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Sulfur dioxide response of *Brettanomyces bruxellensis* strains isolated from Greek wine

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ABSTRACT

Brettanomyces bruxellensis is the most common spoilage wine yeast which can provoke great economic damage to the wine industry due to the production of undesirable odors. The capacity of the species to adapt in various environmental conditions offers a selective advantage that is reflected by intraspecific variability at genotypic and phenotypic level. In this study, microsatellite analysis of 22 strains isolated from Greek wine revealed the existence of distinct genetic subgroups that are correlated with their geographical origin. The response of these strains to increasing levels of sulfur dioxide confirmed the presence of both sensitive and tolerant strains, which belong to distinguished genetic clusters. The genetic categorization of *B. bruxellensis* strains could be used by the winemakers as a diagnostic tool regarding sulfur dioxide sensitivity.

1. Introduction

Wine is a fermented beverage that has been consumed for millennia. The involvement of microorganism is essential for the winemaking process, determining wine quality. Even if nowadays wine production is more and more controlled, the action of certain undesirable microorganism sometimes can't be avoided. *Brettanomyces bruxellensis* (synonym *Dekkera bruxellensis*) is the most known spoilage yeast in wine while it has also been isolated from other fermented beverages like cider, beer and kombucha (Coton et al., 2017; Crauwels et al., 2015; Smith and Divol, 2016; Steensels et al., 2015). In wine, the undesirable character is due to the production of ethyl phenols, associated with medicinal or barnyard odors (Chatonnet Pascal et al., 2006; Curtin et al., 2012; Di Toro et al., 2015; Gilliland, 2013). Nevertheless, the yeast species has also been reported to positively influence sensory complexity of certain type of beers and sometimes even wine (Belda et al., 2017; Licker et al., 2000; Steensels et al., 2015).

Molecular techniques are useful to obtain a representative image of the species diversity. The most frequent methods are PCR based techniques like restriction fragment length polymorphism (PCR–RFLP) (Mitrakul et al., 1999) and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) (Renouf et al., 2006), which are used for species identification. Furthermore, fingerprinting techniques are liable for intraspecific species characterization like random amplified polymorphism (RAPD), amplified fragment length polymorphism (AFLP), pulsed field electrophoresis (REA-PFGE), amplification of internal transcribed spacer (ITS) and microsatellite markers analysis (Albertin et al., 2014; Avramova et al., 2018a, 2018b; Miot-Sertier and Lonvaud-Funel, 2007; Mitrakul et al., 1999; Oelofse et al., 2009).

Plethora of studies have been performed to assess the diversity of the *B. bruxellensis* isolated from wine, focusing on genetic, physiological and phenotypic level (Agnolucci et al., 2009; Crauwels et al., 2015; Fugelsang and Zoecklein, 2003; Harris et al., 2008; Oelofse et al., 2009). Phenotypes of interest for the spoilage species include the production of ethyl phenols (Berbegal et al., 2018; Chatonnet Pascal et al., 2006; Dias et al., 2003; Oelofse et al., 2009; Romano et al., 2008), the preference for nutrients (Borneman et al., 2014; Conterno et al., 2010; Crauwels et al., 2015; Joseph et al., 2013), the impact of antimicrobial agents (Barata et al., 2008; Curtin et al., 2012; Louw et al., 2016) or the capacity to enter in a Viable But Non Culturable (VBNC) state when exposed to severe wine conditions (Agnolucci et al., 2009; Capozzi et al., 2016). Sulfur dioxide is widely used due to its antioxidant and antimicrobial properties though only the molecular form (mSO_2) can have antiseptic action in wine (Divol et al., 2012) and it seems that the effective inhibitory dose of mSO_2 to *B. bruxellensis* cells is strain dependent (Avramova et al., 2018a,b; Capozzi et al., 2016; Curtin et al.,

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2012; Conterno et al., 2010; Vigentini et al., 2013) Moreover it's possible that the isolates of the same genetic cluster to show similar response to sulfur dioxide treatment, suggesting adaptation mechanism via selective pressure imposed by human practices (Avramova et al., 2018a, 2018b; Curtin et al., 2012). Furthermore, the ploidy level variation within the species was shown to be correlated to phenotypic diversity (Avramova et al., 2018a; Hellborg and Piškur, 2009). Polyploidy is considered as costly from a resource energy viewpoint and the fact that some isolates maintain a polyploidy state strongly suggests the existence of a selective advantage for the species (Comai, 2005). The most recent study on this subject was realized by Avramova et al. (2018b), showing that some triploid groups of *B. bruxellensis* presented higher resistance to sulfur dioxide comparing to other groups, mostly diploids.

This work proposes the first genotypic and phenotypic analysis of *B. bruxellensis* strains isolated from Greek wine. 22 strains were originated from 3 different geographical regions of Greece and were genotyped using microsatellite markers analysis. The effect of increasing SO_2 amount on their growth was measured. A comparison of the Greek *B. bruxellensis* strains with others strains isolated from various regions and niches around the world was also performed.

2. Materials and methods

2.1. Yeast strains

The 46 wine samples purchased from the local market and originating from different areas of Greece were tested for the presence of *Brettanomyces*. Firstly 20 ml of wine sample were centrifuged (12000 rpm, 10 min), and the pellet was concentrated twenty times. Then 100 μl of the resuspended samples were spread on agar medium according to previous studies (Rodrigues et al., 2001) with some modifications. More precisely the isolation medium contained: glucose $10\,g\,L^{-1}$, peptone $5\,g\,L^{-1}$, malt extract $3\,g\,L^{-1}$, yeast nitrogen base $3\,g\,L^{-1}$, bromocresol green $100\,mg\,L^{-1}$, p-coumaric acid $0.1\,g\,L^{-1}$ adjusted to pH 5.4 and filter-sterilized. Agar $20\,g\,L^{-1}$ was added after autoclave sterilization. After cooling at about 45–50 °C the antibiotics (cycloheximide $10\,mg\,L^{-1}$ and chloramphenicol $100\,mg\,L^{-1}$) and ethanol (6%) were added. The plates were inoculated at 27 °C for 12 days. All typical colonies (white or pale green, turning the medium yellow and giving characteristic smell) were examined microscopically and those that had the characteristic morphology of *Brettanomyces* cells, were selected, transferred in YM broth (yeast extract, $3\,g\,L^{-1}$; malt extract, $3\,g\,L^{-1}$; peptone, $5\,g\,L^{-1}$; dextrose, $10\,g\,L^{-1}$) and streaked twice in YM agar ($20\,g\,L^{-1}$). A well separated colony was transferred in YM broth plus calcium carbonate ($5\,g\,L^{-1}$) and maintained in agar slants at 4 °C until tested.

