1988. *Candida and candidosis: a review and bibliography*. 2nd edition. *Candida and candidosis: a review and bibliography*. 2nd edition.
- Oelofse, A., Lonvaud-Funel, A., du Toit, M., 2009. Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production. *Food Microbiology* 26, 377–385. <https://doi.org/10.1016/j.fm.2008.10.011>
- Okagaki, L.H., Strain, A.K., Nielsen, J.N., Charlier, C., Baltes, N.J., Chrétien, F., Heitman, J., Dromer, F., Nielsen, K., 2010. Cryptococcal Cell Morphology Affects Host Cell Interactions and Pathogenicity. *PLOS Pathogens* 6, e1000953. <https://doi.org/10.1371/journal.ppat.1000953>
- Olsen, R.-A., Bunikis, I., Tiukova, I., Holmberg, K., Lötstedt, B., Pettersson, O.V., Passoth, V., Källner, M., Vezzi, F., 2015. De novo assembly of *Dekkera bruxellensis*: a multi technology approach using short and long-read sequencing and optical mapping. *Gigascience* 4, 56. <https://doi.org/10.1186/s13742-015-0094-1>
- Otto, S.P., 2007. The Evolutionary Consequences of Polyploidy. *Cell* 131, 452–462. <https://doi.org/10.1016/j.cell.2007.10.022>
- Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* 20, 289–290. <https://doi.org/10.1093/bioinformatics/btg412>
- Park, H., Bakalinsky, A.T., 2000. SSU1 mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* 16, 881–888. [https://doi.org/10.1002/1097-0061\(200007\)16:10<881::AID-YEA576>3.0.CO;2-3](https://doi.org/10.1002/1097-0061(200007)16:10<881::AID-YEA576>3.0.CO;2-3)
- Park, H. (Sunmoon U., Bakalinsky, A.T. (Oregon S.U., 2004. Evidence for Sulfite Proton Symport in *Saccharomyces cerevisiae*. *Journal of Microbiology and Biotechnology*.
- Park, S., Perlin, D.S., 2005. Establishing Surrogate Markers for Fluconazole Resistance in *Candida albicans*. *Microbial Drug Resistance* 11, 232–238. <https://doi.org/10.1089/mdr.2005.11.232>
- Pascal Ribéreau-Gayon, Denis Dubourdieu, Bernard Donèche, Aline Lonvaud, Philippe Darriet et al., 2017. *Traité d’oenologie - Tome 1*.
- Passoth, V., Blomqvist, J., Schnürer, J., 2007. *Dekkera bruxellensis* and *Lactobacillus vini* Form a Stable Ethanol-Producing Consortium in a Commercial Alcohol Production Process. *Appl. Environ. Microbiol.* 73, 4354–4356. <https://doi.org/10.1128/AEM.00437-07>
- Peer, Y.V. de, Mizrahi, E., Marchal, K., 2017. The evolutionary significance of polyploidy. *Nature Reviews Genetics* 18, nrg.2017.26. <https://doi.org/10.1038/nrg.2017.26>
- Pérez-Ortín, J.E., Querol, A., Puig, S., Barrio, E., 2002. Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. *Genome Res.* 12, 1533–1539. <https://doi.org/10.1101/gr.436602>

- Pérez-Torrado, R., Rantsiou, K., Perrone, B., Navarro-Tapia, E., Querol, A., Cocolin, L., 2017. Ecological interactions among *Saccharomyces cerevisiae* strains: insight into the dominance phenomenon. *Sci Rep* 7. <https://doi.org/10.1038/srep43603>
- Peris, D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J.P., Alexander, W.G., Hittinger, C.T., 2014. Population structure and reticulate evolution of *Saccharomyces eubayanus* and its lager-brewing hybrids. *Mol Ecol* 23, 2031–2045. <https://doi.org/10.1111/mec.12702>
