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Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers

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Abstract

Yeast isolates from orange fruit and juice in a spontaneous fermentation were identified and classified by two molecular techniques. The first was analysis of the restriction pattern generated from the polymerase chain reaction (PCR)-amplified 5.8S rRNA gene and the two internal transcribed spacers (ITS) using specific primers. The second technique was sequence analysis of the ITS regions using the same two primers. Nine different restriction profiles were obtained from the size of the PCR products and the restriction analyses with three endonucleases (*Cfo*I, *Hae*III and *Hin*fI). These groups were identified as *Candida tropicalis*, *Clavispora lusitaniae*, *Hanseniaspora uvarum*, *Pichia anomala*, *Pichia fermentans*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, and *Trichosporon asahii*. Checking against identification according to morphological, physiological and biochemical traits corroborated this molecular identification. A total concordance was found in the identification with PCR-restriction fragment length polymorphism of the ITS region after analysing certified yeast strains from two different culture collections. Consequently, a rapid and reliable identification of the yeast populations was achieved by using molecular techniques.

Keywords: Yeast identification; Restriction fragment length polymorphism-polymerase chain reaction analysis; Sequence analysis; 5.8S internal transcribed spacer region

1. Introduction

Traditionally, yeasts have been identified and classified by morphological and physiological traits [1,2]. These methods are laborious and time-consuming. Moreover, these characteristics are influenced by culture conditions and can provide uncertain results [3]. Simplified biochemical methods have also been developed, based on fermentation and assimilation characteristics [4]. In addition, many methods based on molecular biology have been applied to the identification of yeasts. DNA-based methods have the advantage of being independent of gene expression [5]. These methods include DNA–DNA hybridisation [6], electrophoresis of whole-cell protein [7], electrophoretic karyotyping [8], restriction fragment length polymorphism (RFLP) [9], (mtDNA) restriction analysis [10] and DNA fingerprinting [11]. A disadvantage of these techniques is that they are rather time-consuming. In this direction, methods based on the polymerase chain reaction (PCR) have been shown to be the most appropriate tools for rapid yeast identification. Amplified fragments flanked by repeated sequences [12] and PCR fingerprinting [13] have been demonstrated to discriminate between different yeast species, but only a few species have been investigated. Since 1990, random amplified polymorphic DNA has been used to identify and classify yeast species [14]. Another technique derived from PCR is the study of the nucleotide divergence in the 5′-end of the large subunit

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Abbreviations: ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

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(26S) ribosomal DNA gene (rDNA region D1/D2) [15]. This rDNA region is sufficiently variable to allow reliable identification of yeast species [16]. More recently, the differences in the rRNA internal transcribed spacer (ITS) have been used to identify yeast species [17–19]. The ITS region, including the 5.8S rRNA gene (coding and conserved) and two flanking variable and non-coding regions ITS1 and ITS2, show low intraspecific variability, and high interspecific polymorphism. From the conserved sequences of 18S and 28S rRNA genes at ends of the ITS region two universal primers can be obtained [20]. The subsequent restriction analysis of the amplicons allows the identification of yeasts even though several species may be present simultaneously [21].

The aim of this study was to identify yeast strains present in a spontaneous fermentation of orange juice using molecular methods. The two techniques used, restriction and sequence analysis of the PCR-amplified region between 18S and 28S rRNA genes, were tested for the rapid identification of yeast populations present in the juice.

2. Materials and methods

2.1. Yeast strains

Isolation of the yeasts from spontaneously fermenting oranges and orange juice at three different harvest times produced a total of 100 different isolates. Samples for the isolation and enumeration of yeasts were taken every 24 h during fermentation. Aliquots of 25 μl, 50 μl and 100 μl of juice (before 48 h) and of two dilutions (10^{-5} and 10^{-6}) were spread onto plates of YPD agar (yeast extract 1%, peptone 2%, glucose 2%, agar 2%, pH 6.5; Difco Laboratories, Detroit, MI, USA) supplemented with ampicillin (100 ng μl^{-1}) to avoid bacterial growth. Plates were incubated at 27°C. The colonies selected by colony differentiation were isolated on new plates to be analysed by PCR-RFLP of the ITS region.

