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The uses of AFLP for detecting DNA polymorphism, genotype identification and genetic diversity between yeasts isolated from Mexican agave-distilled beverages and from grape musts

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ABSTRACT

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Aims: The objectives were to determine the variability and to compare the genetic diversity obtained using amplified fragment length polymorphism (AFLP) markers in analyses of wine, tequila, mezcal, sotol and raicilla yeasts.

Methods and Results: A molecular characterization of yeasts isolated from Mexican agave musts, has been performed by AFLP marker analysis, using reference wine strains from Italian and South African regions.

Conclusions: A direct co-relation between genetic profile, origin and fermentation process of strains was found especially in strains isolated from agave must. In addition, unique molecular markers were obtained for all the strains using six combination primers, confirming the discriminatory power of AFLP markers.

Significance and Impact of the Study: This is the first report of molecular characterization between yeasts isolated from different Mexican traditional agave-distilled beverages, which shows high genetic differences with respect to wine strains.

Keywords: agave yeasts, amplified fragment length polymorphism, genetic diversity, mezcal, tequila.

INTRODUCTION

Mexico is recognized for the production of various alcoholic beverages obtained from the distillation of fermented juice of different species of agave plants. The most well known is tequila obtained from the *Agave tequilana* Weber var. azul, but other beverages like mezcal, sotol and raicilla can be mentioned too. Mezcal is produced with different *Agave* species like *Agave angustifolia*, *Agave salmiana* and *Agave potatorum*. The production process is more rudimentary than tequila and the fermentation step is carried out with juice and bagasse without inoculation. Sotol and raicilla are obtained from *Dacilirium* and *Agave maximiliana* respectively using process similar to mezcal.

Traditionally, the identification of yeast species has been based on assimilation and fermentation tests and morphological traits (Kurtzman and Fell 1998). Based on these methods, Lachance (1995) identified 13 yeast species involved in the fermentation of tequila. However these methods are not able to detect differences at the strain level inside the same species, which can affect the efficiency of the process and aroma production. Recent progress in molecular biology has led to the development of new methods for yeast identification and characterization based on molecular techniques. DNA-based methods have the advantage of being independent of gene expression.

For yeast identification and classification some authors have used internal transcribed spacer (ITS) regions

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(Pramateftaki *et al.* 2000), or a rapid method like restriction fragment length polymorphism (Esteve-Zarzoso *et al.* 1999), electrophoretic karyotyping (Versavaud *et al.* 1995; Giudici *et al.* 1998) and more recently a PCR method based on the variation of introns in the mitochondrial gene COX1 (López *et al.* 2002). In other cases a combination of different techniques is used, for example Fernández-Espinar *et al.* (2001) combined mtDNA restriction analysis with the electrophoretic karyotyping and PCR amplification of δ sequences in the study of the authenticity of commercial wine yeast strains by molecular techniques.

Amplified fragment length polymorphism (AFLP) analysis is a technique through which selected fragments from the digestion of total plant DNA are amplified by the PCR (Vos *et al.* 1995). The AFLP technique allows the identification of a higher number of polymorphic bands, is highly reproducible and uses small amounts of DNA (Polanco and Ruiz 2002). The resulting DNA fingerprinting provides a multiplex ratio, defined as the number of information points analysed per experiment, much higher than for other types of molecular markers (Powell *et al.* 1996).

The AFLP technology has been widely applied in plant studies (Breyne *et al.* 1999), in animals (Otsen *et al.* 1996) and more recently in micro-organisms (de Barros Lopes *et al.* 1999). However, only a few studies of genetic variation in yeasts have been carried out using AFLP markers so far (de Barros Lopes *et al.* 1999; Azumi and Goto-Yamamoto 2001).

The objectives of the present study are: (i) to develop a set of AFLP markers in bulk analysis, (ii) to determine their variability, (iii) to apply them for genome analysis, distinguishing between yeast genotypes, and (iv) to compare the genetic diversity obtained using these markers in analyses of wine and tequila, mezcal, sotol and raicilla yeasts.

MATERIALS AND METHODS

Yeast strains

Yeasts were obtained from different collections: University of Basilicata, Wine Microbiology Laboratory collection (Italy), University of Stellenbosch collection (South Africa) and CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Edo. de Jalisco) collection (Mexico). The origin of the yeasts used and the fermentation process from which they were isolated are described in Table 1.

