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Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine

M. Caruso, A. Capece, G. Salzano and P. Romano

Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Università degli Studi della Basilicata, Campus Macchia Romana, 85100 Potenza, Italy

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Aims: *Kloeckera apiculata* and *Saccharomyces cerevisiae* yeast species are dominant, respectively, at the early and at the following stages of wine fermentation. In the present study, PCR fingerprinting and NTS region amplification and restriction were applied as techniques for monitoring yeast population performing Aglianico of Vulture grape must fermentation.

Methods and Results: Thirty *S. cerevisiae* and 30 *K. apiculata* strains were typed by PCR fingerprinting with $(GAC)_5$ and $(GTG)_5$ primers and by complete NTS region amplification followed by restriction with *Hae*III and *Msp*I enzymes. *S. cerevisiae* strains generated two patterns with $(GAC)_5$ primer, while $(GTG)_5$ primer yielded a higher genetic polymorphism. Conversely, in *K. apiculata* Aglianico wine strains $(GAC)_5$ and $(GTG)_5$ primers generated the same profile for all strains. Restriction analysis of the amplified NTS region gave the same profile for all strains within the same species, except for one strain of *S. cerevisiae*.

Conclusions: The PCR fingerprinting technique was useful in discriminating at strain level *S. cerevisiae*, particularly with the primer $(GTG)_5$. RFLP patterns generated from the NTS region of the two species can be more easily compared than the patterns resulting from PCR fingerprinting, thus RFLP is more suitable for the rapid monitoring of the species involved in different stages of fermentation.

Significance and Impact of the Study: The molecular techniques used allow discrimination of *S. cerevisiae* at strain level and monitoring of the ratio of *S. cerevisiae*/*K. apiculata* during the fermentation process. Thus, their application can assure technological adjustments in a suitable time.

INTRODUCTION

Over the last decade molecular techniques for the identification and typing of micro-organisms have been widely studied. Thus, the applicability of molecular techniques, such as the RAPD method (Baleiras Couto *et al*. 1994) and restriction enzyme analysis of PCR-amplified small subunits (Baleiras Couto *et al*. 1995) have been demonstrated as a tool for taxonomic purposes providing information on the species level.

Among fermented foods, the wine produced by spontaneous fermentation is the result of the activity of yeasts that originate from the grapes. The variety and composition of this yeast flora in the must can vary according to the geographical location (Constantí *et al*. 1997), climatic conditions (Parrish and Carroll 1985) and grape variety (Schütz and Gafner 1994). During the early stages of grape must fermentation, yeast species with low fermentative activity, mainly apiculate yeasts belonging to the genus *Kloeckera*, achieve a population of 10^7 cfu ml^{-1} in 4 d both in spontaneous and inoculated fermentation (Heard and Fleet 1985) and are dominant. As the fermentation progresses, these yeasts become suppressed with increasing alcohol content, leaving *Saccharomyces cerevisiae* strains to predominate and complete the process.

Since the achievement of the microbiological stability and quality of the wine requires the monitoring of the yeast population (Querol *et al*. 1992), there is a growing demand

Correspondence to: M. Caruso. Dip. Biologia, Difesa and Biotecnologie Agro-Forestali, Universita` degli Studi della Basilicata, Campus Macchia Romana, 85100-Potenza, Italy (e-mail: romano@unibas.it).

for typing techniques which enable differentiation at the strain level. Among the other techniques, electrophoretic karyotyping (Blondin and Vezinhet 1988; Petering *et al.* 1988) and mitochondrial DNA polymorphism (Vezinhet *et al.* 1990) are useful in differentiating wine yeast strains. However, these approaches do not find an easy application in the wine industry because they are complex and laborious techniques when compared to PCR-typing techniques (Henriques *et al.* 1991; Baleiras Couto *et al.* 1996).

The present work investigates molecular techniques to determine genetic polymorphism in autochthonous strains of *Kloeckera apiculata* and *Saccharomyces cerevisiae* from Aglianico wine, in the Basilicata region, Southern Italy. PCR fingerprinting by oligonucleotides $(GAC)_5$ and $(GTG)_5$ was tested for intraspecies-specific patterns. In addition, restriction enzyme analysis of PCR-amplified fragments from the complete NTS region will be used in intraspecies differentiation.