- Petrova, B., Cartwright, Z.M., Edwards, C.G., 2016. Effectiveness of chitosan preparations against *Brettanomyces bruxellensis* grown in culture media and red wines. *OENO One* 50, 49–56. <https://doi.org/10.20870/oeno-one.2016.50.1.54>
- Peynaud, E., Domercq, S., 1956. Sur les brettanomyces isolés de raisins et de vins. *Archiv. Mikrobiol.* 24, 266–280. <https://doi.org/10.1007/BF00419012>
- Phister, T.G., Mills, D.A., 2003. Real-Time PCR Assay for Detection and Enumeration of *Dekkera bruxellensis* in Wine. *Appl. Environ. Microbiol.* 69, 7430–7434. <https://doi.org/10.1128/AEM.69.12.7430-7434.2003>
- Pilkington, B.J., Rose, A.H., 1988. Reactions of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to Sulphite. *Microbiology* 134, 2823–2830. <https://doi.org/10.1099/00221287-134-10-2823>
- Piškur, J., Ling, Z., Marcet-Houben, M., Ishchuk, O.P., Aerts, A., LaButti, K., Copeland, A., Lindquist, E., Barry, K., Compagno, C., Bisson, L., Grigoriev, I.V., Gabaldón, T., Phister, T., 2012. The genome of wine yeast *Dekkera bruxellensis* provides a tool to explore its food-related properties. *International Journal of Food Microbiology* 157, 202–209. <https://doi.org/10.1016/j.ijfoodmicro.2012.05.008>
- Prosperi, M.C.F., Ciccozzi, M., Fanti, I., Saladini, F., Pecorari, M., Borghi, V., Giambenedetto, S.D., Bruzzone, B., Capetti, A., Vivarelli, A., Rusconi, S., Re, M.C., Gismondo, M.R., Sighinolfi, L., Gray, R.R., Salemi, M., Zazzi, M., Luca, A.D., 2011. A novel methodology for large-scale phylogeny partition. *Nature Communications* 2, 321. <https://doi.org/10.1038/ncomms1325>
- Put, H.M., De Jong, J., Sand, F.E., Van Grinsven, A.M., 1976. Heat resistance studies on yeast spp. causing spoilage in soft drinks. *J. Appl. Bacteriol.* 40, 135–152.
- Querol, A., Barrio, E., Ramón, D., 1992. A Comparative Study of Different Methods of Yeast Strain Characterization. *Systematic and Applied Microbiology* 15, 439–446. [https://doi.org/10.1016/S0723-2020\(11\)80219-5](https://doi.org/10.1016/S0723-2020(11)80219-5)
- Querol, A., Bond, U., 2009. The complex and dynamic genomes of industrial yeasts. *FEMS Microbiology Letters* 293, 1–10. <https://doi.org/10.1111/j.1574-6968.2008.01480.x>
- Rainieri, S., Kodama, Y., Nakao, Y., Pulvirenti, A., Giudici, P., 2008. The inheritance of mtDNA in lager brewing strains. *FEMS Yeast Research* 8, 586–596. <https://doi.org/10.1111/j.1567-1364.2008.00363.x>
- Ramsey, J., Schemske, and D.W., 1998. Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants. *Annual Review of Ecology and Systematics* 29, 467–501. <https://doi.org/10.1146/annurev.ecolsys.29.1.467>
- Rancati, G., Pavelka, N., 2013. Karyotypic changes as drivers and catalyzers of cellular evolvability: A perspective from non-pathogenic yeasts. *Seminars in Cell & Developmental Biology, WASP/WAVE proteins: expanding members and functions & The role of ploidy variation on cellular adaptation* 24, 332–338. <https://doi.org/10.1016/j.semcdb.2013.01.009>
- Rankine, B.C., Pocock, K.F., 1969. Influence of yeast strain on binding of sulphur dioxide in wines, and on its formation during fermentation. *J. Sci. Food Agric.* 20, 104–109. <https://doi.org/10.1002/jsfa.2740200210>