In addition, the type strains of *Saccharomyces sensu stricto* wine species, obtained from Centraalbureau voor Schimmelcultures (the Netherlands), were used as reference strains: CBS1171 for *Saccharomyces cerevisiae*, CBS380 for *Saccharomyces bayanus*, CBS432 for *Saccharomyces paradoxus*, CBS1538 for *Saccharomyces pastorianus*.

DNA extraction

Yeasts were grown in YPD medium (10 g l^{-1} of yeast extract, 20 g l^{-1} of glucose and 20 g l^{-1} of peptone) at 30°C in a mechanical shaker (250 rev min^{-1}) for 12 h. For each yeast medium the equivalent to 2000×10^6 cells was collected and then centrifuged at 2270 *g* for 15 min. Frozen cells (−20°C) were disrupted by 1 min treatment in a Braun

Table 1 List of strains used in the present work

Strains	Genera	Region	Isolation source
20EI5	*Kloeckera*	Basilicata (South Italy)	Red wine, Aglianico grape
20EII5	*Kloeckera*	Basilicata (South Italy)	Red wine, Aglianico grape
7EI3	*Kloeckera*	Basilicata (South Italy)	Red wine, Aglianico grape
4LBI3	*Saccharomyces*	Basilicata (South Italy)	Red wine, Aglianico grape
AGME997	*Saccharomyces*	Basilicata (South Italy)	Red wine, Aglianico grape
NDAMII2	*Saccharomyces*	Sicily (South Italy)	Red wine, Nero d'Avola grape
FIMA3	*Saccharomyces*	Campania (South Italy)	White wine, Fiano grape
VIN13	*Saccharomyces*	Cape town (South Africa)	Wine
N96	*Saccharomyces*	Cape town (South Africa)	Wine
SO2	*Candida*	Chihuahua (North Mexico)	Sotol, *Dacilirium* and *Agave angustifolia* fermented juice
SOM	*Saccharomyces*	Chihuahua (North Mexico)	Sotol, *Dacilirium* and *Agave angustifolia* fermented juice
OFF1	*Candida*	Guerrero (South Mexico)	Mezcal, *Agave cupreata* fermented juice
CHA	*Saccharomyces*	Guerrero (South México)	Mezcal, *Agave cupreata* fermented juice
RG1	*Saccharomyces*	Jalisco (Central México)	Raicilla, *Agave maximiliana* fermented juice
TE4	*Kloeckera*	Jalisco (Central México)	Tequila, *Agave tequilana* fermented juice
GU4	*Saccharomyces*	Jalisco (Central Mexico)	Tequila, *Agave tequilana* fermented juice
MG	*Saccharomyces*	Jalisco (Central Mexico)	Tequila, *Agave tequilana* fermented juice
AR5	*Saccharomyces*	Jalisco (Central Mexico)	Tequila, *Agave tequilana* fermented juice

Homogenizer (Braun Biotech International, Bethlehem, PA, USA). The cell-free extract was treated according to the Kit G1N-70 (Gen Elute Mammalian Genomic DNA miniprep kit, Sigma). DNA concentration was analysed by electrophoresis on 1% agarose gel in Tris–acetic acid–EDTA buffer and stained with ethidium bromide.

Restriction analysis of 18S rDNA amplified

The primer pair NS1/ITS4 (White *et al.* 1990) was used to amplify the 18S rDNA, including ITS region, of *Saccharomyces* strains. The PCR reaction was performed in 30-μl reaction volumes containing 50 ng of DNA template, 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris–HCl (pH 9·0), 1% Triton X-100, 0·2 mmol l^{-1} of each dNTPs, 2 mmol l^{-1} $MgCl_2$, and 20 ng of each primer and 1 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR conditions were: an initial denaturing step of 2 min at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 3 min, and a final extension step of 7 min at 72°C. Amplification products were digested with two restriction enzymes, *Hae*III and *Msp*I (Promega) separately following the supplier's instructions. The restriction fragments were separated on 1·5% agarose gel, with TBE buffer and adding ethidium bromide (Sigma) at a final concentration of 0·5 μg ml^{-1}. Molecular weights were estimated by comparison against a DNA molecular weight marker VI (Roche, Monza, Italy) by using the software Diversity Database (Bio-Rad).