MATERIALS AND METHODS

Micro-organisms

Thirty strains of *Saccharomyces cerevisiae* and 30 strains of *Kloeckera apiculata*, belonging to the culture collection of Basilicata University were analysed. The strains were isolated during grape must fermentations, performed spontaneously in the laboratory. The grape samples were collected aseptically from 10 different vineyards of Aglianico of Vulture cultivar in the Basilicata region, Southern Italy. The isolates were identified at the species level according to Kurtzman and Fell (1998).

DNA extraction

Pure cultures of each strain were grown on 10 ml of YPD broth (1% yeast extract, 2% bacteriological peptone, 2% glucose) at 25°C for 24–48 h. DNA isolation from the strains was carried out according to Valente *et al.* (1996).

PCR fingerprinting

The microsatellite oligonucleotide primers $(GAC)_5$ and $(GTG)_5$ (Gibco Brl) described by Baleiras Couto *et al.* 1996) were used. PCR-amplified DNA was separated on a 1·2% (w/v) agarose gel by electrophoresis, using TBE (Tris-borate 0·045 mol l^{-1}, EDTA 0·001 mol l^{-1}, pH 8) buffer and adding ethidium bromide (Sigma) at a final concentration of 0·5 μg ml^{-1}. The gel was visualized under u.v. light and photographed by Polaroid 667 camera system. A 100-bp DNA ladder marker (Gibco Brl) was used as the size standard.

Amplification and restriction analysis of NTS region

The complete NTS region was amplified with the primers NTS F and nts R (Gibco Brl). The sequence of the primers were: 5′-TGA ACG CCT CTA AGY CAG AAT C for NTS F and 5′-TTA TAC TTA GAC ATG CAT GGC for NTS R. Final concentrations in the PCR mixture were described by Baleiras Couto *et al.* 1995. The PCR program utilized was as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 57°C and 3 min at 72°C; then the mixture was heated at 72°C for 2 min and subsequently cooled at 4°C.

The PCR products were analysed on 0·8% agarose gel, containing 0·5 μg ml^{-1} ethidium bromide, and then digested with restriction enzymes. The digestion took place in a 20-μl volume with 10 μl of processed PCR amplification mixture. The restriction enzymes *Msp*I and *Hae*III (Boehringer Mannheim, Mannheim, Germany) were applied under the conditions recommended by the manufacturer. Digestion products were analysed on a 2% agarose gel using 0·5 × Tris-borate-EDTA buffer and visualized by staining with ethidium bromide. Gene Ruler$^{TM.}$ DNA ladder mix (MBI Fermentas, Lithuania) and a 100-bp DNA ladder marker (Gibco BRL) were used as molecular marker.

Cluster analysis

Molecular patterns were analysed with the software Gel Compar, version 4·0 (applied maths), using the UPGMA algorithm.

RESULTS

Thirty *S. cerevisiae* and 30 *K. apiculata* strains isolated at different stages of fermentation of Aglianico grape musts were typed by PCR fingerprinting with $(GAC)_5$ and $(GTG)_5$ primers and by NTS amplification, followed by restriction. In our study, *S. cerevisae* strains generated two different patterns with the microsatellite primer $(GAC)_5$, as shown in Fig. 1(a). One pattern was generated with 20 strains and the other with 10 strains. The second pattern was characterized by bands of lower molecular size. PCR fingerprinting of *S. cerevisiae* strains with $(GTG)_5$ yielded a higher genetic polymorphism, although the patterns obtained are quite similar (Fig. 1b). The cluster analysis of the data set, based on the presence or absence of major bands, allowed the recognition of four main groups, branching at a similarity value of 60%. Two strains appeared significantly separated from the other clusters of *S. cerevisiae* Aglianico strains, they are 5EIII10 and 8LA6 (Fig. 2).