2.2. Identification of yeasts

Conventional yeast identification was carried out following the criteria described by Barnett et al. [2] based on their morphological and physiological characteristics. The physiological tests were: ability to ferment glucose, lactose, sucrose, galactose, melibiose, maltose, melezitose, starch, cellobiose, α-D-methyl-glucoside and raffinose; ability to use as sole source of carbon for aerobic growth glucose, galactose, L-arabinose, L-rhamnose, sucrose, melibiose, lactose, raffinose, melezitose, starch, erythritol, mannitol, α-D-methyl-glucoside, trehalose, maltose, cellobiose, D-arabinose, D-ribose, D-xylose, *mio*-inositol, 50% and 60% glucose; ability to use nitrogen compounds; urea hydrolysis test.

2.3. Microbial type cultures

Certified yeast strains of various species for controls were obtained from the Spanish Type Culture Collection (CECT) and German Collection of Microorganisms and Cell Cultures (DSMZ). Strains studied were: *Candida tropicalis* (CECT 1440); *Clavispora lusitaniae* (DSMZ 70102); *Hanseniaspora uvarum* (CECT 10885); *Pichia anomala* (CECT 1114); *Pichia fermentans* (CECT 11773); *Rhodotorula mucilaginosa* (CECT 11010); *Saccharomyces cerevisiae* (CECT 1971) *Saccharomyces unisporus* (CECT 10682); *Trichosporon asahii* (CECT 11852).

2.4. DNA isolation

Pure cultures of each isolate were grown in 10 ml of YPD at 27°C for 12 h at 250 rpm on an orbital shaker. The cells were then collected by centrifugation at $1500 \times g$ for 10 min at room temperature. The cells were resuspended in 10 ml of distilled water and centrifuged as described above. Collected cells were resuspended in 1 ml of extraction buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris–HCl, pH 8, 10 mM EDTA) and transferred to a microcentrifuge tube. After centrifuging at $1500 \times g$ for 10 min the pellet was crushed with a disposable pestle (a conical grinder, exactly fitting the tube). After removing the pestle, 0.2 ml of extraction buffer, 0.2 ml of equilibrated phenol, pH 6, and a pinch of sterile sea sand were added. The conical grinder was rotated by hand until total homogeneity was achieved. Then 0.2 ml of chloroform:isoamyl alcohol (24:1) was added, and centrifuged at $1500 \times g$ for 10 min after mixing by vigorous inversion. NaCl in a final concentration of 0.2 M and 2 volumes of absolute EtOH were added to the supernatant. After leaving the mixture for 1 h at −20°C, it was centrifuged at $12\,000 \times g$ for 10 min at 4°C. The pellet was then washed with EtOH 70%, and left for the EtOH to evaporate. Finally the dry precipitate was resuspended in 20 μl of TE (10 mM Tris–HCl, 1 mM EDTA) or double-distilled water. To verify the quality of DNA extraction, electrophoresis in 0.7% (w/v) agarose mini-gel using TBE (Tris-borate 0.045 M, EDTA 0.001 M, pH 8) buffer was performed. The amount of DNA obtained in this way was approximately 300 ng μl^{-1}. The genomic DNA for PCR was diluted to 5–25 ng μl^{-1}.

2.5. PCR amplification

The amplification reactions of the 5.8S-ITS region were carried out under the following conditions: each 50 μl reaction mixture contained 5–25 ng template DNA; 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.5 μM of each primer; and 1 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, Sweden). The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TC-

CTCCGCTTATTGATATGC-3′) described by [22] were used to amplify this region. Amplification was performed in a Perkin Elmer Thermocycler 2400 programmed as follows: initial denaturation at 94°C for 5 min; 30 s 94°C for denaturing, 30 s at 57°C for annealing, 1 min at 72°C for synthesis, repeated for 35 cycles; and a final extension step of 5 min at 72°C. Amplified samples were kept at −20°C until further use. Aliquots of 10 μl of amplified product were separated electrophoretically in 1.4% (w/v) agarose gels in TBE buffer at 100 V constant voltage for 1 h, stained with ethidium bromide and photographed under transilluminated UV light (Fotodyne).

