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Genetic diversity and physiological traits of *Brettanomyces bruxellensis* strains isolated from Tuscan Sangiovese wines

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ABSTRACT

Eighty four isolates of *Brettanomyces bruxellensis*, were collected during fermentation of Sangiovese grapes in several Tuscan wineries and characterized by restriction analysis of 5.8S-ITS and species-specific PCR. The isolates were subsequently analysed, at strain level, by the combined use of the RAPD-PCR assay with primer OPA-02 and the mtDNA restriction analysis with the *Hinf*I endonuclease. This approach showed a high degree of polymorphism and allowed to identify seven haplotypes, one of them being the most represented and widely distributed (72 isolates, 85.7%). Physiological traits of the yeasts were investigated under a wine model condition. Haplotypes clustered into two groups according to their growth rates and kinetics of production of 4-ethylphenol and 4-ethylguaiacol. Hexylamine was the biogenic amine most produced (up to 3.92 mg l^{-1}), followed by putrescine and phenylethylamine. Formation of octapamine was detected by some haplotypes, for the first time.

1. Introduction

Wine alterations of biological origin are caused by the activity of bacteria and yeasts. Advances in wine technology and the implementation of Good Manufacturing Practices throughout the winemaking process have significantly decreased the risk of wine spoilage by bacteria, but spoilage by yeast contamination still remains a potential threat. Indeed, yeasts are so far the most feared contaminants affecting the wine quality. The common spoilage effects are film or sediment formation, cloudiness or haziness, gas production and off-odours and off-tastes at all stages of winemaking. Particularly for products aged in wood barrels, there has been an increased interest in spoilage by yeasts in the genera Brettanomyces/Dekkera.

Brettanomyces bruxellensis (teleomorph *Dekkera bruxellensis*) is the most representative in wines among the species of this genus. *B. bruxellensis* is able to produce 4-vinylphenol and 4-ethylphenol from p-coumaric acid, whereas 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid. Recent studies (Silva et al., 2005; Farina et al., 2007) revealed that *B. bruxellensis* strains varied in their production of phenolic substances in wine. Such volatile substances smell of stable, wet-horse and other stinks (Chatonnet et al., 1997; Gerbaux et al., 2000) that are generally considered strongly detractive for the product. In wines spoiled by *B. bruxellensis* high amounts of acetic acid can be found as well (Romano, 2007; Vigentini et al., 2008). However the behaviour of this yeast species in terms of off-flavour production, at the strain level, is not yet fully understood, both because only few systematic studies on *B. bruxellensis* strains of different origin have been performed and because the cultural media used have been poorly standardized or they had little oenological significance.

Some studies showed that *B. bruxellensis* is able to produce neuroactive and vasoactive amines, mainly phenylethylamine from phenylalanine at an average quantity of 10 mg l^{-1} and lower amounts of other biogenic amines (BA) (Caruso et al., 2002). Moreover the ability of *Brettanomyces* yeasts to generate biogenic amines seems to be strain dependent (Vigentini et al., 2008). These compounds can be metabolically synthesized and degraded in animals, plants and microorganisms, and are found in a wide variety of foods such as fish, meat, cheese, wine, beer and other fermented foods (Silla Santos, 1996). The occurrence of BA (i.e. histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermine and spermidine) in wine has been linked to the amino acid decarboxylase activity of lactic acid bacteria, such as *Oenococcus oeni*, which is used as a starter during malolactic fermentation (Coton et al., 1999). Genetic determinants of some decarboxylases have been identified in *S. cerevisiae* (White Tabor and Tabor, 1985) and in *O. oeni* (Marcobal et al., 2006; Moreno-Arribas et al., 2003) whereas they are unknown for *B. bruxellensis*.

In oenological practices, the isolation and enumeration of *B. bruxellensis* are carried out by using selective/differential microbiological media. However, these protocols have a limited efficacy, due

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to the low growth rate of these yeasts and their low cell density, with respect to other microbial species present in the must-wine. Furthermore the stress conditions that may occur during wine fermentations can induce the cells to enter into Viable-Not-Culturable state (VNC) (du Toit et al., 2005) and therefore they might not be detected by cultural analyses.