The reference strain YMC 2.7 of *B. bruxellensis* was kindly provided by the Greek Agricultural Organization Demeter, while 6 more strains of *B. bruxellensis* and 1 strain of *Saccharomyces cerevisiae* came from the CRBO collection (Centre de Ressources Biologiques Oenologie, Institut des Sciences de la Vigne et du Vin, France). The 22 Greek strains isolated in this study belong to the Laboratory of Food Microbiology and Hygiene, Department of Food Science and Technology in the Faculty of Agriculture, Forestry and Natural Environments, Aristotle University of Thessaloniki (Table 1).

2.2. DNA extraction

The DNA extraction was carried out according to Granchi (Granchi et al., 1999) with some slight modifications. Cells from stock cultures were grown in YM broth and then streaked on YM agar. After incubation at 27 °C a well separated colony was transferred with a sterile loop in 1 ml of saline water in Eppendorf tube, centrifuged (10000 rpm, 5 min) and the supernatant was discarded. The cells were suspended in lysis buffer (0.25% SDS, 0.05N NaOH), heated at 95 °C for 10 min, vortexed for 5 s. 180 μl Tris-HCl (10 mM Tris-HCl, pH 8.5) were added and vortexed again for 15–20 s. After a final centrifugation (12000 rpm, 20 min, 4 °C) the supernatant was transferred to a new sterile Eppendorf tube, stored at −20 °C, until use as DNA template.

2.3. PCR of rDNA ITS region

The isolates were further identified at species level on the basis of their ITS size as previously developed by Granchi (Granchi et al., 1999). The PCR analysis was carried out with ITS1 (5′ TCCGTAGGTGAACCT GCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primers. PCR was performed in a total volume of 25 μl containing 20 ng of template DNA, 20 pmol of each primer, 0.2 mM of dNTPs (mix of four), 2 U of Taq DNA polymerase (Bioline), and 1,5 mM $MgCl_2$. Amplification was achieved in a thermal cycler (PTC-100 MJ Research, Waltham, MA, USA) as follow: an initial denaturation step at 95 °C for 5 min, then 35 cycles at 95 °C for 1 min, 52 °C for 2 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Amplicons (8 μl sample + 2 μl tracking dye) were separated in gel (1.5% agarose) with TBE (0.5X) in presence of ethidium bromide ($5\ \mu g\ ml^{-1}$). Electrophoresis was performed in the same buffer at 100V for 5 h (SCIE-PLAS Horizontal Electrophoresis Units H4-SET, England) and gel image was captured under UV light (DNR BioImaging Systems, Mini BIS Pro, Israel). The expected size of PCR product with primers ITS1 and ITS4 for *B. bruxellensis* is 459 bp and was determined using a standard size marker (100 bp ladder, BioLabs, England) and the control strain (*B.bruxellensis* YMC2.7).

2.4. SDS-PAGE of whole cell proteins

B. bruxellensis isolates of Greek origin were grouped according to their whole cells proteins extract profile on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The treatment of cells for extraction of proteins for SDS-PAGE was achieved according to previous protocol (Kushnirov, 2000). More precisely 1 ml of actively grown strains in YM broth were centrifuged (8000 rpm for 15 min, Sigma 3K centrifuge, Sigma Chemical Co. St. Louis, MO), then suspended in 0.01 M potassium-phosphate buffer (pH 6.5), and washed twice. After the washing steps, the cells were resuspended in 100 μl distilled water added with 100 μl NaOH (0.2 M) and incubated for 5 min at room temperature. Cells were centrifuged, resuspended in 50 μl SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue), boiled for 3 min and centrifuged again. SDS-PAGE performed according to the method of Laemmli (1970). About 6 μl supernatant was loaded per lane in Tris-HCl gel slabs (140 mm × 160 mm × 1.5 mm), composed of 12% and 4% (w/v) acrylamide for separating and stacking gels respectively. The running buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.6. The gels were run at 80 mA for about 4 h under constant voltage. Gels were stained with brilliant blue R-250 0.25% (w/v) in water:ethanol:acetic acid (40:50:10) and destained with a solution containing water:ethanol:acetic acid (65:25:10). The registration of the protein electrophoretic patterns, the normalization of the densitometric traces as well as the grouping of strains were based on Pearson's correlation coefficient (r). Unweighted Pair Group Method using Average Linkages (UPGMA) cluster analysis was performed by the techniques described by Pot et al. (1994) on gel images (MiniBIS Pro, DNRBio-Imaging Systems, Jerusalem, Israel) using the software package of Gel Compar (version 4.6, Applied Maths, Belgium).

2.5. Microsatellite analysis

The DNA used for the microsatellite analysis for the Greek isolates was extracted as previously described. The 12 pairs of primers corresponding to the developed microsatellite markers for *B. bruxellensis* species were used. The PCR condition for the microsatellite markers

Table 1
The yeast strains used for the phenotypic characterization in this study.