2.6. Restriction analysis

Aliquots (10 μl) of PCR products were digested without further purification with 1 U of restriction enzyme in 20 μl reaction volume, using the manufacturer's instructions and conditions. The restriction enzymes used were *Cfo*I, *Hae*III and *Hin*fI. RFLP products were analysed by horizontal electrophoresis in 3% (w/v) agarose gels. DNA molecular mass marker (1-kb+ DNA ladder; Life Technologies, Barcelona, Spain) was used as standard. All electrophoreses were carried out with 15×7-cm gels on a Wide Mini-Sub Cell GT unit (15×7-cm tray with 20 wells; Bio-Rad Laboratories, Madrid, Spain) at 70 V for 1 h. After electrophoresis, the gels were stained with ethidium bromide and photographed under transilluminated UV light.

2.7. Automated DNA sequencing and data analysis

Both strands of the ITS1 and ITS2 regions were sequenced after PCR amplification using the ITS1 and ITS4 primers, and each sequence was repeated at least in duplicate. PCR products were purified using a microcolumn (MicroSpin S-400 HR columns, Amersham Pharmacia Biotech, Spain). Sequencing was carried out using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems).

The sequences were edited with Microsoft Word 97 and assembled using the CLUSTALW program [23]. The assembled sequences were aligned and compared with all available sequence databases of DNA through Internet using Basic Local Alignment Search Tool (BLAST) [24]. Manual comparisons of the sequences were realised with CLUSTALW [23]. The percentage similarity among the fragments was calculated with BLAST and SEAVIEW [25]. The restriction analysis of the sequences was made with the program pDRAW32 (Ljeld Olsen/AcaClone Software, a freeware program available in the webpage http://www.crosswinds.net/–acaclone).

3. Results and discussion

One hundred yeast isolates from the initial stages of spontaneous orange juice fermentation were identified according to ITS polymorphisms. The ITS1 and ITS4 primers amplified the region between 18S rRNA and 28S rRNA. The isolates showed different PCR product sizes, ranging from 370 bp to 880 bp (Table 1). The PCR products digested with *Cfo*I, *Hae*III and *Hin*fI enzymes were analysed for all the isolated strains, whereby nine different profiles were obtained. A roman numeral designates each group (Table 1). All restriction fragments were different for each enzyme in each of the strains studied. For comparison, PCR-RFLP of the ITS region was simultaneously applied to certified strains from the CECT and the DSMZ. Additionally, the nine groups were identified using morphological, physiological and biochemical characteristics based on the Barnett keys [2].

Four of the nine groups were identified after comparing the molecular masses of the restriction products with those obtained by the CECT [18]. These four groups were *Saccharomyces cerevisiae* (I), *Candida tropicalis* (II), *Rhodotorula mucilaginosa* (VII) and *Pichia fermentans* (IX). Group VIII showed the same pattern as *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* of the CECT database after using the three enzymes. However, after a new restriction analysis with *Dde*I endonuclease, group VIII was identified as *Hanseniaspora uvarum*. Group III showed the

Table 1
Number of isolates per group, and identification of the nine groups according to the lengths (in bp) of the 5.8S-ITS region amplified by PCR and of the fragments obtained after digestion with three restriction endonucleases

Group	Number of isolates	Species	Amplified product	Restriction fragments		
				*Cfo*I	*Hae*III	*Hin*fI
I	35	*Saccharomyces cerevisiae*	880	385+365	320+230+180+130	360+350+180
II	17	*Candida tropicalis*	510	270+240	430+80	270+270
III	12	*Clavispora lusitaniae*	370	210+180	370	180+160
IV	10	*Trichosporon asahii*	530	270+270	500	240+220+50
V	2	*Saccharomyces unisporus*	750	340+320+80	550+120	400+350
VI	1	*Pichia anomala*	650	630	630	320+270
VII	1	*Rhodotorula mucilaginosa*	600	310+230+80	390+210	220+220+70+50
VIII	17	*Hanseniaspora uvarum*	750	320+310+105	750	350+200+180
IX	5	*Pichia fermentans*	450	170+100+100+80	340+90	250+200

same number and size of fragments as *Yarrowia lipolytica* of the CECT database after digestion with *Cfo*I and *Hae*III, but two bands instead of one appeared when *Hin*fI was used. The remaining groups (IV, V and VI) were not found to correspond with yeasts in the ITS database.

