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ORIGINAL ARTICLE

Genetic and phenotypic diversity of autochthonous *Saccharomyces* spp. strains associated to natural fermentation of 'Malvasia delle Lipari'

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Abstract

Aims: Characterize from both genetic and phenotypic standpoints the indigenous strains of *Saccharomyces* spp. associated with natural fermentation of 'Malvasia delle Lipari'.

Methods and Results: A total of 192 yeast isolates were obtained from completed fermentation of a mix of 'Malvasia delle Lipari' (92%) and 'Corinto nero' (8%) grapes in two wineries in Salina Island (Sicily, Italy). Fifty-one *Saccharomyces* spp. isolates were characterized using ITS–PCR, random amplified polymorphic DNA-PCR and mitochondrial DNA restriction fragment length polymorphism and 12 biotypes were identified. Representative strains of each biotype, tested for their physiological traits, exhibit different killer activity, fermentation vigour, production of hydrogen sulphide and show similar β-glucosidase and proteolytic activity.

Conclusions: It is possible to cluster in different groups naturally occurring indigenous biotypes of *Saccharomyces cerevisiae* from 'Malvasia delle Lipari' on the basis of molecular profiles.

Significance and Impact of the Study: Deeper insight on indigenous wine yeast of a conserved environment. The knowledge gained might offer a contribution to the selection of autochthonous wine yeast as starters for controlled fermentations.

Introduction

Malvasia is a sweet wine obtained from the fermentation of a mix of 'Malvasia delle Lipari' and 'Corinto nero' grapes (92% and 8%, respectively). The grapes are generally harvested in September and exposed to sunlight on rush mats for about 10–15 days, to concentrate sugars up to about 300 g l^{-1}.

This wine is usually produced by natural fermentation induced by autochthonous yeast originating from grapes and winery equipment (Mortimer and Polsinelli 1999) and very little is known about the microbiology of this wine-making process. Fermentation is slowly performed and naturally stopped when the alcohol concentration inhibit yeast activity. The fermentation stops when about alcohol reaches 14% (v/v), leaving partially nonfermented sugar quantities (Bambara and Nicosia 1959).

Typicality and often superior quality of many wines derive from several factors, including the presence of yeast strains particularly fit for the fermentation of certain grapes (Schütz and Gafner 1993). These wild yeast are the result of a natural selection developed by numerous factors linked to environment, local traditions, agronomic management and winery practices (Guillamón *et al.* 1994). For the above reasons, the interest in yeast selected from a natural blastomycetic microbiota and exhibiting particular physiological traits, upgrading the peculiarity of grape variety (Pramateftaki *et al.* 2000), has increased during recent years.

Molecular biology techniques have proven to be very useful to identify *Saccharomyces* species and strains, allowing more accurate analysis of the indigenous microflora present in spontaneous fermentations. In addition, these techniques allow to monitor the prevalence of commercial yeast strains in inoculum-driven fermentations (Fernández-Espinar *et al.* 2001).

In the present study, PCR amplification and restriction pattern analysis of the ITS region (Granchi *et al.* 1999) were used for species characterization of yeast associated with natural fermentation of 'Malvasia delle Lipari'. Differentiation of *Saccharomyces* strains was achieved by amplification of yeast DNA with random primer [random amplified polymorphic DNA (RAPD) analysis] and mitochondrial DNA (mtDNA) restriction enzyme analysis (Baleiras Couto *et al.* 1996). Subsequently, the different yeast strains isolated were physiologically characterized, i.e. killer activity, fermentation vigour, β-glucosidase, proteolytic activities and production of H_2S.

Materials and methods

Strains isolation

Sampling was carried out in two wineries (A and B) located in Salina Island (Sicily). The musts in these cellars had never been inoculated with active dry yeast. In winery A, yeast strains were isolated when fermentation in stainless steel tank was completed. The must density was 1·1294 and the initial sugar and SO_2 concentrations were about 290 g l^{-1} and 145 mg l^{-1}, respectively. Wine density was 1·0253 and ethanol content was 14·46 (v/v). In winery B, yeast strains were isolated when fermentation in barrel was completed. The must density was 1·1302 and the initial sugar and SO_2 concentrations were about 330 g l^{-1} and 150 mg l^{-1}, respectively. Wine density was 1·0431 and ethanol content was 13·83% (v/v). The samples, at an appropriate dilution, were inoculated in triplicate on WL nutrient Agar (Oxoid, Unipath Ltd, Milan, Italy), which allows to discriminate yeast species by colony morphology and colour (Cavazza *et al.* 1992). A total of 192 strains, exhibiting *Saccharomyces* morphology on WL agar and randomly selected from plates, were purified by repeated streaking on the same medium. Fifty-one of these were further used for the characterization study.