Species	Name	Geographical Region	Matrix
Brettanomyces bruxellensis	15.1	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.2	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.3	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.4	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.5	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.6	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.7	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.8	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.11	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.12	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.13	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.15	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.16	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.17	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	15.18	Kavala. Greece	dry red wine
Brettanomyces bruxellensis	33.1	Aigialeia. Greece	sweet red wine
Brettanomyces bruxellensis	33.2	Aigialeia. Greece	sweet red wine
Brettanomyces bruxellensis	33.3	Aigialeia. Greece	sweet red wine
Brettanomyces bruxellensis	33.4	Aigialeia. Greece	sweet red wine
Brettanomyces bruxellensis	45.1	Agio Oros. Greece	dry red wine
Brettanomyces bruxellensis	45.2	Agio Oros. Greece	dry red wine
Brettanomyces bruxellensis	45.3	Agio Oros. Greece	dry red wine
Brettanomyces bruxellensis	1911-MX-V1	Bordeaux. France	dry red wine
Brettanomyces bruxellensis	AWRI1499	McLaren Valley Australia	dry red wine
Brettanomyces bruxellensis	AWRI1608	Margaret River. Australia	beer
Brettanomyces bruxellensis	L0424	Bordeaux. France	dry red wine
Brettanomyces bruxellensis	KOM1449	France	kombucha
Brettanomyces bruxellensis	UWOPS 94–239.3	Mexico	tequila
Saccharomyces cerevisiae	Zymaflore X5	Bordeaux France	dry red wine

amplification and the amplicons analysis were followed according to Avramova (Avramova et al., 2018a). Microsatellite sizes were used to draw a dendrogram using Neighbour Joining (NJ) clustering and Bruvo's distance, using *poppr* and ape R packages. Bruvo's distance was chosen as it is one of the few distance allowing mixed ploidy levels (Bruvo et al., 2004; Kamvar et al., 2014; Paradis et al., 2004). The Greek microsatellite dataset was mixed with a dataset encompassing 1488 isolates previously published (Avramova et al., 2018a).

2.6. Sulfur dioxide response assay

For the sulfate resistance experiment liquid YNB-based medium was used (yeast nitrogen base 6.7 $g\,L^{-1}$, D-glucose 2.5 $g\,L^{-1}$, D-Fructose 2.5 $g\,L^{-1}$, ethanol 6% (v/v), pH adjusted to 3.5 with phosphoric acid 1M). The sterilization was realized by filtration (0.22 µm pore filter of Millipore). Increasing SO_2 levels were reached by the addition of potassium metabisulfite (PMB, $K_2S_2O_5$) (Termo Fischer Scientifc) in YNB-based medium. Initially a stock solution of YNB medium containing 5 $g\,L^{-1}$ of total SO_2 was prepared. The free SO_2 levels were calculated by titration and the corresponding molecular SO_2 was estimated as previously described (Louw et al., 2016). Finally the volume from the stock SO_2 solution was calculated and added to YNB medium for the kinetics growth assay.

The 28 B. *bruxellensis* strains and the *Saccharomyces cerevisiae* strain (Table 1) were pre-cultured on YPD medium for 96 h at 27 °C. Then the strains were dispensed in 96-well microtiter plate at a final volume of 200 µl at an initial optical density (OD600 nm) between 0.1 and 0.2 All strains were inoculated in 4 YNB-media containing increasing molecular SO_2 concentrations (0. 0.2, 0.4 and 0.6 $mg\,L^{-1}$). The microtiter plates were placed in an automated turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd., Raisio, Finland) at an incubation temperature of 27 °C. Optical density (OD) measurements were taken every 6 h using the wideband filter (420–580 nm) of the instrument until all the strains in the tested conditions reached the stationary phase. The plate was agitated for 10 s before each measurement. Each combination of strain per media was realized in triplicate.

2.7. Statistical analysis

To assess whether some significant differences exist between isolates from different groups, three parameters were calculated from the growth kinetics. For lag phase calculation (h), for each growth curve, the initial OD was considered (time = 0h). The time at which OD became superior to 10% of the initial OD was considered for lag phase calculation. Linear interpolations between OD measurements were performed to accurately determine the lag phase. For maximal growth rate (noted max. growth rate, number of cellular divisions per hour), we calculated population doubling per hour as followed:

$\log2((OD_{t+1}-OD_t)/(t_{t+1}-t_t))$ with OD_{t+1} and OD_t the OD between two points time (t + 1 and t); t_{t+1} and t_t the corresponding time in hours. Population doubling was calculated between each time points, and the maximal was considered to be the maximal growth rate. Finally, max. OD stands for maximal observed OD (420–580 nm). For these parameters, the mean coefficient of variation was low, indicating a reproducible estimation: 0.127 for lag phase, 0.0003 for max. growth rate, and 0.0018 for max. OD. For each group and each parameter, a Kruskal-Wallis test (non-parametric) was performed to identify significance groups (alpha = 5%) using the *agricolae* package (Mendiburu Delgado, 2009).

3. Results

3.1. Diversity of Greek Brettanomyces bruxellensis strains

The 23 isolates from Greek wine were named according to their region of origin. The isolates from Kavala region start with the number 15, from Aigialeia with the number 33 and from Agio Oros with the number 45. Additionally 6 model strains (1911-MX-V1, AWRI1499, AWRI1608, L0424, KOM1449 and UWOPS 94–239.3) were also used as representatives of the four main genetic clusters of the species: Kombucha 2N, Wine 2N, Beer 3N and Wine 3N, as shown in the work of Avramova et al. (2018a) (Table 1).