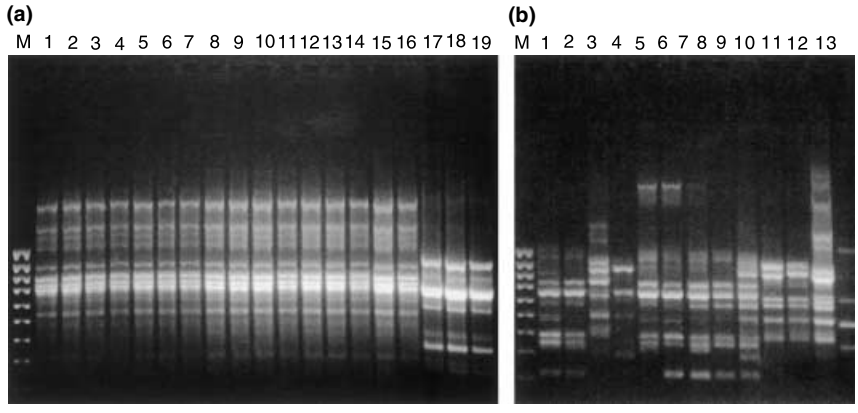

Fig. 1 PCR fingerprinting of *Saccharomyces cerevisiae* strains primed with the microsatellites $(GAC)_5$ (a) and $(GTG)_5$ (b) M = 100 bp DNA ladder. Lanes: 1 = 2LI6; 2 = 3LI1; 3 = 7LI3; 4 = 9LI2; 5 = 14LBI3; 6 = 6LBI1; 7 = 16EIII5; 8 = 18EIII3; 9 = 1LBI3; 10 = 7EIII10; 11 = 8EIII11; 12 = B51; 13 = 5EIII10

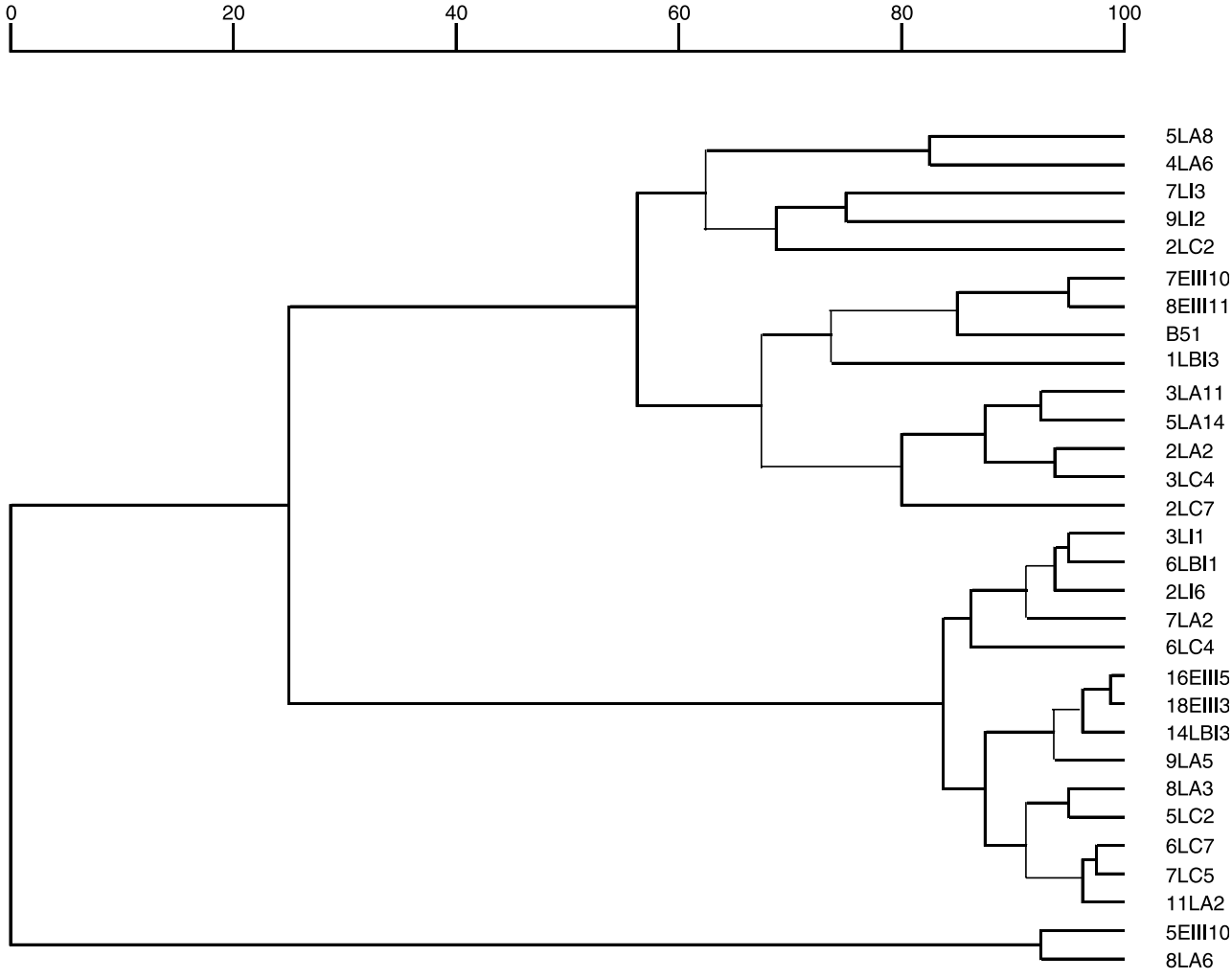

Fig. 2 Cluster analysis showing the similarity values between 30 *Saccharomyces cerevisiae* strains on the basis of genetic profiles determined by PCR fingerprinting with $(GTG)_5$

Conversely, in *K. apiculata* Aglianico strains, the microsatellites $(GAC)_5$ and $(GTG)_5$ generated the same profile for all the strains (Fig. 3), consequently these primers can not be used to obtain intraspecific discrimination in this species.

