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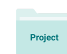

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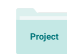

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RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts

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Abstract

The PCR amplification and subsequent restriction analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was applied to the identification of yeasts belonging to the genus *Saccharomyces*. This methodology has previously been used for the identification of some species of this genus, but in the present work, this application was extended to the identification of new accepted *Saccharomyces* species (*S. kunashirensis*, *S. martiniae*, *S. rosinii*, *S. spencerorum*, and *S. transvaalensis*), as well as to the differentiation of an interesting group of *Saccharomyces cerevisiae* strains, known as flor yeasts, which are responsible for ageing sherry wine. Among the species of the *Saccharomyces sensu lato* complex, the high diversity observed, either in the length of the amplified region (ranged between 700 and 875 bp) or in their restriction patterns allows the unequivocal identification of these species. With respect to the four sibling species of the *Saccharomyces sensu stricto* complex, only two of them, *S. bayanus* and *S. pastorianus*, cannot be differentiated according to their restriction patterns, which is in accordance with the hybrid origin (*S. bayanus* × *S. cerevisiae*) of *S. pastorianus*. The flor *S. cerevisiae* strains exhibited restriction patterns different from those typical of the species *S. cerevisiae*. These differences can easily be used to differentiate this interesting group of strains. We demonstrate that the specific patterns exhibited by flor yeasts are due to the presence of a 24-bp deletion located in the ITS1 region and that this could have originated as a consequence of a slipped-strand mispairing during replication or be due to an unequal crossing-over. A subsequent restriction analysis of this region from more than 150 flor strains indicated that this deletion is fixed in flor yeast populations.

Abbreviations: ITS – Internal Transcribed Spacer; 5.8S-ITS – 5.8S rRNA gene and internal transcribed spacers1 and 2; mtDNA – mitochondrial DNA

Introduction

Molecular genetic methods have started to have a major impact on yeast identification and characterisation compared to traditional methods based on phenotypic characteristics. Among these molecular methods, those based on the analysis of restriction fragment length polymorphism of the DNA that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) and the non-coding ITS (internal transcribed spacers) and IGS (intergenic spacer) regions appear to be useful for the detection of many yeast and fungal species (e.g., Hopfer et al. 1993; Molina et al. 1993; Redecker et al. 1997; Wyder & Puhan 1997). The interest in these molecules for species identification comes from the concerted fashion in which they evolve showing a low

intraspecific polymorphism and a high interspecific variability (Li 1997).

Esteve-Zarzoso et al. (1999) successfully applied restriction analysis of the rDNA region spanning the 5.8S rRNA gene and flanking internal transcribed spacers 1 and 2 (from now 5.8S-ITS region) to differentiate 132 yeast species belonging to 25 different genera. The 5.8S-ITS region exhibits far greater interspecific differences than the 18S and 26S rRNA genes (Cai et al. 1996; James et al. 1996; Kurtzman 1992, 1993) often allowing the differentiation of closely related species. In addition, its smaller size compared to the 18S and 26S rRNA genes simplify the methodology. The interest in this technique is evident for the identification of species of biotechnological interest because of its relative ease of manipulation and the high reproducibility. This is the case with the genus *Saccharomyces* which includes species used in the food industry (Rosini et al. 1982; Vaughan-Martini & Martini 1989) and others of medical importance (Clemons et al. 1994; McCusker et al. 1994).

The genus *Saccharomyces* has undergone innumerable taxonomic changes over the years. At present, 16 species of the genus are accepted according to the last taxonomic reviews (James et al. 1997; Vaughan-Martini & Martini 1998). These species are currently classified into three groups established by van der Walt (1970): *Saccharomyces sensu stricto*, formed by the species *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*; *Saccharomyces sensu lato*, including the species *S. barnettii*, *S. castellii*, *S. dairenensis*, *S. exiguus*, *S. kunashirensis*, *S. martiniae*, *S. rosinii*, *S. servazzii*, *S. spencerorum*, *S. transvaalensis* and *S. unisporus* and a third group formed by *Saccharomyces kluyveri*.