First of all, we wanted to confirm that the Greek isolates from the

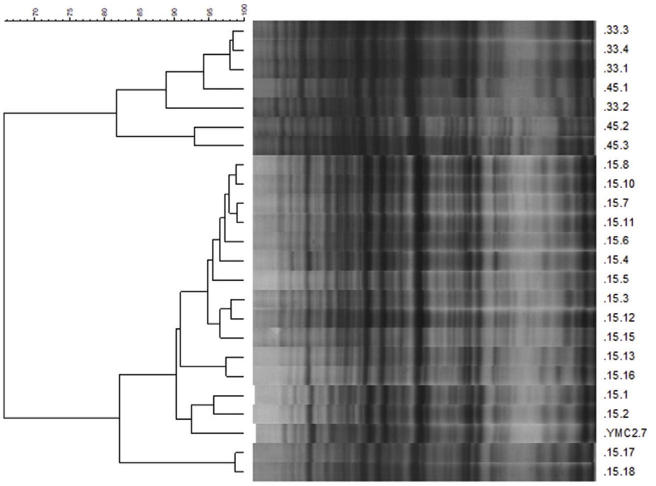

Fig. 1. Dendrogram of the whole cell protein profile of 24 *Brettanomyces bruxellensis* isolates. The YMC2.7 was used as a reference strain while the others were isolated and examined in this study. The grouping of the strains was realized using the Pearson product moment correlation coefficient (r) and the software package of Gel Compar.

agar medium belonged to *B. bruxellensis* species. The molecular identification was realized by the amplification of the rDNA-ITS region. The electrophoresis in 1.5% agarose gel of rITS-PCR amplicons, displayed one band, corresponding to 459 bp approximately, for all the 23 isolates as well as the reference strain YMC 2.7 (data not shown). This is the expected product size for *B. bruxellensis* amplicon using the ITS1-ITS4 primers (Granchi et al., 1999). In order to further investigate how the identified isolates were grouped, SDS electrophoresis of whole cells proteins was performed. The dendrogram profile of the proteins revealed the existence of two main clusters (Fig. 1). The first one contained the 4 isolates of the Group 33 and the 3 isolates of the Group 45, sharing a similarity of 81.6%. The second cluster contained all the isolates from the Group 15 and the reference strain, demonstrating a similarity of 81.7% (Fig. 1). Finally the 23 isolates were genotyped with 12 primer pairs amplifying microsatellite regions, eight of those being specific for the species (Albertin et al., 2014). As all the isolates responded positively to the tested techniques, our results confirm the *B.bruxellensis* species specificity. To go further, the intraspecific diversity of the species was assessed through microsatellite analysis profile. 22 B. *bruxellensis* strains out of the 23 isolates from Greek wines gave reliable microsatellite genotyping results. The isolates 15.16 and 15.17 gave the same profile for all 12 markers and were considered as clones. A dendrogram was drawn (Fig. 2), taking into account the Greek strains as well as 1488 isolates previously characterized (Avramova et al., 2018a). The Greek isolates clustered into two previously described genetic groups (Fig. 2A). More precisely, the isolates from Kavala (15 strains) and Agio Oros (3 strains) were part of the so-called “Kombucha 2N” group, that contained mostly diploid isolates from kombucha and wine. By contrast, the 4 strains from Aigialeia were grouped with the “Wine 3N” cluster colored in red (Fig. 2B). The Kombucha 2N and Wine 2N groups were characterized as sensitive to sulfur dioxide while Wine 3N was shown to contain resistant/tolerant isolates (Avramova et al., 2018b, 2018a). It has to be noted that the Greek strains belonging to Kombucha 2N cluster were isolated from red dry wine while the Aigialeia strains (Wine 3N) were isolated from red sweet wine.

3.2. Sulfur dioxide effect on the growth of Greek B. bruxellensis strains

Our genetic analysis revealed that the strains of *B. bruxellensis* isolated from Greek wines were grouped based on their geographical region and their matrix of isolation. We then wanted to investigate a phenotypic trait of oenological interest and we studied their response to increasing amounts of sulfur dioxide. The 22 strains isolated from dry and sweet red wines of three different geographical regions of Greece were tested for their growth kinetics in YNB-based medium with increasing mSO_2 concentrations from 0 to 0.6 mg L^{-1}. Additionally the 6 already characterized strains for their sulfur sensitivity (1911-MX-V1, AWRI1499, AWRI1608, L0424, KOM1449 and UWOPS 94–239.3) were also used as models strains representatives of the four main genetic clusters: Kombucha 2N, Wine 2N, Beer 3N and Wine 3N (Fig. 2B). The first condition, with no added antimicrobial agent will be referred as the control medium. Isolate 15.1 couldn't grow in any tested condition so for the phenotyping part we included 21 Greek strains. The growth curves of each strain in the four tested medium were measured by optical density every six hours until the stationary phase was reached (Fig. 3). The growth kinetics of the 21 strains revealed the existence of three behaviors. The Group 15, colored in light green, was the most affected by the presence of SO_2, showing decreased growth from the lowest tested concentration of mSO_2 (0.2 mg L^{-1}). The Group 33 represented by 4 strains showed similar growth whatever the concentration of the antimicrobial agent was. The addition of SO_2 in the growth medium has an intermediate effect for the three strains of the Group 45. The three strains of this subgroup could resist the presence of 0.2 mg L^{-1} mSO_2 by presenting a growth curve almost the same as in the control medium but when increased doses of SO_2 were applied, no more growth was observed.

Based on the growth curves and in order to study further the impact of mSO_2 in the culture media, three growth parameters were analyzed for each resulting subgroup: the lag phase, the maximum growth rate and the maximum absorbance (Fig. 4). The duration of the lag phase differentiated the three subgroups. The Group 15 showed great variability especially for the higher tested amounts of SO_2. The strain the less capable of sulfite adaptation was 15.6 which could not grow at all in the presence of 0.2 mg L^{-1} SO_2. By contrast, all the strains of the Group 33 had short lag phases in presence of SO_2, indicating sulfite adaptation, with an average lag phase for the 4 strains of 14.75 h in presence of the highest concentration of mSO_2 (0.6 mg L^{-1}). Finally, the Group 45 expressed significant increase of the duration of lag phase after the addition of 0.4 mg L^{-1} and 0.6 mg L^{-1} mSO_2.

