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An optimized protocol for the production of interdelta markers in *Saccharomyces cerevisiae* by using capillary electrophoresis

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ABSTRACT

The amplification of genomic sequence blocks flanked by delta elements of retrotransposon origin has proved to be a very convenient method for molecular characterization of *Saccharomyces cerevisiae* strains. Fluorescent automated capillary electrophoresis (CE) was used to detect interdelta marker (IDM) patterns in *S. cerevisiae*, using the ABI Prism 3130 Genetic Analyzer. Main experimental parameters were studied and the optimal conditions for IDM amplification and samples run on the CE apparatus were determined. Fingerprints from fluorescent-labelled IDM produced using CE with the same sample analyzed by agarose electrophoresis (AE) were compared. The CE analysis was able to distinguish 43 different IDM profiles among 45 *S. cerevisiae* isolates with a discriminating capacity of 99.8%, whereas the AE analysis of the same samples allowed the identification of 27 different patterns (discriminatory power equal to 96%). Detection of fluorescent IDM was fast and reliable, and it facilitated data comparison. For the first time in our knowledge, the fluorescent CE proved to be well suited for IDM fingerprinting. Moreover, it could be routinely applied for the molecular differentiation of *S. cerevisiae* strains.

1. Introduction

Identification at strain level of *Saccharomyces cerevisiae* is a fundamental step to investigate the biodiversity of this yeast and to examine population dynamics during the fermentative process (Lopes et al., 2002; Granchi et al., 2003; Pulvirenti et al., 2001; Cappello et al., 2004; Lopandic et al., 2008). Intraspecies classification of *S. cerevisiae* is also essential for the selection of a fermentation starter (Dequin, 2001; Lopes et al., 2007) and for unequivocally distinguishing clinical isolates responsible for infections in immunodepressed patients (McCullough et al., 1998). Numerous polymerase chain reaction (PCR)-based methods have been suggested for such purposes: random amplified polymorphic DNA (RAPD) (McGrath et al., 1998; Perez et al., 2001), genome amplification by using intron-specific primer pairs (de Barros Lopes et al., 1996; Lopez et al., 2003), fingerprinting of microsatellite markers (Ayoub et al., 2006) and finally the amplification of genomic sequence blocks flanked by delta elements of retrotransposon origin (Ness et al., 1993; Legras and Karst, 2003). Interdelta typing has proved to be a very convenient method that can advantageously replace the other methods for molecular characterization of *S. cerevisiae* strains (Schuller et al., 2004). However, some problematic aspects of interdelta marker (IDM) typing need to be cited, since it is often possible to observe weakly amplified bands, which may render difficult the interpretation of the obtained results after electrophoresis on agarose gel (Fernandez-Espinar et al., 2001; Schuller et al., 2004). A major analytical improvement has been obtained in recent years by the use of fluorescent primers and automatic sequencers to analyze amplified fragment-length polymorphism (AFLP) in several species (Ovilo et al., 2000; Terefework et al., 2001; Grando et al., 2003). When compared with gel-based analytical methods, the use of capillarity systems and automated analysis increases data throughput, scoring and reliability, decreasing the overall experimental error (Papa et al., 2005). In the present report, we describe for the first time a highly reproducible protocol for IDM detection in *S. cerevisiae*, using the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA). The proposed method showed that it increased the efficiency of amplification and enhanced the resolution power of IDM profiles, proving that it is an excellent system for large-scale identification of *S. cerevisiae* strains.

2. Materials and methods

2.1. DNA extraction

Enological yeast strains used in this work are listed in Table 1. Yeast cells were routinely cultured in YPD medium (1% w/v yeast extract, 1% w/v bactopeptone, 2% w/v glucose) in agitation for 24 h at 28 °C. Genomic DNA was extracted using the protocol described by Hoffman and Winston (1987) with few modifications. Briefly, yeasts were harvested by centrifugation (6700 *g*, for 5 min) from 2 ml of fresh culture. The sediment was suspended in a 200 µl breaking buffer [2% (v/v)

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Table 1
Saccharomyces cerevisiae strains used in the study.

Geographic area	Grape	Strain
Gioia del Colle	Primitivo	G1, G2, G3, G4, G5, G6, G7, G, G9, G10, G11, G12
C. S. Marco	Negroamaro	2P1, 2P2, 2P3, 2P4, 2P5, 2P6, 2P7, 2P8, 2P9, 2P10
Torchiarolo	Negroamaro	B1
Melissano	Negroamaro	8M, 8M1, 8M2, 8M3, 8M4, 8M5, 8M6, 8M7, 8M8, 8M9, 8M10
Melissano	Negroamaro	11M, 11M1, 11M2, 11M3, 11M4, 11M5, 11M6, 11M7, 11M8, 11M9, 11M10

Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8.0, 0.1 mM EDTA] and then 0.2 g glass beads (0.45–0.6 mm, Sigma) were added to it. Cells were disrupted by vortexing for 2 min, the Proteinase K (0.28 mg/ml) was added and the mixture was incubated at 60 °C for 1 h. Samples were kept on ice for 5 min and, after the addition of 400 µl phenol/chloroform/isoamyl alcohol (25:24:1 v/v) were vortexed twice for 3 min. After the addition of 200 µl of TE buffer (100 mM Tris-Cl, 10 mM EDTA pH 8.0) the samples were centrifuged for 5 min at 14,000 *g*. The aqueous layer was collected and transferred to a new tube containing one volume of chloroform/isoamyl alcohol (24:1 v/v). The mixture was submitted to a centrifugation step as above, the aqueous phase was transferred to a clean tube and the DNA was precipitated by the addition of two volumes of absolute ethanol. The samples were incubated at −20 °C for 1 h and subsequently centrifuged at 14,000 *g* for 3 min at 4 °C. The nucleic acid pellet was dissolved in 400 µl TE buffer and was treated with RNAse A (75 µg/ml) for 5 min at 37 °C. Then, after the addition of 10 µl of 4 M ammonium acetate and 1 ml of ethanol, the mixture was centrifuged as above and the dried DNA pellet was suspended in a 200 µl of sterile water.