Several studies have reported new techniques for the rapid detection and identification of *Brettanomyces* yeasts (Hayashi et al., 2007; Röder et al., 2007), also directly from wine (Cocolin et al., 2004; Delaherche et al., 2004) and from different grape varieties (Renouf and Lonvaud-Funel, 2007; Agnolucci et al., 2007). Molecular methods such as random amplified polymorphism DNA (RAPD) PCR (Mitrakul et al., 1999; Martorell et al., 2006; Miot-Sertier and Lonvaud-Funel, 2007), mitochondrial DNA (mtDNA) restriction analysis (Martorell et al., 2006), amplified fragment length polymorphism (AFLP) analysis (Curtin et al., 2007), restriction enzyme analysis and pulse field gel electrophoresis (REA–PFGE) (Miot-Sertier and Lonvaud-Funel, 2007) have been applied to characterize *Brettanomyces* isolates from different parts of the world. The resulting biodiversity is lower with respect to other wine yeasts.

Sangiovese is a red wine grape variety originated in Italy and is the primary grape used in Tuscany to produce Chianti and other wines such as Brunello di Montalcino, Vino Nobile di Montepulciano and Morellino di Scansano, along with "Super Tuscan" wines like Tignanello. These wines are usually aged in wood barrels where the spoilage by *B. bruxellensis* yeasts can lead to relevant economic losses.

In the present study genetic diversity within *Brettanomyces* strains, isolated during winemaking with Sangiovese grapes in several Tuscan wineries of the Chianti area, was investigated by analysing the 5.8S-ITS region and by species-specific PCR. Differentiation of *B. bruxellensis* was achieved by DNA amplification with random primer (RAPD analysis) and mitochondrial DNA (mtDNA) restriction analysis. The identified strains were then physiologically characterized for their ability both to grow and survive under a wine model condition by cultural tests and to produce volatile phenols and biogenic amines.

2. Materials and methods

2.1. Yeast strains collection

Strains of *B. bruxellensis* used as reference were: CBS (Centraalbureau Voor Schimmelcultures) 4601, 4481, 4459 (South Africa wines isolates), 2499 (French wine isolate) and 72 (Belgium Lambic beer isolate).

Wild, non-*Saccharomyces* yeasts were isolated from red wines samples taken during the vinification of Sangiovese grapes in different Tuscan wineries of Chianti area (vintage 2002–2004) (Table 1). Wine samples, at an appropriate sample dilution, were spread on plates containing DBDM agar (Dekkera/Brettanomyces Differential Medium) (Rodrigues et al., 2001) and WL nutrient agar (Cavazza et al., 1992) (Oxoid) supplemented with 100 mg l^{-1} cycloheximide (Sigma-Aldrich) and 60 mg l^{-1} chloramphenicol (Sigma-Aldrich). A modified MRS agar medium (De Man et al., 1960) (Fluka), named MRS-TJCC, was also used, containing 20 g l^{-1} Tomato Juice Broth (Fluka), 5 g l^{-1} L-malic acid (Fluka), 100 mg l^{-1} cycloheximide and 60 mg l^{-1} chloramphenicol. Plates, for this medium, were incubated anaerobically in jars (Anaero *Gen*™, Oxoid) at 28 °C. Colonies exhibiting *Brettanomyces* typical microscopic morphology were randomly selected from plates and purified by repeated streaking on the same medium. A total of 84 isolates were collected and stored at −80 °C (Collection of the Department of Crop Biology, University of Pisa) in glycerol 20% (v/v).

Table 1
Brettanomyces bruxellensis isolates included in this study

Number of isolates	Isolated from	Winemaking process	Strains designation
10	Radda in Chianti (SI)	AF in concrete vats, MLF and ageing in wood barrels	BD
5	Castellina in Chianti (SI)	AF, MLF and ageing in steel vats	BF
24	Certaldo (FI)	AF in concrete vats, MLF and ageing in wood barrels	T
20	Greve in Chianti (FI)	AF in steel vats, MLF and ageing in wood barrels	N
25	Terriciola (PI)	AF in concrete vats, MLF and ageing in wood barrels	L

Alcoholic fermentation (AF); Malolactic fermentation (MLF).