The maximum growth rate was another growth parameter tested for the 21 B. *bruxellensis* strains. The growth rate of 14 strains of Group 15 was mainly decreased in the presence of more than 0.2 mg L^{-1} mSO_2. As for all the tested growth parameters, the maximum growth rate was not affected by the presence of the antimicrobial agent for Group 33. Heterogeneity was observed within the Group 45 for the growth rate in the presence of SO_2, especially for the two highest concentrations. The strain the most resistant of this group was 45.3 which showed a growth rate decreased 2 times in the presence of 0.6 mg L^{-1} mSO_2 according to the control condition (0 mg L^{-1}) while for the other two strains of the group the decrease was higher, 3 times for 45.2 and 10 times for 45.1.

The parameter that clearly differentiated the three subgroups was the maximum absorbance. The group 15, in light green represented by the 14 isolates from Kavala, was the most sensitive as all the added SO_2 amounts significantly decreased the maximum absorbance according to the control condition without the presence of sulfite (Fig. 4). The isolates from Group 45, Agio Oros, colored in dark green, could not grow when the level of mSO_2 was superior to 0.4 mg L^{-1}. By contrast, the maximum absorbance was not altered at all for the 4 strains of the Group 45 in the tested growth conditions.

3.3. Genotypic and phenotypic correlation of Brettanomyces bruxellensis strains isolated from various geographical regions

Since the 21 strains of *B. bruxellensis* isolated from Greek wines were distinguished in three main subgroups, their phenotypic profile was

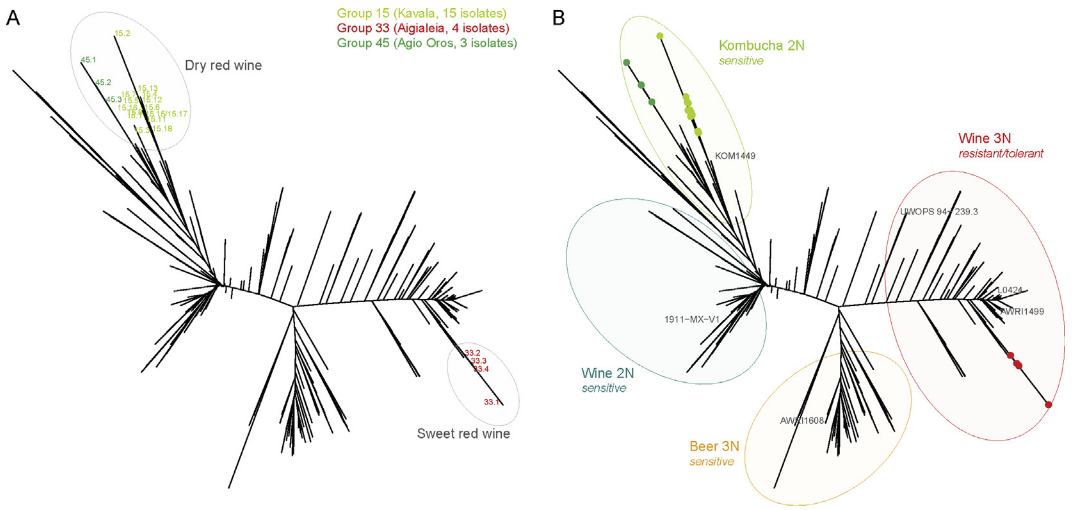

Fig. 2. Genetic position of 22 isolates from Greece within B. *bruxellensis* population. Dendrogram of 1510 isolates of *B. bruxellensis* using 12 microsatellite markers. 22 isolates from Greece were genotyped for this paper, all other isolates were genotyped in previous work (Avramova et al., 2018b and unpublished data). A. The 22 Greek isolates are represented by different colours and are grouped based on their geographical origin and wine matrix. B. Four main genetic clusters are represented by different colours. Six representative strains (AWRI1499, AWRI1608, L0424, KOM1449, UWOPS 94–239.3, 1911-MX-V1) were also included in subsequent sulfite phenotyping. The two dendrograms were produced through poppr package, using Bruvo's distance and NJ clustering. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