- Renouf, V., 2009. Evidence for differences between *B. bruxellensis* strains originating from an enological environment. *International Journal of Wine Research* 95. <https://doi.org/10.2147/IJWR.S4612>
- Renouf, V., Lonvaud-Funel, A., 2007. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiological Research* 162, 154–167. <https://doi.org/10.1016/j.micres.2006.02.006>
- Rex, J.H., Rinaldi, M.G., Pfaller, M.A., 1995. Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* 39, 1–8. <https://doi.org/10.1128/AAC.39.1.1>
- Richards, K.D., Goddard, M.R., Gardner, R.C., 2009. A database of microsatellite genotypes for *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* 96, 355–359. <https://doi.org/10.1007/s10482-009-9346-3>
- Romano, A., Perello, M. c., Revel, G. de, Lonvaud-Funel, A., 2008. Growth and volatile compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *Journal of Applied Microbiology* 104, 1577–1585. <https://doi.org/10.1111/j.1365-2672.2007.03693.x>
- Romano, A., Perello, M.C., Lonvaud-Funel, A., Sicard, G., de Revel, G., 2009. Sensory and analytical re-evaluation of “Brett character.” *Food Chemistry* 114, 15–19. <https://doi.org/10.1016/j.foodchem.2008.09.006>
- Rozpędowska, E., Hellborg, L., Ishchuk, O.P., Orhan, F., Galafassi, S., Merico, A., Woolfit, M., Compagno, C., Piškur, J., 2011. Parallel evolution of the make–accumulate–consume strategy in *Saccharomyces* and *Dekkera* yeasts. *Nat Commun* 2, 302. <https://doi.org/10.1038/ncomms1305>
- Safadi, R.A., Weiss-Gayet, M., Briolay, J., Aigle, M., 2010. A polyploid population of *Saccharomyces cerevisiae* with separate sexes (dioecy). *FEMS Yeast Research* 10, 757–768. <https://doi.org/10.1111/j.1567-1364.2010.00660.x>
- Salinas, F., Mandaković, D., Urzua, U., Massera, A., Miras, S., Combina, M., Angelica Ganga, M., Martínez, C., 2010. Genomic and phenotypic comparison between similar wine yeast strains of *Saccharomyces cerevisiae* from different geographic origins. *Journal of Applied Microbiology* 108, 1850–1858. <https://doi.org/10.1111/j.1365-2672.2010.04689.x>
- Sam Crauwels, J.S., 2015. *Brettanomyces Bruxellensis*, Essential Contributor in Spontaneous Beer Fermentations Providing Novel Opportunities for the Brewing Industry. *Brewing Science* 68, 110–121.
- Sampaio, J.P., Gonçalves, P., 2008. Natural Populations of *Saccharomyces kudriavzevii* in Portugal Are Associated with Oak Bark and Are Sympatric with *S. cerevisiae* and *S. paradoxus*. *Appl. Environ. Microbiol.* 74, 2144–2152. <https://doi.org/10.1128/AEM.02396-07>
- Sanna, V., Pretti, L., 2015. Effect of wine barrel ageing or sapa addition on total polyphenol content and antioxidant activities of some Italian craft beers. *Int J Food Sci Technol* 50, 700–707. <https://doi.org/10.1111/ijfs.12666>
- Schacherer, J., Shapiro, J.A., Ruderfer, D.M., Kruglyak, L., 2009. Comprehensive polymorphism survey elucidates population structure of *Saccharomyces cerevisiae*. *Nature* 458, 342–345. <https://doi.org/10.1038/nature07670>
- Schifferdecker, A.J., Dashko, S., Ishchuk, O.P., Piškur, J., 2014. The wine and beer yeast *Dekkera bruxellensis*. *Yeast* 31, 323–332. <https://doi.org/10.1002/yea.3023>