The PCR products amplification of the non-transcribed spacer region (NTS) of all the strains within the same species yielded identical molecular size (results not shown). In order to augment polymorphism and compare profiles of the two species, the amplifications were digested with the enzymes *Hae*III and *Msp*I. Regarding *S. cerevisiae*, only one strain was found to differ from all the others, exhibiting a unique profile with both the enzymes used (Fig. 4), whereas all the *K. apiculata* strains generated the same profile with both the enzymes (Fig. 5).

DISCUSSION

Microsatellites are highly polymorphic in all population studied, so they are extensively used as markers for genome mapping (Debrauwere *et al.* 1997). Previous works reported that microsatellites in *Escherichia coli* and *S. cerevisiae* are unstable and that some trinucleotides are characterized by a particularly high level of instability (Sia *et al.* 1997), thus determining also intraspecific variability. In *S. cerevisiae* Aglianico strains the primers utilized, especially $(GTG)_5$, allowed to discriminate at the strain level, while in *K. apiculata* Aglianico strains can not be used to obtain intraspecific discrimination.

By using the amplification of the non-transcribed spacer region (NTS), we obtained an identical profile for all strains

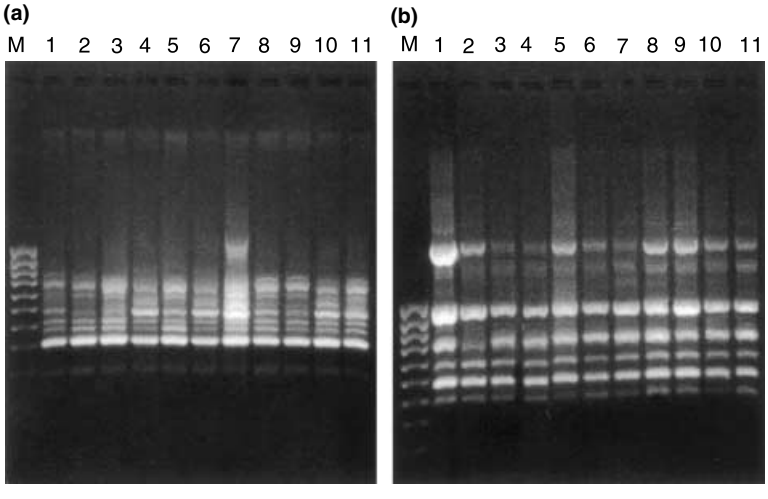

Fig. 3 PCR fingerprinting of *Kloeckera apiculata* strains primed with the microsatellites $(GAC)_5$ (a) and $(GTG)_5$ (b) M = 100 bp DNA ladder. Lanes: 1 = 2EII4; 2 = 4EIII5; 3 = 6EIII2; 4 = 8EI3; 5 = 10EII2; 6 = 13EI5; 7 = 14EIII1; 8 = 15EI3; 9 = 16EI4; 10 = 17EII2; 11 = 18EI2