2.2. DNA amplification

The following parameters were estimated for the optimization of the PCR reaction: i) reaction volume, ii) amount of DNA template and iii) quantity of primers. The optimal reaction volume was evaluated by testing different final-volume amounts corresponding to 10, 20 and 50 µl. Different primer concentrations (0.10, 0.15, 0.25, 0.50 and 0.75 µM) and DNA template (0.5, 2, 4, 6 and 8 ng/µl) were tested. PCR amplifications were carried out using 0.5 units/reaction EuroTaq DNA Polymerase (Euroclone, Italy), reaction buffer [16 mM $(NH_4)_2S0_4$, 67 mM Tris HCl pH 8.8, 0.01% Tween-20, 0.15 mM of $MgCl_2$] and 0.2 mM of each dNTP. The primers were those described by Legras and Karst (2003), i.e. delta12: 5′-TCAACAATGGAATCCCAAC-3′ and delta21 5′-CATCTTAACACCGTATATGA, with the exception that the delta21 primer was 5′-dye-labelled with 6-Carboxyfluorescein (6-FAM, MWG-Operon, Germany). Amplifications were performed with a thermal cycler under the following conditions: 4 min at 95 °C (1 cycle), 30 s at 95 °C, 30 s at 46 °C and 90 s at 72 °C (35 cycles). A final extension step was carried out at 72 °C and the effect of different incubation periods (5, 15, 30 and 45 min) was assessed.

2.3. Agarose gel and capillary electrophoresis

Amplification products were separated by electrophoresis in 2% (w/v) agarose gels containing 1 µg/µl ethidium bromide, at 80 V for 1 h in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). Capillary electrophoresis (CE) of the amplified fragments was carried out on an ABI Prism 3130 Genetic Analyzer using the default AFLP run module of the ABI Prism 3130 (Applied Biosystems, USA), which adopted the following default parameters: run voltage: 15 kV; injection voltage: 1.2 kV; injection time: 16 s; run time: 20 min; temperature: 60 °C; and laser power: 15. The GeneScan-600 LIZ (Applied Biosystems, USA), which is composed of standard labelled fragment population ranging from 20 to 600 bp, was used as a size standard. The following parameters were evaluated for the optimization of the CE assay: sample dilution ($10^1, 10^{-1}, 10^{-2}$ and 10^{-3}) and size standard volume (0.5, 0.3, 0.1 and 0.005 µl) to be loaded on the microplate, injection time duration (15, 25, 40 and 50 s) and run time (20, 30 and 40 min).

2.4. Reproducibility and reliability

The reproducibility of the proposed method was validated by multiple testing ($n=5$) as follows. The genomic DNA, extracted from four distinct *S. cerevisiae* isolates, was used as template in five independent PCR reactions (see Section 2.3) and the resulting interdelta fragments were separately analyzed either by agarose electrophoresis (AE) or by CE. Protocol reliability and data quality were confirmed by applying the optimized protocol on a population composed of 45 wild *S. cerevisiae* isolates. To compare the discriminating capacity of the two electrophoresis methods, the index of discrimination (D) based on Simpson's index of diversity as described by Hunter and Gaston (1988), was used:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j\left(n_j - 1\right)$$

where N is the total number of isolates in the sample population, s is the total number of types described, and n_j is the number of isolates belonging to the jth type. Confidence intervals (CI_{95}) for D were determined as previously reported (Grundmann et al., 2001).

2.5. Data analysis

Agarose gels were scanned with a Gel Doc 1000 apparatus (Bio-Rad, USA) and analyzed with Molecular Analyst Fingerprinting (Bio-Rad, USA). The Delta-FAM patterns obtained by CE were analyzed with GeneMapper software version 4.0 (Applied Biosystem, USA), taking into consideration the fragment population ranging from 60 to 600 bp. Data were reported in a binary format with "1" for the presence of a band/peak and "0" for its absence. A matrix of similarities between each pair of individuals was created using the Dice's similarity index, S (Dice, 1945). The similarity matrix was employed to construct a UPGMA dendrogram, using the SAHN-clustering and TREE programs contained in the statistical package NTSYS-2.1 (Applied Biostatistics, USA). A cophenetic matrix was derived from the similarity matrix to test goodness-of-fit of the clusters, by comparing the two matrices using the Mantel matrix correspondence test (Mantel, 1967) in the MXCOMP program of the NTSYS-pc package (Rohlf, 1993). Gel images and dendrograms were produced by respectively using Adobe Photoshop (Adobe, USA) and Draw (OpenOffice.org, USA).

3. Results

In order to carry out the optimization of experimental parameters for IDM amplification and analysis by CE, four different *S. cerevisiae* strains, already characterized for their molecular and oenological properties (F. Grieco, unpublished results), were chosen as model. A scaled down procedure of the canonical PCR reaction was assessed, by performing amplification reactions in a smaller volume and by using lower primer concentrations, with respect to those commonly employed (data not shown). Control reactions were assembled in different final volumes (50, 20 and 10 µl) and no noticeable differences were observed. Then, the lowest tested volume (10 µl) was chosen as optimal. When different primer pair concentrations were evaluated, primer addition $\leq$0.20 µM produced faint DNA profiles, whereas satisfactory and reproducible results were obtained using higher primer concentrations, i.e. $\geq$0.25 µM. The optimal amount of DNA template was then established and the obtained results showed that template addition $\leq$4 ng/µl produced faint and