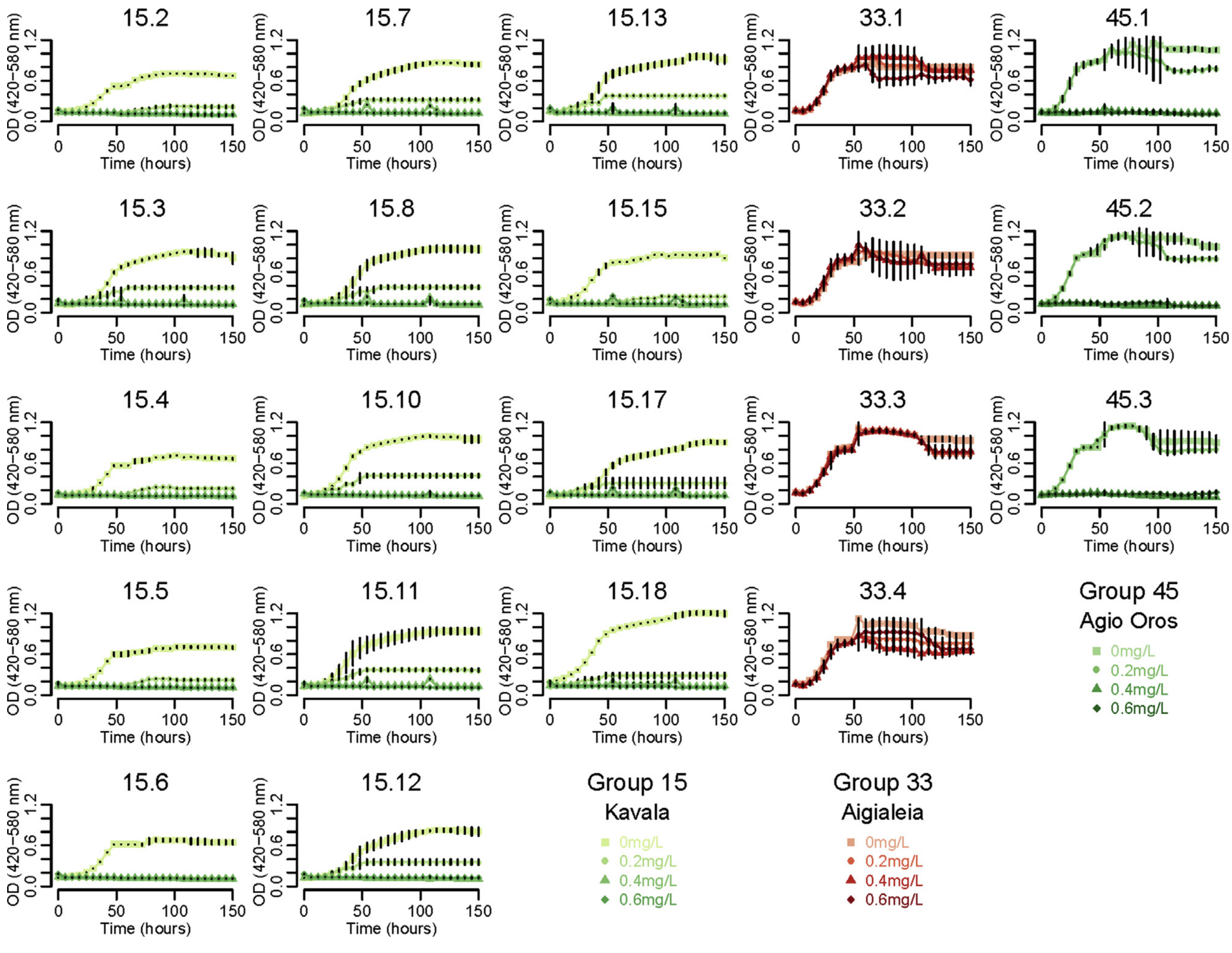

Fig. 3. Growth kinetics of *B. bruxellensis* strains for increasing sulfite concentrations. The growth kinetics curves of 21 Greek isolates (1 isolated could not grow in any tested condition) were measured using OD (420–580 nm) in media containing 0.0, 0.2, 0.4 and 0.6 mg L^{-1} sulfur dioxide every 6 h until the stationary phase was reached in the control medium. The three main genetic subgroups, Group 15, Group 33 and Group 45 are presented in different colours. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