Restriction polymorphisms in the 5.8S-ITS region have previously been applied to the identification of the species forming the *Saccharomyces sensu stricto* group by Huffman et al. (1992) and Molina et al. (1992). In recent work, McCullough et al. (1998) have used this method, for medical purposes, for the differentiation of the 10 genotypically distinct *Saccharomyces* species accepted by Naumov et al. (1993) and three clinical isolates (*Candida glabatra*, *Candida albicans* and *Blastomyces dermatitidis*). Their results are in accordance with the previous investigations carried out by Messner & Prillinger (1995) for *Saccharomyces* species assignment using the entire ribosomal transcription unit.

In the present paper, we have extended the RFLP analysis of the 5.8S-ITS region to the newly accepted *Saccharomyces* species (*S. kunashirensis, S. martiniae, S. rosinii*, *S. spencerorum*, and *S. transvaalensis*) studying the utility of this technique as a fast method for the identification of all the species included within the genus *Saccharomyces*. An additional purpose of the present study was to assess whether this method could be used to differentiate a group of *Saccharomyces* yeasts known as flor yeasts, since they form a film ('flor') responsible for sherry wine ageing (Martínez et al. 1997). The *Saccharomyces* flor yeasts were originally considered as different species (*S. beticus*, *S. cheresiensis* and *S. montuliensis*), but later incorporated as synonyms within the taxon *S. cerevisiae* (Kreger-van Rij 1984; Barnett et al. 1990, 1992; Vaughan-Martini & Martini 1998). These yeasts exhibit some particular metabolic capabilities that allow them to survive under extreme conditions (alcohol content over 15%, level of sugar negligible) compared to other *S. cerevisiae* wine yeasts. Whether these properties are the reflection of genetic differences at the molecular level has been very poorly studied. Martínez et al. (1995) attempted the classification of different flor yeasts directly isolated from sherry wines according to their chromosomal pattern and mitochondrial DNA polymorphism and observed that flor yeasts show a higher degree of polymorphism in their mtDNA but a lower variation in their nuclear genome compared to other wine *S. cerevisiae* strains. These differences found at the molecular level indicated the possibility of reconsidering the classification of these yeasts as *S. cerevisiae*.

Materials and methods

Yeast strains

A total of 29 strains belonging to the genus *Saccharomyces* were analysed. Strain designations and their isolation sources are listed in Table 1.

PCR reaction, digestion and sequencing

DNA was isolated according to Querol et al. (1992) and diluted to 1–50 ng/μl. The 5.8S-ITS region was amplified in a Progene thermocycler (Techne, Cambridge, UK). Primer pairs used to amplify the ITS region its1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and its4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were described elsewhere (White et al. 1990). Amplification reactions containing 8 μl of the DNA diluted

Table 1. Saccharomyces strains analysed in the present study

Species	Designation		Isolation source
	CECT	CBS	
Saccharomyces sensu stricto			
S. bayanus	1369		Wine
	$1969^{T a}$	395^{T}	Juice of *Ribes nigrum*
S. cerevisiae	1485		Wine
	1883		Wine
	1942^{NT}	1171^{NT}	Beer (top yeast)
S. paradoxus	1939^{NT}	432^{NT}	Tree exudate
	11143	829	Soil
S. pastorianus	1940^{NT}	1538^{NT}	Beer
	1970	1503	Beer (bottom yeast)
Saccharomyces flor yeasts			
	11756		Winery
	11757		Winery
	11758		Winery
	11759		Winery
	11760		Winery
	11761		Winery
	11762		Winery
	11763		Winery
	11764		Winery
Saccharomyces sensu lato			
S. castellii	11356^{T}	4309^{T}	Soil
S. dairenensis	11345^{T}		Dry fruit of *Diospyros* sp.
S. exiguus	1119^{NT}	379^{NT}	Unknown
S. kunashirensis	11346^{T}	7662^{T}	Soil
S. martinae	11351^{T}	6334^{T}	Fermenting mushroom
S. rosinii	11357^{T}	7127^{T}	Soil
S. servazzii	11353^{T}	4311^{T}	Soil
S. spencerorum	11347^{T}	3019^{T}	Soil
S. transvaalensis	11354^{T}	2186^{T}	Soil
S. unisporus	10682^{T}	398^{T}	Unknown
Saccharomyces kluyveri	11039^{T}	3082^{T}	*Drosophila pinicola*