AFLP procedure

The AFLP fingerprinting (Vos *et al.* 1995) was performed using the Analysis System for Microorganisms and AFLP Microorganism Primer Kit (Gibco BRL Life Technologies, Carlsbad, CA, USA) according to the protocol supplied by the manufacturer. Primer nomenclature follows the Gibco-BRL Life Technologies Kit. After the addition of an equal volume (10 μl) of sequencing dye (98% formamide, 10 mmol $^{-1}$ EDTA, 0·025% xylene cyanol, 0·025% bromophenol blue), the samples were heated at 94°C for 3 min and chilled on ice. A 4-μl aliquot of each sample was subjected to electrophoresis on a denaturing 6% polyacrylamide gel containing 7·5 mol $^{-1}$ urea and a 0·05x TBE running buffer (45 mmol l^{-1} Tris–borate, 1 mmol l^{-1} EDTA, pH 8) at 60 W for 1·5 h, in a 30 × 40 cm manual sequencing apparatus (Bio-Rad). Amplified fragments were visualized using the silver staining method (Bassam *et al.* 1991).

Data analysis

The AFLP markers were scored as presence (1) or absence (0) of a band, and the data obtained were used in a triangular matrix. The data matrix was then used to generate a genetic similarity index (GS) (Nei and Li 1979), using NTSYS 16 (Rohlf 1993). Cluster analysis was carried out based on genetic distance (GD = 1 − GS), using UPGMA (unweighted pair-group method using arithmetic averages) (Sneath and Sokal 1973). The resulting clusters were represented as a dendrogram and viewed in the program Tree View 15 [Roderic D.M. Page (1998); available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html]. The robustness of the dendrogram was assessed with the WinBoot program (Yap and Nelson 1996).

RESULTS

The *Saccharomyces* wild strains (Table 1) were identified at the species level by analysis of 18S rDNA, including the ITS region (Redepovi *et al.* 2002). The size of the amplified fragment was about 1900 bp. This amplification product was digested separately with the restriction enzymes *Hae*III and *Msp*I and Table 2 reports the approximate length of the restriction fragments observed after digestion for the type strains. Analysing these data, the enzyme *Hae*III produced two different restriction patterns: one for *S. cerevisiae* and *S. paradoxus*, another for *S. bayanus* and *S. pastorianus*. After restriction with *Msp*I, *S. cerevisiae*, *S. bayanus* and *S. pastorianus* showed restriction fragments of the same molecular size, whereas *S. paradoxus* exhibited a different restriction pattern. The wild strains, after restriction with *Hae*III, exhibited the profile recorded for *S. cerevisiae* and *S. paradoxus*, whereas with the endonuclease *Msp*I showed the pattern recorded for *S. cerevisiae*, *S. bayanus* and *S. pastorianus*.

Table 2 Molecular sizes of the fragments obtained after digestion of 18S rDNA of *Saccharomyces* strains

	Restriction enzymes	
Type strains	*Hae*III	*Msp*I
S. cerevisiae (CBS1171)	780, 410, 310, 200	1020, 505, 250, 125
S. bayanus (CBS380)	780, 605, 275, 135	1020, 505, 250, 125
S. paradoxus (CBS432)	780, 410, 310, 200	1500, 285, 115
S. pastorianus (CBS1538)	780, 605, 275, 135	1020, 505, 250, 125

Table 3 Average number of fragments obtained from six selective primer combinations to detect AFLPs among 18 yeast strains

Primer pair	Total no. of fragments	% Polymorphism
C/C	37	15·8
C/G	56	38·3
C/T	35	21·5
AC/G	75	11·1
AC/C	80	18·6
C/A	16	42·6

The genetic analysis was performed using a total of six AFLP primer combinations. Table 3 summarizes the percentage of polymorphism in the strains studied. Each primer combination produced an average of 49·8 amplification products per strain (80 with AC/C to 16 with C/A). The polymorphism with each AFLP fingerprinting ranged from 11·1 to 42·6% indicating a middle marker index (Table 3). The total number of unique molecular markers specific for each species ranged from 1 to 3, when the six-primer combinations were used. The unique markers identified can be used to generate specific probes for the different strains.