- Schifferdecker, A.J., Siurkus, J., Andersen, M.R., Joerck-Ramberg, D., Ling, Z., Zhou, N., Blevins, J.E., Sibirny, A.A., Piškur, J., Ishchuk, O.P., 2016. Alcohol dehydrogenase gene *ADH3* activates glucose alcoholic fermentation in genetically engineered *Dekkera*

- bruxellensis yeast. *Appl Microbiol Biotechnol* 100, 3219–3231. <https://doi.org/10.1007/s00253-015-7266-x>
- Schuelke, M., 2000. An economic method for the fluorescent labeling of PCR fragments. *Nat Biotech* 18, 233–234. <https://doi.org/10.1038/72708>
- Schuller, D., Valero, E., Dequin, S., Casal, M., 2004. Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol Lett* 231, 19–26. [https://doi.org/10.1016/S0378-1097\(03\)00928-5](https://doi.org/10.1016/S0378-1097(03)00928-5)
- Schütz, M., Gafner, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Letters in Applied Microbiology* 19, 253–257. <https://doi.org/10.1111/j.1472-765X.1994.tb00957.x>
- Selmecki, A., Forche, A., Berman, J., 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* 313, 367–370. <https://doi.org/10.1126/science.1128242>
- Selmecki, A.M., Maruvka, Y.E., Richmond, P.A., Guillet, M., Shores, N., Sorenson, A.L., De, S., Kishony, R., Michor, F., Dowell, R., Pellman, D., 2015. Polyploidy can drive rapid adaptation in yeast. *Nature* 519, 349–352. <https://doi.org/10.1038/nature14187>
- Serpaggi, V., Remize, F., Grand, A.S.-L., Alexandre, H., 2010. Specific identification and quantification of the spoilage microorganism *Brettanomyces* in wine by flow cytometry: A useful tool for winemakers. *Cytometry* 77A, 497–499. <https://doi.org/10.1002/cyto.a.20861>
- Serpaggi, V., Remize, F., Recorbet, G., Gaudot-Dumas, E., Sequeira-Le Grand, A., Alexandre, H., 2012. Characterization of the “viable but nonculturable” (VBNC) state in the wine spoilage yeast *Brettanomyces*. *Food Microbiology* 30, 438–447. <https://doi.org/10.1016/j.fm.2011.12.020>
- Shapiro, R., 1977. Genetic effects of bisulfite (sulfur dioxide). *Mutation Research/Reviews in Genetic Toxicology* 39, 149–175. [https://doi.org/10.1016/0165-1110\(77\)90020-3](https://doi.org/10.1016/0165-1110(77)90020-3)
- Shearer, C.A., 1995. Fungal competition. *Can. J. Bot.* 73, 1259–1264. <https://doi.org/10.1139/b95-386>
- Sicard, D., Legras, J.-L., 2011. Bread, beer and wine: Yeast domestication in the *Saccharomyces sensu stricto* complex. *Comptes Rendus Biologies, On the trail of domestications, migrations and invasions in agriculture* Dominique Job, Georges Pelletier, Jean-Claude Pernollet 334, 229–236. <https://doi.org/10.1016/j.crv.2010.12.016>
- Sipiczki, M., 2008. Interspecies hybridization and recombination in *Saccharomyces* wine yeasts. *FEMS Yeast Res.* 8, 996–1007. <https://doi.org/10.1111/j.1567-1364.2008.00369.x>
- Sniegowski, P.D., Dombrowski, P.G., Fingerman, E., 2002. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Res* 1, 299–306. <https://doi.org/10.1111/j.1567-1364.2002.tb00048.x>
- Soltis, P.S., Marchant, D.B., Van de Peer, Y., Soltis, D.E., 2015. Polyploidy and genome evolution in plants. *Current Opinion in Genetics & Development, Genomes and evolution* 35, 119–125. <https://doi.org/10.1016/j.gde.2015.11.003>