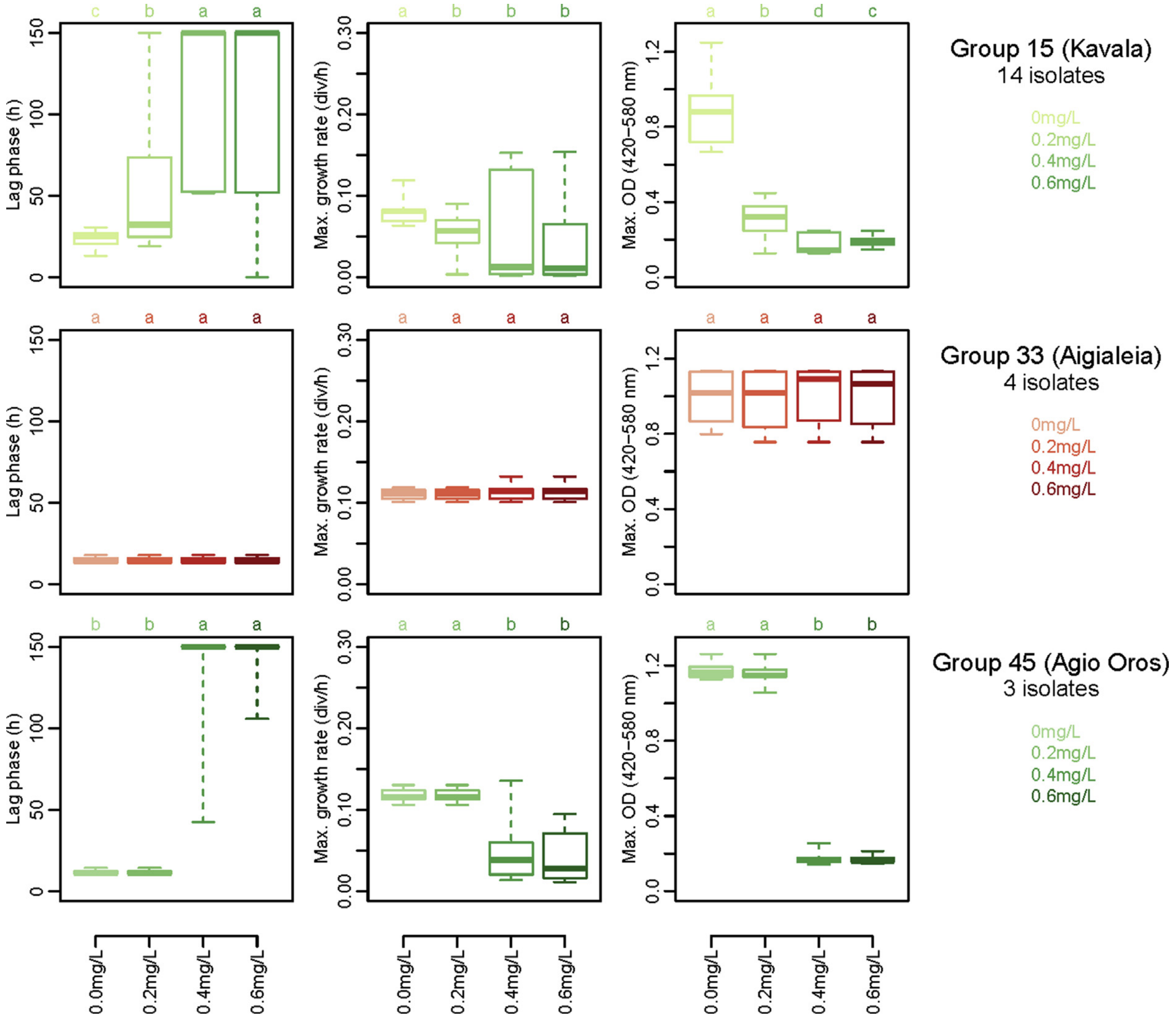

Fig. 4. Growth parameters of *B. bruxellensis* groups for increasing sulfite concentrations.”Growth was measured using OD (420–580 nm) in media containing 0.0, 0.2, 0.4 and 0.6 mg L^{-1} sulfur dioxide. Isolates from Kavala, Aigialeia and Agio Oros (14, 4 and 3 isolates respectively) were considered together. Reference strains were studied for Wine 3N (AWWRI1499, L0424, UWOPS 94–239.3), Kombucha 2N (KOM1449), Wine 2N (1911-MX-V1), Beer 3N (AWRI1608) and *S. cerevisiae* (X5). Three parameters were calculated: lag phase (h), maximal growth rate (noted max. growth rate, number of cellular divisions per hour); maximal OD (max. OD 420–580 nm). For each group and each parameter, a Kruskal-Wallis test (alpha = 5%) was performed to identify significance groups, shown as top letters.

compared to the 6 model strains. To establish whether all the tested strains from the same genetic cluster (see the dendrogram, Fig. 2B) presented similar phenotype, the growth kinetics curves in increasing mSO_2 amounts were compared (Fig. 5). The Group 15 (14 strains), the Group 45 (3 strains) and the strain KOM1449 were part of the genetic cluster Kombucha 2N. As shown in Fig. 5 these strains could not grow when the concentration of mSO_2 was 0.4 mg L^{-1} and 0.6 mg L^{-1}. Similar behavior regarding the presence of sulfite was observed for the strains 1911-MX-V1 and AWRI1608, belonging to the genetic clusters “Wine 2N” and “Beer 3N” respectively, previously shown to be sensitive to sulfite treatment. On the opposite, the strains of the Wine 3N genetic cluster could grow in any of the added sulfur dioxide concentrations, albeit with varying lag-phase. This tolerant group included the strains from the Group 33 and strain AWRI1449 that presented identical growth curves whatever the concentration of mSO_2. The two other strains, L0424 and UWOPS94–239.3, showed decrease growth in presence of 0.6 mg L^{-1} mSO_2 but their maximal populations still remained important (> 0.6 OD). A *Saccharomyces cerevisiae* wine strain was used as reference and showed a sensitive behavior, with no more growth for 0.4 mg and 0.6 mg L^{-1} mSO_2.

4. Discussion

B. bruxellensis can resist during all the winemaking steps, but is mostly known for growing and expressing the ethyl phenol character during stuck alcoholic or malolactic fermentation and during aging in oak barrels. The genetic and physiological characterization of *B. bruxellensis* strains is a way for understanding and unrevealing the resistance mechanism to antimicrobial agents and persistence capacity to various niches (Borneman et al., 2014; Conterno et al., 2010; Curtin et al., 2012; Hellborg and Piškur, 2009; Renouf et al., 2006; Wedral et al., 2010). This is the first study that focuses on the strains of *B. bruxellensis* isolated from Greek wine.

Initially the *B. bruxellensis* strains were grouped by SDS-PAGE according to their similarity of protein profile in two distinct clusters. All isolates from Kavala grouped in one cluster (similarity value 81.6%) and isolates from Aigialeia and Agio Oros were clustered together in another group (similarity value 81.7%). Since SDS-PAGE can be considered as a phenotypic traits analysis, we further performed microsatellite analysis to determine the population genetic structure (Albertin et al., 2015; Capece et al., 2005). The genotyping of 22 isolates from Greek wines with 12 microsatellites markers revealed the

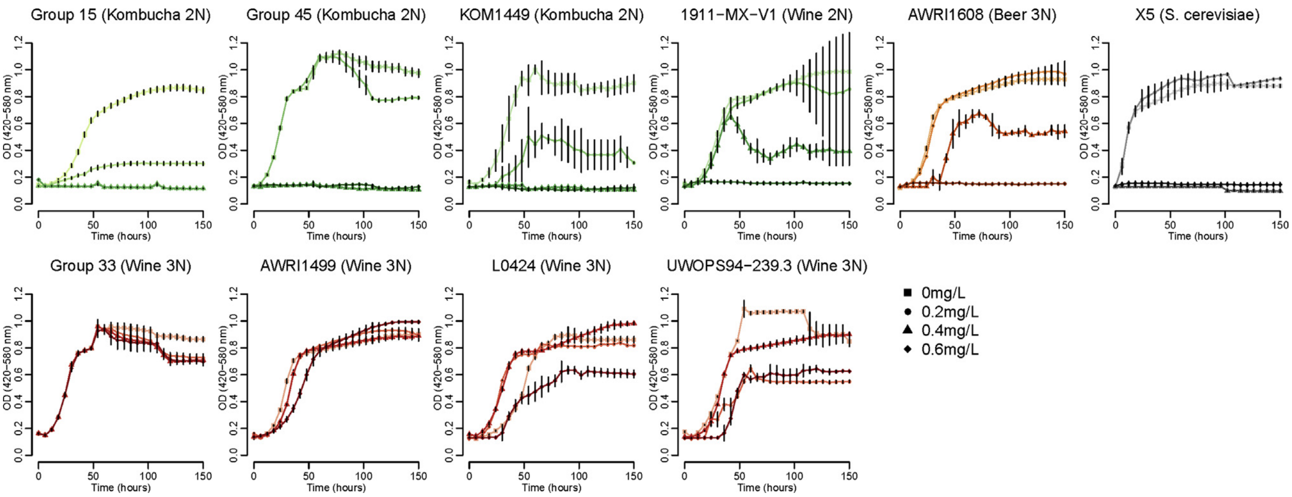

Fig. 5. Growth kinetics of yeast strains for increasing sulfite concentrations. Growth was measured using OD (420–580 nm) in media containing 0.0, 0.2, 0.4 and 0.6 mg L − 1 sulfur dioxide. The three main genetic subgroups, Group 15, Group 33 and Group 45 as well as six main genetic clusters represented by representative strains (AWRI1499, AWRI1608, L0424, KOM1449, UWOPS 94–239.3, 1911-MX-V1) were studied. Error bars represent standard errors. One strain of *Saccharomyces cerevisiae* was used as a reference of another wine species.