The dendrogram obtained after UPGMA cluster analysis of the genetic distance data is shown in Fig. 1. The cophenetic correlation coefficient obtained was 0·95 and bootstrap

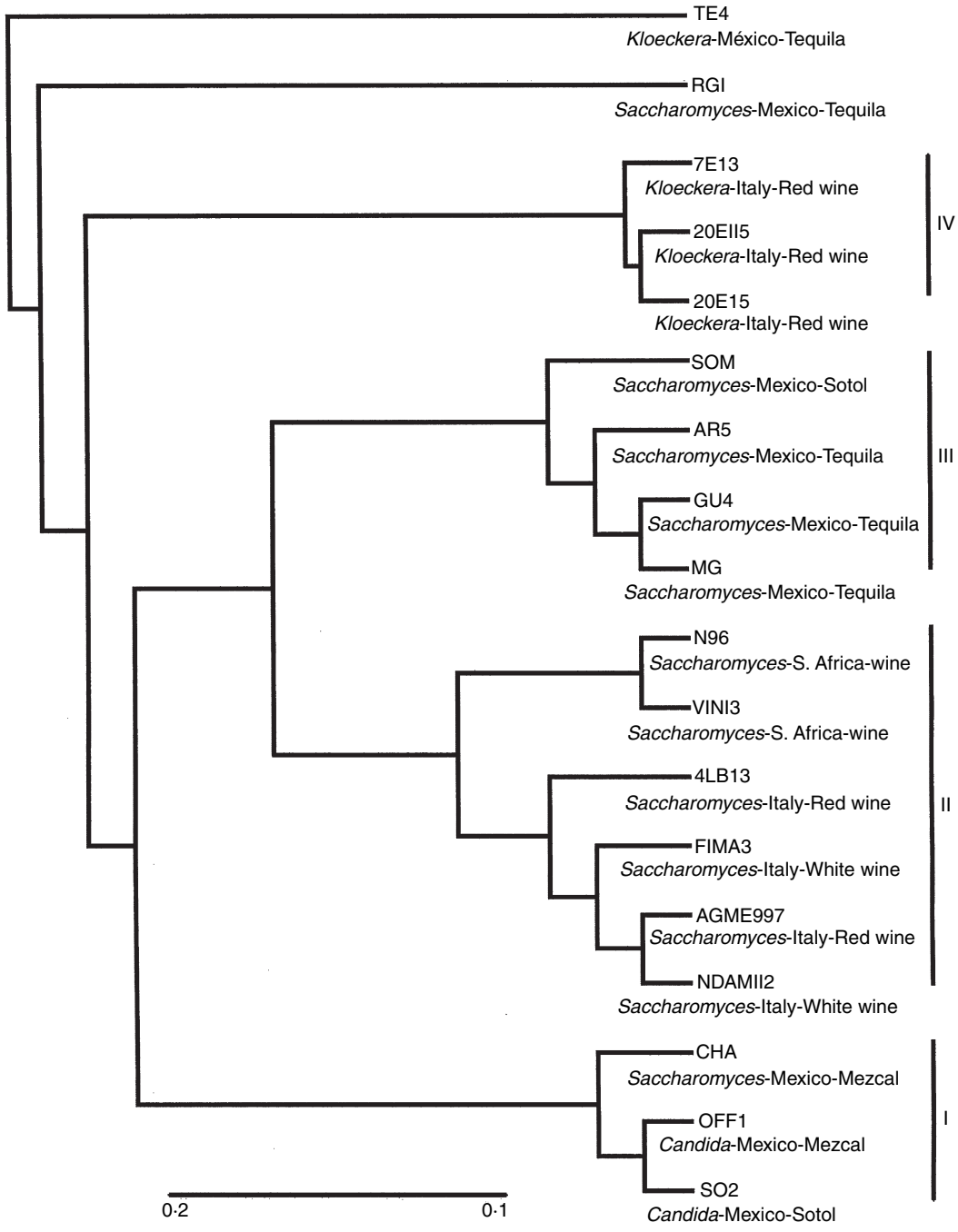

Fig. 1 UPGMA cluster analysis of AFLP generated by six primer combinations on wine, tequila, mezcal, sotol and raicilla yeasts. Scale depicts genetic diversity estimates (GDEs)

analysis revealed that most of the branches in the dendrogram have bootstrap values of 69·3–100%. The cophenetic correlation coefficient showed a good fit between the dendrogram and the original similarity matrix. Moreover, high bootstrap values validated the robustness of the branching pattern obtained.