- Souza, R.B. de, Santos, B.M. dos, Souza, R. de F.R. de, Silva, P.K.N. da, Lucena, B.T.L., Morais, M.A. de, 2012. The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation. *J Ind Microbiol Biotechnol* 39, 1645–1650. <https://doi.org/10.1007/s10295-012-1167-0>
- Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.-M., Landschoot, A.V., Vuyst, L.D., Vandamme, P., 2014. The Microbial Diversity of Traditional Spontaneously

- Fermented Lambic Beer. PLOS ONE 9, e95384. <https://doi.org/10.1371/journal.pone.0095384>
- Stebbins, 1940. The Significance of Polyploidy in Plant Evolution. *The American Naturalist* 74, 54–66. <https://doi.org/10.1086/280872>
- Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H., Verstrepen, K.J., 2015. *Brettanomyces* yeasts — From spoilage organisms to valuable contributors to industrial fermentations. *International Journal of Food Microbiology* 206, 24–38. <https://doi.org/10.1016/j.ijfoodmicro.2015.04.005>
- Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen, K.J., 2014. Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiol Rev* 38, 947–995. <https://doi.org/10.1111/1574-6976.12073>
- STRATFORD, M., MORGAN, P., ROSE, A.H., 1987. Sulphur Dioxide Resistance in *Saccharomyces cerevisiae* and *Saccharomyces ludwigii*. *Microbiology* 133, 2173–2179. <https://doi.org/10.1099/00221287-133-8-2173>
- Sturm, J., Grossmann, M., Schnell, S., 2006. Influence of grape treatment on the wine yeast populations isolated from spontaneous fermentations. *J. Appl. Microbiol.* 101, 1241–1248. <https://doi.org/10.1111/j.1365-2672.2006.03034.x>
- Taillandier, P., Joannis-Cassan, C., Jentzer, J.-B., Gautier, S., Sieczkowski, N., Granes, D., Brandam, C., 2015. Effect of a fungal chitosan preparation on *Brettanomyces bruxellensis*, a wine contaminant. *J. Appl. Microbiol.* 118, 123–131. <https://doi.org/10.1111/jam.12682>
- Teixeira, R., Dopico-García, S., Andrade, P.B., Valentão, P., López-Vilariño, J.M., González-Rodríguez, V., Cela-Pérez, C., Silva, L.R., 2015. Volatile phenols depletion in red wine using molecular imprinted polymers. *J Food Sci Technol* 52, 7735–7746. <https://doi.org/10.1007/s13197-015-1892-2>
- Tempere, S., Schaaper, M.H., Cuzange, E., de Lescar, R., de Revel, G., Sicard, G., 2016. The olfactory masking effect of ethylphenols: Characterization and elucidation of its origin. *Food Quality and Preference* 50, 135–144. <https://doi.org/10.1016/j.foodqual.2016.02.004>
- Teoh, A.L., Heard, G., Cox, J., 2004. Yeast ecology of Kombucha fermentation. *International Journal of Food Microbiology* 95, 119–126. <https://doi.org/10.1016/j.ijfoodmicro.2003.12.020>
- Tessonnière, H., Vidal, S., Barnavon, L., Alexandre, H., Remize, F., 2009. Design and performance testing of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. *Int. J. Food Microbiol.* 129, 237–243. <https://doi.org/10.1016/j.ijfoodmicro.2008.11.027>
- The origin of ethylphenols in wines - Chatonnet - 1992 - *Journal of the Science of Food and Agriculture - Wiley Online Library [WWW Document]*, n.d. URL <http://onlinelibrary.wiley.com/doi/10.1002/jsfa.2740600205/abstract;jsessionid=DDF05AA5E37FCFD91A32F927D35CEB41.f03t02> (accessed 7.13.17).