In order to check the identity of the five groups identified by restriction analysis of the ITS region, and identify the other four groups isolated in this study, the PCR products from the nine groups were sequenced. The sequences were compared with available DNA sequence databases using BLAST and CLUSTALW programs. Nine species-specific ITS region DNA sequences have been submitted to GenBank (Table 2). For comparative analysis, the nine sequences were analysed for their predicted restriction patterns with the pDRAW32 program.

The five groups identified by RFLP analysis of the 5.8S rRNA and ITS1 and ITS2 (I, II, VII, VIII and IX) were again identified as *S. cerevisiae* (I), *C. tropicalis* (II), *R. mucilaginosa* (VII), *H. uvarum* (VIII) and *P. fermentans* (IX) after comparing their sequences to the GenBank database (Table 2). The 781-nucleotide sequence corresponding to group I showed a similarity of about 98% with the *S. cerevisiae* 5.8S and ITS DNA sequences found in the GenBank database. However, the discrepancy in group I with the enzyme *Cfo*I, between restriction analysis with only two bands and sequence analysis with four fragments (data not shown), revealed differences between these two techniques. The same sequence analysis with *Cfo*I was carried out for the *S. cerevisiae* 5.8S rRNA gene region from the GenBank database (Table 2). One of the sequences (Z95943) presented four bands with molecular masses similar to ours. The remaining sequences showed only two fragments by ITS sequence analysis. Due to these differences in the sequence it was thought that these isolates could be different strains of the same species. These results are in accordance with those obtained by Esteve-Zarzoso et al. [18], where the *S. cerevisiae* strain CECT 1971 included in the yeast database showed two bands of 385 and 365 bp [18]. However, a recent study with *S. cerevisiae*

Table 2
Length of region 5.8S-ITS sequence of nine groups and homology with GenBank sequences

Group	Species	Sequenced fragment (nt)	Homology (%)	GenBank accession number
I	*Saccharomyces cerevisiae*	781		AF321540 (this study)
I	*Saccharomyces cerevisiae*	756	99	Z95943
I	*Saccharomyces cerevisiae*	777	99	Z95935
I	*Saccharomyces cerevisiae*	773	99	Z95942
I	*Saccharomyces cerevisiae*	780	98	Z95938
I	*Saccharomyces cerevisiae*	780	98	Z95940
I	*Saccharomyces cerevisiae*	773	99	Z95939
I	*Saccharomyces cerevisiae*	753	98	U09327
I	*Saccharomyces cerevisiae*	759	98	Z95929
II	*Candida tropicalis*	523		AF321539 (this study)
II	*Candida tropicalis*	529	98	AF268095
II	*Candida tropicalis*	524	97	L47112
III	*Clavispora lusitaniae*	364		AF321541 (this study)
III	*Clavispora lusitaniae*	323	99	AF172262
III	*Clavispora lusitaniae*	383	97	AF009215
III	*Clavispora lusitaniae*	255	98	AF218970
IV	*Trichosporon asahii*	537		AF322110 (this study)
IV	*Trichosporon asahii*	358	100	AF245218
IV	*Trichosporon asahii*	454	99	AB018014
IV	*Trichosporon asahii*	454	99	AB018013
V	*Saccharomyces unisporus*	680		AF321542 (this study)
V	*Saccharomyces unisporus*	729	99	D89896
V	*Saccharomyces unisporus*	643	99	AJ229065
VI	*Pichia anomala*	605		AF321543 (this study)
VI	*Pichia anomala*	560	97	AF270936
VI	*Pichia anomala*	375	96	AF218991
VII	*Rhodotorula mucilaginosa*	611		AF321544 (this study)
VII	*Rhodotorula mucilaginosa*	702	98	AB026012
VII	*Rhodotorula mucilaginosa*	702	98	AB025998
VII	*Rhodotorula mucilaginosa*	702	98	AB025988
VII	*Rhodotorula mucilaginosa*	702	98	AB025979
VIII	*Hanseniaspora uvarum*	680		AY027507 (this study)
VIII	*Hanseniaspora uvarum*	710	100	AJ271031
IX	*Pichia fermentans*	444		AY027508 (this study)
IX	*Pichia fermentans*	307	80	AF218969
IX	*Pichia fermentans*	307	79	AF218998