DNA extraction from isolated yeast strains

DNA was isolated following the method of Zilio *et al.* (1998). For amplification of the ITS region, DNA samples were spectrophotometrically quantified and tested for purity according to Maniatis *et al.* (1989).

5·8S-ITS PCR and restriction analysis

The 5·8S-ITS region was amplified by a GeneAmp System 2004 (Applied Biosystems, Milan, Haly) using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990) as reported by Agnolucci *et al.* (2007). 5·8S-ITS restriction analysis were carried out according to Fernández-Espinar *et al.* (2000).

RAPD–PCR assay

The oligonucleotides M13 (5′-GAGGGTGGCGGTTCT-3′) and RF2 (5′-CGGCCCCTGT-3′) were used singly in two series of RAPD amplifications as described by Andrighetto *et al.* (2000).

mtDNA restriction analysis

Seven microlitres of the DNA extracted from isolated yeast strains was digested with *Rsa*I endonuclease, according to the instructions of the supplier (Roche Diagnostics, Mannheim, Germany), which is reported to show the highest variability for intraspecific screening of yeast strains belonging to the same species (Comi *et al.* 2000). The profiles obtained were analysed with SYN-TAX 5.0 software package (Scientia Publishing, Budapest, Hungary; Podani 1993) and a dendogram was obtained by UPGMA analysis.

Physiological traits

The 'killer activity assay' was determined according to Stumm *et al.* (1977). Killer activity was scored positive when the strain was surrounded by a clear zone of growth inhibition bonded by stained cells. The fermentation vigour assay was performed by using MNS medium as described by Delfini (1995). Screening of β-glucosidase activity was carried out on agar plates with arbutin (Sigma Chemical Co., St Louis, MO, USA) as substrate according to Rosi *et al.* (1994). Proteolytic activity was tested according to the method proposed by Bilinsky *et al.* (1987). Hydrogen sulphide production was monitored using the bismuth-containing indicator medium Bacto BiGGy agar (Oxoid). Plates were incubated at 28°C for 2 days and the production of H_2S was revealed by the darkening of colonies.

Results

Isolation and molecular characterization of yeast strains

Thirty-seven of the 51 strains studied were obtained at the end of stainless steel alcoholic fermentation in the winery A, the remaining 14 strains were isolated at the end of barrels alcoholic fermentation in the winery B. The isolated strains from winery A were designated by numbers and yeast isolated from winery B by capital letters.

For the species identification, the ITS region of 51 isolates was successfully amplified with ITS1–ITS4 primers. A single ITS1-5·8S-ITS2 amplicon, of about 850 bp, was obtained from all isolates used in this work and therefore the isolated yeast were identified as belonging to the genus *Saccharomyces* (Esteve-Zarzoso *et al.* 1999). The ITS amplification products were then subjected to restriction [restriction fragment length polymorphism (RFLP)] analysis using the enzyme *Hae*III and *Hpa*II. Thirty-seven strains displayed a PCR pattern of 320, 250, 180 and 145 bp with *Hae*III and 720 and 120 bp with *Hpa*II, corresponding to those of *Saccharomyces cerevisiae*. Fourteen strains displayed a profile of 490, 225 and 145 bp with *Hae*III and 720 and 120 bp with *Hpa*II, corresponding to *Saccharomyces bayanus* and *Saccharomyces pastorianus* (Fernández-Espinar *et al.* 2000). All the strains identified as *S. bayanus/S. pastorianus* were isolated at the end of alcoholic fermentation in barrels (winery B).

The genetic polymorphism of some isolates was examined by RAPD–PCR amplifications with the primers RF2 and M13. Numerical analysis (UPGMA clustering) of combined RAPD–PCR patterns with the two oligonucleotides allowed to discriminate only between *S. bayanus/S. pastorianus* and *S. cerevisiae*, because within each species profiles the similarity observed was more than 90% (data not shown).