2.2. 5.8S-ITS analysis and species-specific PCR

Yeast cells were grown overnight in 5 ml malt broth medium (Oxoid). Nucleic acid extraction was performed as described by White et al. (1990), with the following modifications: 1.5 ml of fermentation broth was spun down at 10.000 ×*g* for 10 min. The cell pellet was resuspended in 0.2 ml of extraction buffer (2.5 ml SDS 10%, 0.5 g of Triton X-100, 0.5 ml NaCl 5 M, 250 µl Tris 1 M, 50 µl EDTA 0.5 M up to 25 ml of water), with 0.3 g glass beads (0.4–0.5 mm in diameter; Sigma-Aldrich) and 0.2 ml phenol/chloroform/isoamyl alcohol (25:24:1—Fluka). The cells were homogenized by vortex and then centrifuged at 10.000 ×*g* for 5 min. The aqueous phase was collected and the DNA was precipitated with the same volume of isopropyl alcohol (Sigma-Aldrich) and then centrifuged at 10.000 ×*g* for 5 min. The pellet was washed with 70% ethanol and centrifuged at 10.000 ×*g* for 5 min, dried and resuspended in 50 ml of TE (TRIS 10 mM; EDTA 1.0 mM; pH 7.5). Extracted DNA was analysed by PCR amplification with ITS1–ITS4 primers (White et al., 1990; Granchi et al., 1999) and specific primers pB2-ITS 4 for *B. bruxellensis* (Egli and Henick-Kling, 2001) as described by Agnolucci et al. (2007). Reactions were carried out in a 50 µl PCR mixture containing 1x Mg free Buffer Finnzyme (50 mM KCl, 10 mM Tris–HCl pH 8.3, 0.1% Triton X-100), 4 mM $MgCl_2$, 0.2 mM each dNTPs, 0.4 µM of each primer, 2.5 U DyNazyme II DNA polymerase (Finnzyme) and about 80 ng of DNA template. A iCycler® IQ Multicolor Real Time PCR Detection System (Bio-Rad Laboratories) was used with the following amplification conditions: initial denaturation at 94 °C for 1 min followed by 30 cycles at 94 °C for 1 min, 52 °C for 2 min, 72 °C for 1 min, with final extension at 72 °C for 5 min. Amplification products were analysed by electrophoresis on 1.5% (w/v) agarose TBE (0.089 M Tris–borate, 0.089 M boric acid and 0.02 M EDTA pH 8.3) gels. All gels were visualized and captured as TIFF format file by Liscap program for Image Master VDS system (Pharmacia Biotech).

2.3. RAPD-PCR assay

The oligonucleotides OPA-02 (5′-TGC CGA GCT G-3′), OPA-03 (5′-AGT CAG CCA G-3′) and OPA-09 (5′-GGG TAA CGC C-3′) were used singly in three series of RAPD amplifications as reported by Mitrakul et al. (1999). Reactions were carried out in 25 µl PCR mixture containing 1x Buffer Finnzyme (50 mM KCl, 10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 0.125 mM of each dNTPs, 0.4 µM of the primer, 0.5 U DyNazyme II DNA polymerase (Finnzyme) and about 500 ng of template DNA. Cycler conditions were: initial step at 94 °C for 1 min followed by 39 cycles at 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final step at 72 °C for 8 min. RAPD products (15 µl) were separated by electrophoresis on 1.8% (w/v) agarose gels in 1x TBE buffer for 1 h at 120 V. Reference strains were analysed with all primers.

2.4. mtDNA restriction analysis

Yeast cells were grown overnight, in 50 ml flasks containing 5 ml YEPD medium, (1% yeast extract, 2% peptone, 2% glucose) (Oxoid) under agitation at 120 rpm and centrifuged at 8000 ×*g* for 10 min. DNA was isolated according to Querol et al. (1992), modified by using 25 mg/ml of lytic enzyme from *Rhizoctonia solani* (Sigma-Aldrich) in 1 M sorbitol and 0.1 M EDTA pH 7.5 with incubation at 45 °C for 2 h to digest the cell wall (Zilio et al., 1998). 11.5 µl of the DNA extracted from