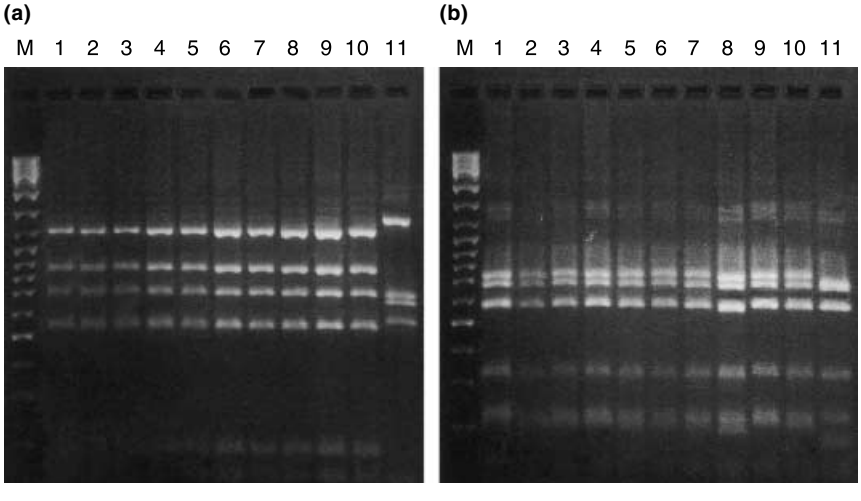

Fig. 4 Restriction digests of PCR-amplified NTS region of *Saccharomyces cerevisiae* with *Hae*III (a) and *MspI* (b) M = Gene Ruler$^{TM.}$ DNA ladder mix. Lanes: 1 = 2LI6; 2 = 3LI1; 3 = 7LI3; 4 = 9LI2; 5 = 14LBI3; 6 = 6LBI1; 7 = 16EIII5; 8 = 18EIII3; 9 = 1LBI3; 10 = 7EIII10; 11 = 8EIII11; 12 = B51; 13 = 5EIII10

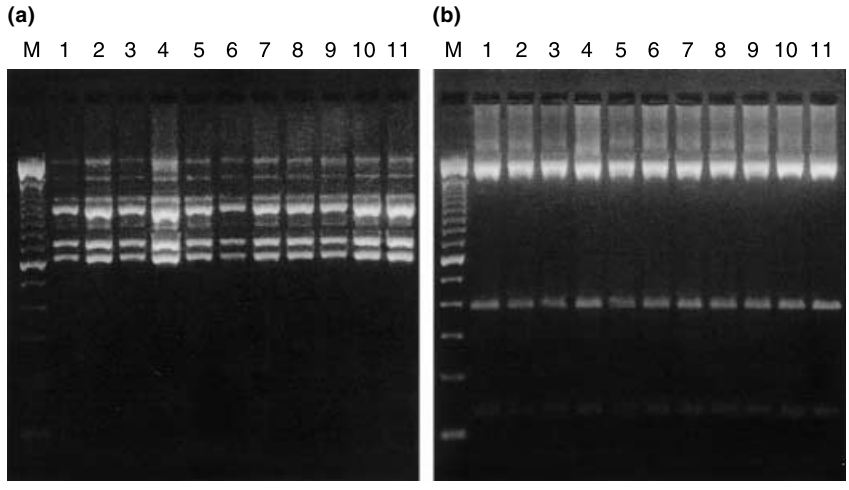

Fig. 5 Restriction digests of PCR-amplified NTS region of *Kloeckera apiculata* with *Hae*III (a) and *MspI* (b) M = 100 bp DNA ladder. Lanes: 1 = 2EII4; 2 = 4EIII5; 3 = 6EIII2; 4 = 8EI3; 5 = 10EII2; 6 = 13EI5; 7 = 14EIII1; 8 = 15EI3; 9 = 16EI4; 10 = 17EII2; 11 = 18EI2

within the same species, according to Baleiras Couto *et al.* (1996). In fact, this region is highly variable mainly between species and proved to be valuable as a population marker (Lachance 1990). Conversely, other workers found a large length variation of the NTS region in strains belonging to *Cryptococcus neoformans* and *Candida krusei* (Fan *et al.* 1995; Carlotti *et al.* 1997).

As regards RFLP technique, contrary to the results of Baleiras Couto *et al.* (1996), which obtained different profiles among *S. cerevisiae* strains, Aglianico *S. cerevisiae* strains yielded a low intraspecific discrimination and *K. apiculata* strains did not differ.

Since the profiles obtained with RFLP analysis are characterized by a low number of bands, the patterns from the two species can be more easily compared than the patterns resulting from PCR fingerprinting with $(GAC)_5$ and $(GTG)_5$ microsatellites. For this reason, RFLP technique can be used for a rapid monitoring of the yeast species involved in different stages of fermentation.

Conversely, PCR fingerprinting, especially with $(GTG)_5$ primer, can be useful to follow the evolution of *S. cerevisiae* population during fermentation and to determine the degree of natural biodiversity.

It must be underlined that the molecular profile of *K. apiculata* strains with $(GAC)_5$ primer is different from those exhibited by *S. cerevisiae* ones. Therefore, the use of this primer, together with the amplification and restriction of complete NTS region, can be applied to rapidly recognize and follow the presence of apiculate yeasts during the fermentation. In this context, the determination of this specific molecular profiles can be applied to follow in time the two dominant wine yeast species. Thus, if an unusual ratio of *S.cerevisiae*/*K.apiculata* occurs, it is possible to perform in a good time technological adjustments, that assure the suitable performance of the fermentation process and the achievement of desirable organoleptic wine characteristics.