[a] Type of *Saccharomyces uvarum* Beijerinck.

to 1-50 ng/μl, 0.5 μM of each primer, 80 μM deoxynucleotides, 1$\times$ buffer and 1 U of DyNAzyme^{TM} II DNA Polymerase (Finnzymes OY, Espoo, Finland) in 100 μl of final volume. The thermal cycling parameters were an initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

The amplified DNAs (10 μl or 0.5-10 μg) were digested without further purification with the following restriction endonucleases: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hind*III, *Hin*fI, *Hpa*II, *Nde*II, *Scr*FI (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instructions. PCR products and their restriction fragments were separated on 1.4% and 3% agarose gels, respectively, with 1$\times$ TAE buffer. After electrophoresis, gels were stained with ethidium bromide and visualised under UV light. A 100-bp DNA ladder marker (Gibco BRL, Gaithersburg, MD) served as the size standard.

For sequencing, PCR product was cleaned with the UltraClean^{TM} PCR Clean-up Kit (Mo Bio Laboratories, Inc., Solana Beach, CA). The purified DNA was directly sequenced by using the Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT), following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer model 310 (ABI PRISM^{TM} 310 Genetic Analyzer, Perkin-Elmer, Norwalk, CT). To sequence both strands, four primers were used: its1 and its4 (as described above), and its 2i (5′-GCT GCG TTC TTC ATC GAT GC-3′) and its 3i (5′-GCA TCG ATG AAG AAC GCA GC-3′).

Phylogenetic inference

For phylogenetic analysis we used sequences from the *Saccharomyces* flor strain CECT11757 (EMBL accession AJ275936), obtained by us, and from other *Saccharomyces sensu stricto* strains available in the EMBL nucleotide sequence database (most of them described in Montrocher et al., 1998; Goddard & Burt 1999). The accession numbers of the strains are indicated between parentheses: *S. bayanus* CBS380^{T} (Z95945), CBS395 (Z95946), CBS424 (Z95947), CBS425 (Z95944), CBS1546 (Z95948), NCYC509 (AJ224320-AJ224321), NCYC686 (AJ224322-AJ224323); *S. cerevisiae* CBS382 (Z95936), CBS400 (Z95939), CBS423 (Z95932), CBS459 (Z95938), CBS1171^{T} (AJ229057), CBS1782 (Z95935), CBS2247 (Z95937), CBS3081 (Z95941), CBS3093 (Z95943), CBS4054 (Z95931), CBS4903 (Z95940), CBS5155 (Z95934), CBS5378 (Z95929), CBS5635 (Z95942), CBS6006 (Z95951), HA6 (U09327), and NCYC361 (Z75721-Z75722); *S. paradoxus* CBS432^{T} (AJ229059, Z75729-Z75730, and Z95933) and *S. pastorianus* CBS1513 (Z95950), CBS1538^{T} (AJ229060 and Z95949), NCYC392 (Z75731-Z75732).

Sequences from the 5.8S-ITS region were aligned using the CLUSTAL X program (a Windows version of the CLUSTAL W program, Thompson et al. 1994). For phylogenetic analysis, we used the maximum-parsimony (MP) method (Fitch 1971). The MP tree was obtained with the program DNAPARS from the PHYLIP package (Felsenstein 1993).