Therefore, restriction analysis of mtDNA was carried out. The use of *Rsa*I endonuclease allowed to differentiate *S. bayanus/S. pastorianus* from *S. cerevisiae* at level of 20% similarity and proved to be discriminant also at intraspecific level, revealing 12 different mtDNA restriction profiles designed with roman numerals from I to XII (Fig. 1a). The resulting dendogram from UPGMA clustering of the patterns obtained with *Rsa*I is illustrated in Fig. 1b. Within the *S. cerevisiae* cluster, 10 biotypes at different levels of similarity from 35% to 100% are present. As regards *S. bayanus/S. pastorianus* isolates, this cluster contained only two distinct biotypes at 90% similarity. The most numerous cluster was cluster II with 18 strains. Clusters III, X and XI included 9, 8 and 6 strains, respectively. All the other clusters were smaller, including 1 or 2 strains.

Physiological characterization of selected strains

A representative strain of each of the 12 identified biotypes was tested for relevant oenological traits. The results show that the different biotypes tested are provided with different levels of killer activity. Two of these (biotypes V and VI) displayed a marked trait, II, III and IV biotypes exhibited low activity, while the remaining did not show any activity. The producer isolates belonged to *S. cerevisiae* species, whereas no *S. bayanus/S. pastorianus* strains displayed this activity.

The fermentation vigour of the 12 biotypes was evaluated, by daily weighing of the flasks in relation to CO_2 loss. Biotypes II, IV, V, VI and IX possess a high fermentation vigour (i.e. more than 14% of ethanol) while the other biotypes, I, III, VII, VIII, X, XI and XII show a fermentative vigour lower than 14% of ethanol (Table 1).

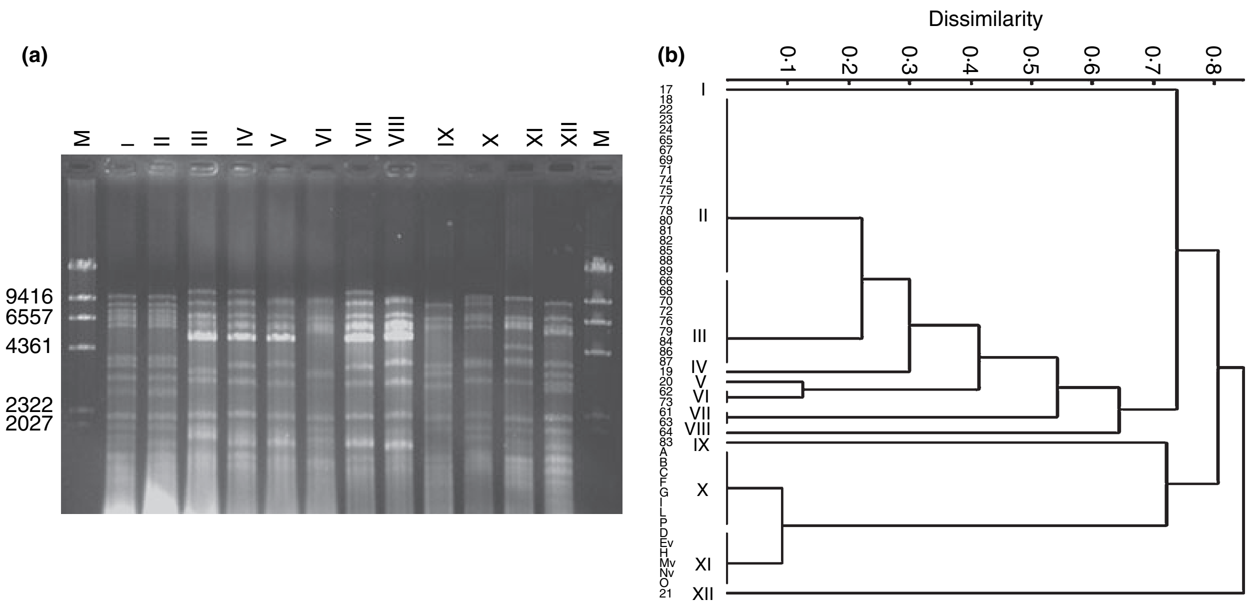

Figure 1 (a) Mitochondrial DNA restriction patterns (I–XII) of strains isolated from 'Malvasia of Lipari' wine. Lane M: λDNA/*Hin*dIII marker size. (b) Dendrogram from UPGMA clustering analysis, based on Jaccard coefficient, of mtDNA *Rsa*I restriction patterns of all 51 isolates.