DISCUSSION

This is the first report of the use of AFLP marker analysis to the characterization and genetic relationships between different genera and species of yeasts isolated from tequila, mezcal, sotol and raicilla musts, and compared with yeasts of wine origin. AFLP is useful for distinguishing among the strains tested and this finding is consistent with that reported by de Barros Lopes *et al.* (1999).

According to the dendrogram, the strains studied can be divided into four principal groups (I, II, III, IV), and two independent strains, which were not closer to any groups (Fig. 1). The position of TE4 and RGI suggests important genetic differences from the other strains.

In group I (Fig. 1) two strains OFF1 and SO2, isolated from different Mexican products, are both *Candida* and are strictly related, indicating in this case that there is no correlation between genetic profile and origin. The third component (CHA) is a *Saccharomyces* strain and in this study it forms a cluster with two *Candida*. There is evidence that *Candida* strains form clusters with other *Saccharomyces* strains (Kurtzman and Robnett 2003). In the case of OFF1 and CHA strains, which were isolated from the same process, they also exhibit a similar physiological behaviour (Arrizon *et al.* 2003). Therefore a possible genetic similarity between these two strains could be solved in the future by multigene sequence analysis (Kurtzman and Robnett 2003).

Group II is composed of only wine *Saccharomyces* strains from Italy and South Africa. In this case a clear clustering related to the geographical origin has been revealed: the two South African strains have the same genetic profile (Fig. 1, group II, subgroup b), whereas the Italian wine strains, even if belonging to the same subgroup a, exhibited a certain genetic diversity, which is in agreement with other reports (Versavaud *et al.* 1995; Caruso *et al.* 2002). However, among *S. cerevisiae* wine strains (4LBI3, AGME997, NDAMII2, FIMA3) the correlation between geographical area and the degree of genetic relatedness is not clear, as found by Versavaud *et al.* (1995).

Group III (Fig. 1) comprises *S. cerevisiae* strains isolated from Mexican agave-distilled beverages. Three of the four strains (GU4, MG, AR5) have the same geographical origin and have been isolated from tequila process. This suggests the existence of a closer correlation between the fermentation process and isolation region among agave *Saccharomyces* strains than among wine ones.

In group IV the three strains from *Kloeckera apiculata* have in common the isolation region. Conversely, strain TE4 (*Kloeckera africana)* did not cluster with the group of *Kloeckera* wine strains (Fig. 1). As dimorphic yeast, *Kloeckera* has similarity with the behaviour of *Hanseniaspora* strains, thus the results from this study are similar to other results found in *Hanseniaspora* species. In the work of Cadez *et al.* (2003), differences were determined in genetic profile obtained by DNA (RAPD)-PCR among *Hanseniaspora* strains isolated from different geographical origin, including some *Hanseniaspora* strains (*Hanseniaspora lachancei*) isolated from fermenting *A. tequilana* juice (Lachance 1995). As regards *Kloeckera* wine strains used in this study, there is a direct relation between AFLP profile, geographical origin and fermentation process.

The comparison of the RG1 strain, the only one isolated from raicilla agave must, with the other agave *Saccharomyces* strains and also with *Saccharomyces* wine strains, emphasizes a considerable genetic difference that might be related to the fermentation process, which has determined the yeast selection.

In this work a general co-relation between AFLP strain profile and isolation origin of strains from fermentation process can be recognized. Agave must fermentation is a relatively short process, which probably can support a more diverse microflora of yeast populations. This could be the result of a specific adaptation, which probably determines different physiological and enological properties, as shown in other reports (Mesa *et al.* 2000; Arrizon *et al.* 2003).

The AFLP analysis was shown to be highly reproducible, as reported elsewhere (Zabeau and Vos 1993; Savelkoul *et al.* 1999). In addition, it was shown to be a powerful tool for demonstrating the relationship between molecular profile, strain origin and fermentation process. Even though in future an extensive characterization must be performed with other wine and Mexican beverage strains, these preliminary results show the importance of using molecular techniques for the characterization of yeast strains used in the beverage industry.

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REFERENCES

Arrizon, J., Fiore, C., Gschaedler, A., Flores, J., Andreotti, G. and Romano, P. (2003) Comparison between wine and agave yeast strains

for traits of technological interest. *23rd International Specialized Symposium on Yeasts*, Budapest, Hungary. pp. 4–21.