Results

Primers its1 and its4 were used to amplify the region corresponding to the 5.8S rDNA gene and the two ITS regions of 20 strains belonging to 15 of the 16 *Saccharomyces* species accepted within the genus *Saccharomyces* in the most recent yeast taxonomic studies (James et al., 1997; Vaughan-Martini & Martini, 1998). All the *Saccharomyces* species are currently classified into the three groups mentioned earlier, namely *Saccharomyces sensu stricto*, *Saccharomyces sensu lato* and *Saccharomyces kluyveri* (van der Walt 1970). The species *Saccharomyces barnettii*, established as a new taxon within the *Saccharomyces sensu lato* group by Vaughan-Martini (1995), is the only species not included in this study because it was not available at the time this work was carried out. Nine flor yeast strains originally classified as *S. beticus*, *S. cheresiensis* and *S. montuliensis*, now considered to be either synonyms or races of *S. cerevisiae*, were also included in this study.

The 5.8S-ITS region amplified by PCR showed a product of approximately 850 bp for all the strains of the species of the *Saccharomyces sensu stricto* group (*S. bayanus*, *S. cerevisiae, S. pastorianus* and *S. paradoxus*) including the flor yeast strains. The 5.8S-ITS region from species belonging to the *Saccharomyces sensu lato* group ranged between 700 and 875 bp. Only two species, *S. kunashirensis* and *S. transvaalensis*, could clearly be identify by the approximate length of the PCR products because they corresponded to the shortest (700 bp) and largest (875 bp) 5.8S-ITS regions, respectively.

PCR products of the 29 strains were digested with the nine restriction endonucleases mentioned earlier. The approximate length of the amplified products and the restriction fragments observed after digestion with the restriction these endonucleases are summarised in Table 2.

Within the *Saccharomyces sensu lato* group an evident diversity was observed either in the size of the PCR amplification products or in the restriction patterns yielded by digestion with several of the enzymes assayed. Among the restriction enzymes tested *Hae*III, *Hpa*II, *Hin*dIII and *Dde*I only digested the PCR products of certain species. Not all these enzymes were useful for identification within the *Saccharomyces sensu lato* group. The remaining endonucleases yielded different restriction patterns for all the species assessed. However, only *Alu*I, *Cfo*I and *Scr*FI were able to discriminate among all the *Saccharomyces sensu lato* species since *Hin*fI and *Nde*II failed to distinguish three species, *S. martiniae*, *S. servazzii* and *S. unisporus*, which, in fact, exhibited the same PCR product length (775 bp) and are phylogenetic-

Table 2. Lengths (in bp) of the 5.8S-ITS region from *Saccharomyces* strains amplified by PCR and of the fragments obtained after digestion with nine restriction endonucleases