Table 1 Physiological traits among the 12 biotypes isolated from 'Malvasia of Lipari' wine

Biotype	Killer activity	Fermentation vigour (%, v/v)	Production of hydrogen sulphide	β-Glucosidase	Proteolytic activity
I	−	11·6	+	−	+
II	+	14·3	+	−	+
III	+	12·4	+	−	+
IV	+	14·10	+	−	+
V	++	13·60	+	−	+
VI	++	13·60	+	−	+
VII	−	12·43	+	−	+
VIII	−	12·48	+	−	+
IX	−	15·41	−	−	+
X	−	11·1	+	−	+
XI	−	12·55	+	−	+
XII	−	12·0	+	−	+

The 12 biotypes were examined for their ability to produce β-glucosidase. None of these was positive.

Proteolytic activity was tested by the caseinolytic activity on Petri dish. All the biotypes showed proteinase production on both YED and YEPD agar media.

Hydrogen sulphide production by *Saccharomyces* strains was estimated using bismuth-containing indicator medium. Colonies producing H_2S become discoloured in a concentration-dependent manner, ranging from off-white, through brown to near-black (Jiranek *et al.* 1995). Most of the tested strains resulted hydrogen sulphide producers, whereas only one of them, IX biotype, gave colourless colonies indicating incapability to produce H_2S. The physiological traits of the 12 biotypes are summarized in Table 1.

Discussion

The *Saccharomyces* spp. biota associated with natural fermentation of 'Malvasia delle Lipari' wine was analysed in two wineries in Salina Island (Sicily) by a molecular and physiological approach. PCR amplification and restriction pattern analysis of ITS region of 51 isolates allowed us to identify *S. cerevisiae* and *S. bayanus/S. pastorianus* within the *Saccharomyces* population. Within the genus, *S. cerevisiae* is the main species found among wine yeast. *Saccharomyces pastorianus* is usually associated with the production of lager beer, whereas *S. bayanus* has been frequently isolated from several European wines (Kishimoto *et al.* 1994). Furthermore, *S. bayanus* is an usually osmotolerant yeast associated with wine making of musts containing high concentrations of sugars, frequently overgrowing *S. cerevisiae* by the end of the fermentation (Sipiczki 2003). The presence of osmotolerant yeast in 'Malvasia delle Lipari' could be expected as these musts have a high sugar content, a typical trait of this sweet dessert wine.

Preliminary experiments, using RAPD analysis as a molecular tool gave unsatisfactory resolution to differentiate the yeast isolates at the intraspecific level. This finding is not in agreement with those reported by Torriani *et al.* (1999), who found a high molecular polymorphisms by both RAPD–PCR and mtDNA restriction of the natural *Saccharomyces* strains from Amarone wine. Cocolin *et al.* (2004) reported that RAPD–PCR with primer M13 was the most powerful method to differentiate among *Saccharomyces* strains isolated from two wineries in Umbria (Italy) in comparison to SAU-PCR and PFGE techniques. The latter evidence suggests that the isolates from 'Malvasia delle Lipari' could be phylogenetically close in comparison to those isolated from Amarone wine and from the two wineries in central Italy. Considering the geographical origin of our isolates, i.e. a little island, this finding is not surprising.

The mitochondrial DNA-RFLP analysis allowed us to detect the presence of 12 different patterns among *S. cerevisiae* and *S. bayanus/S. pastorianus* strains. This would show that this is a better suited approach to detect genetic variability among isolates from 'Malvasia delle Lipari' wine. The degree of variability, measured as the percentage of different strains found *vs* the number of colonies analysed, was high in this study (23%). These results are in agreement with those previously described in other regions of Italy (Guerra *et al.* 1999; Comi *et al.* 2000) where similar biodiversity for different wine production areas was found. Moreover, Vezinhet *et al.* (1992) and Versavaud *et al.* (1995), using the same molecular markers of this work, reported that the strains isolated from the Collio grapes show a large genetic variability in spite of the small geographic region considered in their study. Every yeast strain could indeed have traits of technological importance affecting the course of fermentation and quality traits that might positively or negatively effect chemical traits and wine quality (Zambonelli 1998).