strain CECT 1485 presented three bands of 375, 325 and 150 bp [26]. Both these studies were made with the *Cfo*I enzyme. This phenomenon was encountered again when the group of Granchi obtained two bands after restriction analysis with *Cfo*I and calculated four restriction fragments from the available GenBank ITS region sequence [21].

Groups II (*C. tropicalis*) and VII (*R. mucilaginosa*) presented more repetitive and constant patterns than group I. The homology in the results between our study, the yeast database and the sequencing was very high for the three enzymes. The entire sequence of the group II ITS region (523 nt) was aligned with fragments corresponding to the same region in *C. tropicalis* with a similarity of 97%. The differences between *R. mucilaginosa* and *Rhodotorula glutinis* obtained from the yeast database are very small, the fragments produced with *Hae*III were 425+215 nt and 430+210, respectively [18]. However, the group VII sequence (611 nt) had 98% similarity with that of *R. mucilaginosa*. In this case, with RFLP-PCR of the ITS region it was very difficult to identify the isolated strain. The sequencing permitted correct assignation of the species name of the isolate, because it aligned our sequence with that of *R. mucilaginosa* in the GenBank database for 98%, and no significant similarity with the sequence of *R. glutinis* was found (data not shown).

Group VIII (*H. uvarum*) was identified by both restriction and sequencing techniques. The *Dde*I enzyme was necessary for a total identification of the ITS region by RFLP-PCR, because after cutting with the first three enzymes the pattern was the same for *H. uvarum* and *H. guilliermondii*. After using the fourth enzyme the profile of the isolate of group VIII corresponded to that of *H. uvarum*. Moreover, a total similarity (100%) was found between the complete sequence of 5.8S ITS DNA obtained in this work and that of the GenBank database. The same comparison was made between the sequence of this study and the sequence of *H. guilliermondii* from the GenBank database, where 97% similarity was found.

The entire sequence of group IX (*P. fermentans*) with 444 nt showed 77% similarity with that of *Pichia membranaefaciens*, and 80% similarity with that of *P. fermentans*. The restriction fragments of *P. fermentans* and *P. membranaefaciens* are also very similar for the three enzymes used [18]. It is only possible to distinguish between them after comparing the molecular masses of their PCR products (450 and 500 bp, respectively). In this case, although the sequencing did identify the species, it was verified by the RFLP-PCR technique.

Group III was identified as *C. lusitaniae* instead of *Y. lipolytica* after comparing the PCR sequence of 364 nt in the GenBank database. The sequence similarity between the fragment of group III and the *C. lusitaniae* strains present in GenBank was over 97%. The restriction analyses of a strain of *C. lusitaniae* are presented here for the first time. As a result, it is only possible to compare our restriction analysis with the sequence study of this isolate and with those presented in the GenBank (Table 2). In this comparative study the homology in size and number by both techniques was very close. However, the restriction products with *Cfo*I were different. The three 5.8S-ITS region DNA sequences present in GenBank provided non-coincident fragments. The main difference was in the size of the sequences (383, 255 and 323 nt). The sequence with accession number AF009215 [27] showed the same two fragments as our isolate. This sequence was the most similar in length to our sequence, while the other two sequences were shorter. The number of bands present in the two shorter products with *Cfo*I was three rather than two. These slight differences in the sequences indicate the intraspecific variability in this species. This phenomenon has also been found in several strains belonging to the species *C. lipolytica* [16].