REFERENCES

Baleiras Couto, M.M., van der Vossen, J.M.B.M., Hofstra, H. and Huis in't Veld, J.H.J. (1994) RAPD analysis: a rapid technique for differentiation of spoilage yeasts. *International Journal of Food Microbiology* **24**, 249–260.

Baleiras Couto, M.M., Vogels, J.T.W.E., Hofstra, H., Huis in't Veld, J.H.J. and van der Vossen, J.M.B.M. (1995) Random amplified polymorphic DNA and restriction enzyme analysis of PCR amplified rDNA in taxonomy: two identification techniques for foodborne yeasts. *Journal of Applied Bacteriology* **79**, 525–535.

Baleiras Couto, M.M., Eijsma, B., Hofstra, H., Huis in't Veld, J.H.J. and van der Vossen, J.M.B.M. (1996) Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology* **62**, 41–46.

Blondin, B. and Vezinhet, F. (1988) Identification de souches de levures oenologiques par leurs caryotypes obtenus en électrophorèse en champ pulsé. *Revue Française D'Oenologie* **28**, 7–19.

Carlotti, A., Srikantha, T., Schroppel, K., Kvaal, C., Villard, J. and Soll, D.R. (1997) A novel repeat sequence (CKRS-1) containing a tandemly repeated sub-element (kre) accounts for differences between *Candida krusei* strains fingerprinted with the probe CkF1,2. *Current Genetics* **31**, 255–263.

Constantí, M., Poblet, M., Arola, L., Mas, A. and Guillamón, J.M. (1997) Analysis of yeast populations during alcoholic fermentation in a newly established winery. *American Journal of Enology and Viticulture* **48**, 339–344.

Debrauwere, H., Gendrel, C.G., Lechat, S. and Dutreix, M. (1997) Differences and similarities between various tandem repeat sequences: Minisatellites and microsatellites. *Biochimie* **79**, 577–586.

Fan, N., Chen, L.C., Ragan, M.A., Gutell, R.R., Warner, J.R., Currie, B.P. and Casadevall, A. (1995) The 5S rRNA and the rRNA intergenic spacer of the two varieties of *Cryptococcus neoformans*. *Journal of Medical and Veterinary Mycology* **33**, 215–221.

Heard, G. and Fleet, G. (1985) Growth of natural yeast flora during the fermentation of inoculated wines. *Applied and Environmental Microbiology* **50**, 727–728.

Henriques, M., Sá-Nogueira, I., Giménez-Jurado, G. and van Uden, N. (1991) Ribosomal DNA spacer probes for yeast identification: studies in the genus *Metschnikowia*. *Yeast* **7**, 167–172.

Kurtzman, C.P. and Fell, J.W. (1998) *The Yeasts, a Taxonomic Study*, 4th edn. Elsevier Science B, V., Amsterdam, The Netherlands.

Lachance, M.A. (1990) Ribosomal DNA spacer variation in the cactophylic yeast *Clavispora opuntiae*. *Molecular Biology Evolution* **7**, 178–193.

Parrish, M.E. and Carroll, D.E. (1985) Indigenous yeasts associated with muscadine (*Vitis rotundifolia*) grapes and musts. *American Journal of Enology and Viticulture* **12**, 3–31.

Petering, J., Langridge, P. and Henschke, P. (1988) Fingerprinting wine yeasts. *Australian New Zealand Wine Industry Journal* **3**, 48–52.

Querol, A., Barrio, E. and Ramón, D. (1992) A comparative study of different methods of yeast strain characterization. *Systematic and Applied Microbiology* **15**, 439–446.

Schütz, M. and 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.

Sia, E.A., Jinks-Robertson, S. and Petes, T.D. (1997) Genetic control of microsatellite stability. *Mutation Research* **383**, 61–70.

Valente, P., Gouveja, F.C., de Lamos, G.A., Pimentel, D., van Elsas, J.D. and Mendonça-Hagler, A.N. (1996) PCR amplification of the rDNA internal transcribed spacer region for differentiation of *Saccharomyces* cultures. *FEMS Microbiology Letters* **137**, 253–256.

Vezinhet, F., Hallet, J.N., Valade, M. and Poulard, A. (1990) Ecological survey of wine yeast strains by molecular methods of identification. *American Journal of Enology and Viticulture* **43**, 83–86.