In this study, a representative isolate of each of the identified biotypes was tested for its oenological traits. First, the strains were assayed for the killer activity. Killer phenotype is present in *S. cerevisiae* and in several other yeast genera (Magliani *et al.* 1997). The natural distribution of yeast producing killer toxins and sensitivity to those toxins were demonstrated to be related to phylogeny as well as to ecological habitats of the strains (Ganter and Starmer 1992). Killer phenotype has also been reported as a tool to estimate yeast diversity (Buzzini and Martini 2000). It was recently applied in combination with the mtDNA-RFLP approach as a tool for discrimination among closely related *S. cerevisiae* strains (Lopes *et al.* 2006). In this study, the strains expressing higher killer activity (biotypes V and VI) appear to be phylogenetically close at about 87%, thus partially confirming the data of Lopes *et al.* (2006). These biotypes showed also the same fermentation vigour. The remaining killer strains exhibiting various phenotypes were phylogenetically close too. With respect to qualitative traits, all strains for each biotype were unable to produce the β-glucosidase enzyme and showed a proteolytic activity. This result is in agreement with previous findings that higher β-glucosidase producers are non-*Saccharomyces* species and that most *Saccharomyces* exhibit proteolytic activity (Rosi *et al.* 1994). In addition, except biotype IX, all other biotypes produced hydrogen sulphide. This biotype includes only the strain number 83, belonging to *S. cerevisiae*, although it is phylogenetically distinct from all the other *S. cerevisiae* biotypes (Fig. 1b).

Our study indicates that it is possible to cluster in different groups naturally occurring indigenous biotypes of *S. cerevisiae* from 'Malvasia delle Lipari' on the basis of molecular profiles. Preservation of typicality of this wine could take advantage of this knowledge, ultimately leading to elucidate the oenological peculiarities of the indigenous blastomyceteous biota.

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References

Agnolucci, M., Scarano, S., Rea, F., Toffanin, A. and Nuti, M. (2007) Detection of *Dekkera/Brettanomyces bruxellensis* in pressed Sangiovese grapes by real time PCR. *Ital J Food Sci* **19**, 155–166.

Andrighetto, C., Psomas, E., Tzanetakis, N., Suzzi, G. and Lombardi, A. (2000) Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. *Lett Appl Microbiol* **30**, 5–9.

Baleiras Couto, M.M., Eijsma, B., Hofstra, H., Huis in't Velt, J.H. and Van der Vossen, J.M.B.M. (1996) Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol* **62**, 41–46.

Bambara, G. and Nicosia, G. (1959) Il Malvasia di Lipari. *Vini d'Italia*, 119–125.

Bilinsky, C.A., Russell, I. and Stewart, G.G. (1987) Applicability of yeast extracellular proteinases in brewing: physiological and biochemical aspects. *Appl Environ Microbiol* **53**, 495–499.

Buzzini, P. and Martini, A. (2000) Utilization of differential killer toxin sensitivity patterns for fingerprinting and clustering yeast strains belonging to different genera. *Syst Appl Microbiol* **23**, 450–457.

Cavazza, A., Grado, M.S. and Zini, C. (1992) Rilevazione della flora microbica di mosti e vini. *Vignevini* **9**, 17–20.

Cocolin, L., Pepe, V., Comitini, F., Comi, G. and Ciani, M. (2004) Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. *FEMS Yeast Res* **5**, 237–245.

Comi, G., Maifreni, M., Manzano, M., Lagazio, C. and Cocolin, L. (2000) Mitochondrial DNA restriction enzyme analysis and evaluation of the enological characteristics of *Saccharomyces cerevisiae* strains isolated from grapes of the wine producing area of Collio (Italy). *Int J Food Microbiol* **58**, 117–121.

Delfini, C. (1995) *Scienza e tecnica di microbiologia enologica*. Asti, Italy: Edizioni Il lievito.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* **49**, 329–337.

Fernández-Espinar, M.T., Esteve-Zarzoso, B., Querol, A. and Barrio, E. (2000) RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts. *Antonie van Leeuwenhoek* **78**, 87–97.

Fernández-Espinar, M.T., Lopez, V., Ramon, D., Bartra, E. and Querol, A. (2001) Study of the authenticity of commercial wine yeast strains by molecular techniques. *Int J Food Microbiol* **70**, 1–10.