existence of three distinct genetic groups, Group 15 (15 strains), Group 33 (4 strains) and Group 45 (3 strains). Interestingly the geographical origin of the strains is totally related to the genetic distinction as all the strains of Group 15 were isolated from Kavala, the strains of Group 33 from Aigialeia and the four group 45 strains from Agio Oros. Regions of Group 15 and Group 45 are located in North Greece, while region of Group 33 is located in South Greece. Moreover all the strains isolated from dry wine (Group 15 and Group 45) were genetically close and distinct from the strains isolated from sweet wine (Group 33). Geographical origin and source of isolation are characteristics that have already been correlated with genetic subgroups of *B. bruxellensis* strains (Crauwels et al., 2015; Avramova et al., 2018a). This feature can be related to the evolutionary selection of the species that is driven by human-related factors such as the terroir and the winemaking conditions (Curtin et al., 2012; Di Toro et al., 2015). This local domestication of the strains could be related to the numerous adaptation mechanisms that the spoilage yeast possesses, allowing the survival during all winemaking steps (Conterno et al., 2010; Zuehlke and Edwards, 2013). The resistance to the antimicrobial agent SO_2 is one of them.

Indeed, the genetic and phenotypic analysis of 1488 strains of *B. bruxellensis* isolated from various niches and origins, confirmed the existence of sensitive and tolerant to sulfur dioxide genetic clusters (Avramova et al., 2018b, 2018a). In order to confirm this hypothesis of genotypic and phenotypic correlation, we investigated the impact of increasing concentrations of SO_2 on growth parameters for the Greek isolates. 6 B. *bruxellensis* strains representative of the main clusters described in a previous study (Avramova et al., 2018a) and one *Saccharomyces cerevisiae* strain were also used. Our study confirmed that the four Greek strains of Group 33 from Aigialeia (South of Greece) belonging to the 3N wine genetic cluster exhibited similar behavior to the model strains of the same cluster (AWRI1499, L0424, UWOPS94–239.3). These isolates are triploid and able to grow even when 0.6 mg L^{-1} mSO_2 is present in the medium. It has to be noted that the Greek strains of the Group 33 were isolated from sweet wine where the permitted addition of SO_2 is at least two times more than in dry wine, suggesting that the presence of sulfur dioxide influenced the population structure as well as may offer a selective advantage (Curtin et al., 2012; Avramova et al., 2018a). The number of isolated strains from sweet wine was limited (4 isolates), and had a unique geographical origin. Thus, it would be interesting to increase the strain collection with isolates from various vineyards and type of wines in order to address more formally the relationship between sweet wine and sulfur tolerance.

Under oenological conditions, it was shown that increasing concentration of added sulfur dioxide affects cells permeability and causes damage to the membrane. However, sulfur dioxide antiseptic effect depends on the tested strain and the corresponding population level (Longin et al., 2016). Maybe the capacity to metabolize part of the added sulfur dioxide, mainly through the SAAB (sulfur amino acids biosynthesis) pathway, could be part of the species strain-dependent resistance mechanism (Divol et al., 2012). The exact mechanism to sulfur dioxide resistance has not been clearly deciphered, but several authors suggested that the VBNC (Viable But Non Culturable) state could play an important role for the species tolerance (Longin et al., 2016; Capozzi et al., 2016). It would be interesting from a fundamental viewpoint to test whether higher sulfite concentration (close to 1 mg L^{-1} mSO_2) could stop the growth of the tolerant strains (Riberon Gayon., 2006) as well as how such a high concentration could affect membrane permeability and induction of VBNC state. However, on a practical level, sulfite increase is probably not a pertinent response when resistant isolates are present, as every vineyard country/region has strict guidelines regarding the maximum permitted sulfites levels in wine (Peynaud, 1984), and consumers tend to prefer low-sulfite amounts as well.

The Greek strains from Kavala and Agio Oros (North of Greece), were genetically grouped with Kombucha 2N cluster (Avramova et al., 2018a) and were sensitive to the presence of sulfur dioxide. Even the concentration of 0.2 mg L^{-1} of mSO_2 could stop the growth of the Greek strains from Kavala as well as of the model strain from the same cluster (KOM1449). The strains isolated from Agio Oros (Group 45) were sensitive to higher SO_2 concentrations (0.4 mg L^{-1}). The existence of less tolerant strains which express different physiological characteristics has already been shown (Conterno et al., 2010; Curtin et al., 2013; Louw et al., 2016). In our study, the strain of *Saccharomyces cerevisiae*, usually considered as a species more tolerant than *B. bruxellensis* (Smith and Divol, 2016), presented a sensitive-like behavior regarding the presence of the antimicrobial agent. Nevertheless, it should be taken into consideration that sometimes the growth results in synthetic media may differ from the results in wine, as under real conditions the cells are better adapted (Barata et al., 2008). Moreover the effect of sulfur dioxide does not always provoke the same effect on the microorganism as other parameters can influence its action, like the initial yeast population, the physiological state of the inoculum, the temperature, the medium composition (Dias et al., 2003; Romano et al., 2008; Zuehlke and Edwards, 2013; Louw et al., 2016; Longin et al., 2016; Capozzi et al., 2016).

5. Conclusion

The distinction of *B. bruxellensis* strains isolated from Greek wine based on their origin, wine matrix and sulfur resistance is really promising. The identification of the strains and the subsequent categorization could constitute an important tool for the winemakers in order to define the pertinent oenological practices to limit *B. bruxellensis* spoilage. A future study of a larger strain collection from Greek terroir could be interesting in order to confirm the results of this study and also to consider more parameters like the grape variety, the wine production year and the use of barrels.

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