Groups IV, V and VI, whose profiles after restriction analysis of the ITS region were not found in the yeast ITS database presented by [18], showed very high similarity in their sequences with *T. asahii*, *S. unisporus* and *P. anomala*, respectively (Table 2). The group IV (537 nt) sequence aligned with that of *T. asahii* with 99% similarity. The *Trichosporon* genus has recently been reclassified according to the polymorphisms in the ITS region sequence of its species [28], and a large number of these sequences are included in the GenBank database. Of the total of 19 species included in this genus [29], the sequence presented in our study had over 98% homology with six other species of this genus apart of *T. asahii* (data not shown). These sequences have been reported in two different works [27,30]. Recent studies based on sequencing of the D1/D2 region of the large subunit of rDNA have again shown the great homology among these seven species belonging to the genus *Trichosporon* [31]. For this reason, the strain isolated in this work was identified based on morphological and physiological traits, and was found to be *T. asahii*. Additionally, the restriction profile of the isolate after digestion with the three enzymes was corroborated by comparison with the certified strain *T. asahii* CECT 11852. This is the first time that the ITS region sequence of a yeast isolated from natural juice and corresponding to this genus is presented.

The *P. anomala* group (VI) exhibited great similarities between the restriction analysis products and in the sequence analysis. The sequence fragment was longer (605 nt) than that previously presented [32] (560 nt; accession number AF270936), although the same two primers were used. A different restriction pattern was shown for the three enzymes when our results were compared with those obtained elsewhere [18]. The isolate was, therefore, not identified as *P. anomala*. However, the band patterns of the isolate were totally coincident with those of the *P. anomala* strain obtained by others [21] using the same three enzymes. This variability in the results shows the intraspecific heterogeneity in *P. anomala*, and may prove

to be a problem in the construction of the yeast database.

The *S. unisporus* (V) restriction profile was totally coincident with both techniques used, and with the two *S. unisporus* ITS sequences presented in GenBank. Size and number of the fragments were the same as those obtained in a recent work, where the ITS region of the genus *Saccharomyces*, including *S. unisporus*, was studied [26]. Moreover, analysis with two more restriction enzymes realised in the aforementioned study yielded profiles identical with ours (data not shown).

The composition and evolution of yeast populations during the early fermentation phases of natural juices have been examined before, grape juice being the most widely studied. In wine fermentations several common strains have been found, such as *S. cerevisiae*, *Candida stellata*, *H. uvarum* (or *Kloeckera apiculata*), *Rhodotorula* sp., *P. anomala* and *C. tropicalis* [17,21,33]. In orange juice fermentation similar yeast species have been presented, such as *S. cerevisiae*, *H. uvarum*, *C. tropicalis*, *R. mucilaginosa*, *P. anomala* and *P. fermentans*. In the present work, however, *C. lusitaniae*, *S. unisporus* and *T. asahii* were isolated from the fermentation of a natural juice for the first time. The presence of these three strains had been reported before in clinical samples; besides, orange peel had been indicated as a source of *Trichosporon* sp., and citrus fruit as source of *C. lusitaniae* [2].

The combined use of these two molecular tools, restriction and sequence analysis of the amplified ITS region of the rRNA genes, as a means of yeast identification was demonstrated. Positive results using these techniques separately had already been demonstrated [18,21,26,27], but they were always preceded or accompanied by classical identification. In this study, it was possible to identify all the yeast isolates from spontaneous fermentation by molecular analysis only. It would be interesting to extend the initial yeast database [18] by including the restriction analysis of the ITS region, under the same experimental conditions, as it is a fast, reproducible and cheap technique. In this study we include two new restriction patterns for *T. asahii* and *C. lusitaniae*. For the present, as an alternative to conventional methodology and until the yeast database by restriction analysis is completed, we would recommend either the exclusive use of sequence analysis of the ITS region, or this technique in combination with restriction analysis, as the best means of reliably identifying new yeast strains.

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