Ganter, P.F. and Starmer, W.T. (1992) Killer factor as a mechanism of interference competition in yeasts associated with cacti. *Ecology* **73**, 54–67.

Granchi, L., Bosco, M., Messini, A. and Vincenzini, M. (1999) Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J Appl Microbiol* **87**, 949–956.

Guerra, E., Mannazu, I., Sordi, M., Tangherlini, M., Clementi, F. and Fatichenti, F. (1999) Characterization of indigenous *Saccharomyces cerevisiae* from the Italian region of Marche: hunting for new strains for local wine quality improvement. *Ann Microbiol Enzimol* **49**, 79–88.

Guillamón, J.M., Barrio, E., Huerta, T. and Querol, A. (1994) Rapid characterization of four species of the *Saccharomyces sensu stricto* complex according to mitochondrial DNA patterns. *Int J Syst Bacteriol* **44**, 708–714.

Jiranek, V., Langridge, P. and Henschke, P.A. (1995) Validation of bismuth-containing indicator media for predicting H_2S-producing potential of *Saccharomyces cerevisiae* wine yeasts under enological conditions. *Am J Enol Vitic* **46**, 269–273.

Kishimoto, M., Soma, E. and Goto, S. (1994) Classification of cryophilic wine yeasts based on electrophoretic karyotype, G+C content and DNA similarity. *J Gen Appl Microbiol* **40**, 83–93.

Lopes, C.A., Rodríguez, M.E., Querol, A., Bramardi, S. and Caballero, A.C. (2006) Relationship between molecular and enological features of Patagonian wine yeasts: relevance in selection protocols. *World J Microbiol Biotechnol* **22**, 827–833.

Magliani, W., Conti, S., Gerloni, M., Bertolotti, D. and Polonelli, L. (1997) Yeast killer systems. *Clin Microbiol Rev* **10**, 369–400.

Maniatis, T., Fritsch, E.F. and Sambrook, J., eds (1989) *Molecular Cloning – A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Mortimer, R. and Polsinelli, M. (1999) On the origins of wine yeast. *Res Microbiol* **150**, 199–204.

Podani, J. (1993) *Syn-Tax Computer Programs for Multivariate Data Analysis in Ecology and Systematics*, Version 5.0. Budapest: Scientia Publishing.

Pramateftaki, P.V., Antoniou, P.P. and Typas, M.A. (2000) The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: intraspecific heterogeneity within the intergenic spacer region. *Fungal Genet Biol* **29**, 19–27.

Rosi, I., Vinella, M. and Domizio, M. (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *J Appl Bacteriol* **77**, 519–527.

Schütz, M. and Gafner, J. (1993) Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J Appl Bacteriol* **75**, 551–558.

Sipiczki, M. (2003) *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *Int J Syst Evol Microbiol* **53**, 2079–2083.

Stumm, C., Hermans, J.M.H., Middelbeek, E.J., Croes, A.F. and De Vries, G.J.M.L. (1977) Killer-sensitive relationships in yeasts from natural habitats. *Antonie van Leeuwenhoek* **43**, 125–128.

Torriani, S., Zapparoli, G. and Suzzi, G. (1999) Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. *Antonie van Leeuwenhoek* **75**, 207–215.

Versavaud, A., Courcoux, P., Roulland, C., Dulau, L. and Hallet, J.N. (1995) Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. *Appl Environ Microbiol* **61**, 3521–3529.

Vezinhet, F., Hallet, J.N., Valade, M. and Poulard, A. (1992) Ecological survey of wine yeast strains by molecular methods of identification. *Am J Enol Vitic* **43**, 83–86.

White, T.J., Burns, T.D., Lee, S.B. and Taylor, J.W. (1990) Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal genes. In *PCR Protocols* ed. Innis, M.A., Geleand, D.H., Sninsky, J.J. and White, T.J. pp. 315–322. New York: Academic Press.

Zambonelli, C. (ed.). (1998) *Microbiologia e Biotecnologia dei Vini*. pp. 139–173. Bologna, Italy: Edagricole.

Zilio, F., Lombardi, A., Galeotto, A. and Comi, G. (1998) Profili di restrizione del DNA mitocondriale di ceppi di *Saccharomyces* isolati nella zona di produzione del vino Soave DOC. *Rivista di Viticoltura ed Enologia* **3**, 33–41.