- Thomson, J.M., Gaucher, E.A., Burgan, M.F., De Kee, D.W., Li, T., Aris, J.P., Benner, S.A., 2005. Resurrecting ancestral alcohol dehydrogenases from yeast. *Nat Genet* 37, 630–635. <https://doi.org/10.1038/ng1553>
- Todd, R.T., Forche, A., Selmecki, A., 2017. Ploidy Variation in Fungi – Polyploidy, Aneuploidy, and Genome Evolution. *Microbiol Spectr* 5. <https://doi.org/10.1128/microbiolspec.FUNK-0051-2016>
- Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology* 29, 18–26. <https://doi.org/10.1016/j.fm.2011.08.014>



- Ugarte, P., Agosin, E., Bordeu, E., Villalobos, J.I., 2005. Reduction of 4-Ethylphenol and 4-Ethylguaiaicol Concentration in Red Wines Using Reverse Osmosis and Adsorption. *Am J Enol Vitic.* 56, 30–36.
- Umiker, N. I., Descenzo, R. a., Lee, J., Edwards, C. g., 2013. Removal of *Brettanomyces Bruxellensis* from Red Wine Using Membrane Filtration. *Journal of Food Processing and Preservation* 37, 799–805. <https://doi.org/10.1111/j.1745-4549.2012.00702.x>
- Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., Cocolin, L., 2008. Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. *FEMS Yeast Res* 8, 1053–1062. <https://doi.org/10.1111/j.1567-1364.2008.00364.x>
- Uscanga, M.G.A., Délia, M.-L., Strehaiano, P., 2003. *Brettanomyces bruxellensis*: effect of oxygen on growth and acetic acid production. *Appl Microbiol Biotechnol* 61, 157–162. <https://doi.org/10.1007/s00253-002-1197-z>
- Valdes, J., Tapia, P., Cepeda, V., Varela, J., Godoy, L., Cubillos, F.A., Silva, E., Martinez, C., Ganga, M.A., 2014. Draft genome sequence and transcriptome analysis of the wine spoilage yeast *Dekkera bruxellensis* LAMAP2480 provides insights into genetic diversity, metabolism and survival. *FEMS Microbiol Lett* 361, 104–106. <https://doi.org/10.1111/1574-6968.12630>
- Vieira, M.L.C., Santini, L., Diniz, A.L., Munhoz, C. de F., 2016. Microsatellite markers: what they mean and why they are so useful. *Genet Mol Biol* 39, 312–328. <https://doi.org/10.1590/1678-4685-GMB-2016-0027>
- Vigentini, I., De Lorenzis, G., Picozzi, C., Imazio, S., Merico, A., Galafassi, S., Piškur, J., Foschino, R., 2012. Intraspecific variations of *Dekkera/Brettanomyces bruxellensis* genome studied by capillary electrophoresis separation of the intron splice site profiles. *International Journal of Food Microbiology* 157, 6–15. <https://doi.org/10.1016/j.ijfoodmicro.2012.02.017>
- Vigentini, I., Joseph, C.M.L., Picozzi, C., Foschino, R., Bisson, L.F., 2013. Assessment of the *Brettanomyces bruxellensis* metabolome during sulphur dioxide exposure. *FEMS Yeast Research* 13, 597–608. <https://doi.org/10.1111/1567-1364.12060>
- Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., Foschino, R., Volonterio, G., 2008a. Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine-model conditions. *FEMS Yeast Research* 8, 1087–1096. <https://doi.org/10.1111/j.1567-1364.2008.00395.x>
- Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., Foschino, R., Volonterio, G., 2008b. Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine-model conditions. *FEMS Yeast Res* 8, 1087–1096. <https://doi.org/10.1111/j.1567-1364.2008.00395.x>
- Walt, J.P.V. der, Kerken, A.E.V., 1960. The wine yeasts of the Cape. *Antonie van Leeuwenhoek* 26, 292–296. <https://doi.org/10.1007/BF02539015>