Azumi, M. and Goto-Yamamoto, N. (2001) AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces sensu stricto* and its application to phenetic clustering. *Yeast* **18**, 1145–1154.

de Barros Lopes, M., Rainieri, S., Henschke, P.A. and Langridge, P. (1999) AFLP fingerprinting for analysis of yeast genetic variation. *Int J Syst Bacteriol* **49**, 915–924.

Bassam, B.J., Caetano-Anolles, G. and Gresshoff, P.M. (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Ann Biochem* **196**, 80–83.

Breyne, J.H.A., Rombaut, D., Van Gysel, A., Van Montagu, M. and Gerats, T. (1999) AFLP analysis of genetic diversity within and between *Arabidopsis thaliana* ecotypes. *Mol Gen Genet* **261**, 627–634.

Cadez, N., Poot, G.A., Raspor, P. and Smith, M.Th. (2003) *Hanseniaspora meyeri* sp. nov., *Hanseniaspora clermontiae* sp. nov., *Hanseniaspora lachancei* sp. nov. and *Hanseniaspora opuntiae* sp. nov., novel apiculata yeast species. *Int J Syste Evol Microbiol* **53**, 1671–1680.

Caruso, M., Capece, A., Salzano, G. and Romano, P. (2002) Typing of *Kloeckera apiculata* and *Saccharomyces cerevisiae* strains from Aglianico wine. *Lett Appl Microbiol* **34**, 323–328.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. (1999) Identification of yeasts by RFLP analysis of the 5·8 rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* **49**, 329–337.

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

Giudici, P., Caggia, C., Pulvirenti, A. and Rainieri, S. (1998) Karyotyping of *Saccharomyces* strains with different temperature profiles. *J Appl Microbiol* **84**, 811–819.

Kurtzman, C.P. and Fell, J.W. (1998) *The Yeast, a Taxonomic Study*, 3rd edn. Amsterdam: Elsevier Science.

Kurtzman, C.P. and Robnett, C.J. (2003) Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene analyses. *Fed Eur Microbiol Soc Yeast Res* **3**, 417–432.

Lachance, M.A. (1995) Yeast communities in natural tequila fermentation. *Antonie Van Leeuwenhoek* **68**, 151–160.

López, V., Fernández-Espinar, M.T., Barrio, E., Ramón, D. and Querol, A. (2002) A new PCR-based method for monitoring inoculated wine fermentations. *Int J Food Microbiol* **81**, 63–71.

Mesa, J.J., Infante, J.J., Rebordinos, L., Sánchez, J.A. and Cantoral, J.M. (2000) Influence of the yeast genotypes on enological characteristics of sherry wines. *Am J Enol Viticult* **51**, 15–21.

Nei, M. and Li, W.H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269–5273.

Otsen, M., den Bieman, M., Kuiper, M.T.R., Pravenec, M., Kren, V., Kurtz, T.W., Jacob, H.J., Lankhorst, A. *et al.* (1996) Use of AFLP markers for gene mapping and QTL in the rat. *Genomics* **37**, 289–294.

Polanco, C. and Ruiz, M.L. (2002) AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants. *Plant Sci* **162**, 817–824.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingeny, S. and Rafalski, J.A. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol Breed* **2**, 225–238.

Pramateftaki, P.V., Lanaridis, P. and Typas, M.A. (2000) Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. *J Appl Microbiol* **89**, 236–248.

Redepovi, S., Orli, S., Sikora, S., Majdak, A. and Pretorius, I.S. (2002) Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. *Lett Appl Microbiol* **35**, 305–310.

Rohlf, F.J. (1993) *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System*, Ver. 18. New York: Applied Biostatistics.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. *et al.* (1999) Amplified length polymorphism analysis: the state of art. *J Clin Microbiol* **37**, 3083–3091.

Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy*. San Francisco, CA: W.H. Freeman.

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

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J. *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407–4414.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols* ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. pp. 315–322. San Diego, CA: Academic Press, Inc.

Yap, V. and Nelson, J.R. (1996) *WinBoot: A Program for Performing Bootstrap Analysis of Binary Data to Determine the Confidence Limits of UPGMA-Based Dendrograms*. Manila, Philippines: International Rice Research Institute.

Zabeau, M. and Vos, P. (1993) *Selective Restriction Fragment Amplification; a General Method for DNA Fingerprinting*. European Patent Office, publication 0 534 858 A1.