- Wang, Q.-M., Liu, W.-Q., Liti, G., Wang, S.-A., Bai, F.-Y., 2012. Surprisingly diverged populations of *Saccharomyces cerevisiae* in natural environments remote from human activity. *Mol Ecol* 21, 5404–5417. <https://doi.org/10.1111/j.1365-294X.2012.05732.x>
- Warringer, J., Zörgö, E., Cubillos, F.A., Zia, A., Gjuvsland, A., Simpson, J.T., Forsmark, A., Durbin, R., Omholt, S.W., Louis, E.J., Liti, G., Moses, A., Blomberg, A., 2011. Trait Variation in Yeast Is Defined by Population History. *PLOS Genetics* 7, e1002111. <https://doi.org/10.1371/journal.pgen.1002111>
- Wassmann, K., Benezra, R., 2001. Mitotic checkpoints: from yeast to cancer. *Current Opinion in Genetics & Development* 11, 83–90. [https://doi.org/10.1016/S0959-437X\(00\)00161-1](https://doi.org/10.1016/S0959-437X(00)00161-1)

- Wedral, D., Shewfelt, R., Frank, J., 2010. The challenge of *Brettanomyces* in wine. *LWT - Food Science and Technology, Wine: Parameters impacting composition and quality* 43, 1474–1479. <https://doi.org/10.1016/j.lwt.2010.06.010>
- Wertheimer, N.B., Stone, N., Berman, J., 2016. Ploidy dynamics and evolvability in fungi. *Phil. Trans. R. Soc. B* 371, 20150461. <https://doi.org/10.1098/rstb.2015.0461>
- West, S.A., Griffin, A.S., Gardner, A., 2007. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *Journal of Evolutionary Biology* 20, 415–432. <https://doi.org/10.1111/j.1420-9101.2006.01258.x>
- White, T.C., Holleman, S., Dy, F., Mirels, L.F., Stevens, D.A., 2002. Resistance Mechanisms in Clinical Isolates of *Candida albicans*. *Antimicrob. Agents Chemother.* 46, 1704–1713. <https://doi.org/10.1128/AAC.46.6.1704-1713.2002>
- White, T.C., Marr, K.A., Bowden, R.A., 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* 11, 382–402.
- Wolfe, K.H., Shields, D.C., 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 387, 708–713. <https://doi.org/10.1038/42711>
- Woolfit, M., Rozpędowska, E., Piškur, J., Wolfe, K.H., 2007. Genome Survey Sequencing of the Wine Spoilage Yeast *Dekkera (Brettanomyces) bruxellensis*. *Eukaryotic Cell* 6, 721–733. <https://doi.org/10.1128/EC.00338-06>
- Wright, J.M., Parle, J.N., 1974. *Brettanomyces* in the New Zealand wine industry. *New Zealand Journal of Agricultural Research* 17, 273–278. <https://doi.org/10.1080/00288233.1974.10421007>
- Yarrow, D., Ahearn, D.G., 1971. *Brettanomyces abstinentis* sp.n. *Antonie van Leeuwenhoek* 37, 296–298. <https://doi.org/10.1007/BF02218499>
- Zhu, Y.O., Sherlock, G., Petrov, D.A., 2016. Whole Genome Analysis of 132 Clinical *Saccharomyces cerevisiae* Strains Reveals Extensive Ploidy Variation. *G3: Genes, Genomes, Genetics* 6, 2421–2434. <https://doi.org/10.1534/g3.116.029397>
- Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D.J., Marullo, P., 2014. QTL Dissection of Lag Phase in Wine Fermentation Reveals a New Translocation Responsible for *Saccharomyces cerevisiae* Adaptation to Sulfite. *PLOS ONE* 9, e86298. <https://doi.org/10.1371/journal.pone.0086298>
- Zuehlke, J.M., Edwards, C.G., 2013. Impact of Sulfur Dioxide and Temperature on Culturability and Viability of *Brettanomyces bruxellensis* in Wine. *Journal of Food Protection* 76, 2024–2030. <https://doi.org/10.4315/0362-028X.JFP-13-243R>
- Zuehlke, J.M., Petrova, B., Edwards, C.G., 2013. Advances in the Control of Wine Spoilage by *Zygosaccharomyces* and *Dekkera/Brettanomyces*. *Annual Review of Food Science and Technology* 4, 57–78. <https://doi.org/10.1146/annurev-food-030212-182533>