Species	CECT	5.8S-	Restriction fragments								
	Strain references	ITS	*Alu*I	*Cfo*I	*Dde*I	*Hae*III	*Hind*III	*Hinf*I	*Hpa*II	*Nde*II	*Scr*FI
Saccharomyces sensu stricto											
S. bayanus	1369, 1969^{T}	850	775+75	375+325+150	725+125	495+230+125	850	375+365+110	725+125	455+395	400+320+120
S. cerevisiae	1485, 1883, 1942^{NT}	850	775+75	375+325+150	725+125	325+230+170+125	850	375+365+110	725+125	455+395	400+320+120
S. paradoxus	1939^{NT}, 11143	850	775+75	375+325+150	725+125	325+230+170+125	850	375+365+110	850	455+395	520+320
S. pastorianus	1940^{NT}, 1970	850	775+75	375+325+150	725+125	495+230+125	850	375+365+110	725+125	455+395	400+320+120
Saccharomyces flor yeasts											
	11756, 11757, 11758, 11759, 11760, 11761, 11762, 11763	850	765+75	345+345+150	715+125	325+230+155+125	850	375+365+110	715+125	465+375	380+330+120
	11764	850	775+75	375+325+150	725+125	325+230+170+125	850	375+365+110	725+125	455+395	400+320+120
Saccharomyces sensu lato											
S. castellii	11356^{T}	800	575+225	350+325+100	800	800	800	345+260+130	550+130+120	450+310	350+225+125
S. dairenensis	11345^{T}	800	650+110	330+330+125	600+200	800	700+100	350+345+90	500+200	350+270+180	250+220+180+150
S. exiguus	11192^{NT}	750	525+225	375+300	450+280	500+250	750	350+250+150	600+150	500+210	390+360
S. kunashirensis	11346^{T}	700	425+100+90	300+320	700	500+200	700	350+350	700	430+270	390+310
S. martiniae	11351^{T}	775	700+75	310+225+125+100	775	775	775	400+375	775	450+300	440+335
S. rosinii	11357^{T}	750	600	300+225+100	500+130+120	500+250	750	395+355	750	440+290	430+310
S. servazzii	11353^{T}	775	400+250+125	310+200+175+90	650+125	335+250+190	775	400+375	775	450+300	335+200+180
S. spencerorum	11347^{T}	750	375+225+125	310+310	675	450+200+100	575+175	410+340	725	425+325	440+300
S. transvaalensis	11354^{T}	875	390+270+125+90	415+310	750+125	525+350	875	400+350+125	875	575+250	440+390
S. unisporus	10682^{T}	775	310+250+110	350+310+115	425+225+125	500+110	775	400+375	775	450+300	425+325
Saccharomyces kluyveri											
S. kluyveri	11039^{T}	700	700	320+210+90+80	510+190	400+210+90	700	350+230+120	650	425+270	410+160+90

ally closely related (James et al. 1996; Kurtzman & Robnett 1998).

The species *S. kluyveri* showed a PCR amplification fragment of 700 bp indistinguishable from the PCR fragment obtained for the species *S. kunashirensis*. With the exception of *Hin*dIII, which only cuts the 5.8S-ITS region from *S. dairenensis* and *S. spencerorum*, the use of any of the other eight enzymes, allows differentiation of *S. kluyveri* from *S. kunashirensis* and, of course, from the remaining *Saccharomyces sensu lato* species.

With respect to the *Saccharomyces sensu stricto* group, the restriction enzyme *Hae*III yielded restriction patterns that separated the four sibling species into two groups formed by the pairs *S. bayanus*/*S. pastorianus* and *S. cerevisiae*/*S. paradoxus*. Only *Scr*FI and *Hpa*II distinguished *S. paradoxus* from the other three sibling species. Two *Scr*FI sites are present within the 5.8S-ITS region of the strains of *S. bayanus*, *S. cerevisiae* and *S. pastorianus*, but only one is present within this region from *S. paradoxus* strains. In the case of the enzyme *Hpa*II, no restriction site is recognised within the 5.8S-ITS region of *S. paradoxus* strains but one is present within this region from the other *Saccharomyces sensu stricto* species. When the PCR products were digested with *Alu*I, *Cfo*I, *Dde*I, *Hin*fI and *Nde*II the four species exhibited the same patterns. The endonuclease *Hin*dIII does not cut the 5.8S-ITS region from these species.

Within the species *S. cerevisiae*, restriction fragments were different for the strains isolated from the film formed during the sherry wine ageing (flor yeast strains) since the restriction analysis of the 5.8S-ITS rDNA region allowed their differentiation from type and reference strains of the four sibling species (*S. bayanus, S. cerevisiae*, *S. paradoxus* and *S. pastorianus*). The use of one of the following enzymes: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hpa*II, *Nde*II or *Scr*FI, allows differentiation of a pattern specific for this group of flor yeasts. In all cases, the lengths of the 5.8S-ITS restriction fragments from the flor yeasts are slightly different than those from the other strains of *S. cerevisiae* and sibling species of the *sensu stricto* group. These length differences were observed after restriction analysis and subsequent electrophoresis, but were negligible in the conditions used to separate undigested PCR products. The exception was strain CECT11764, which exhibited the same restriction patterns as the non-flor *S. cerevisiae* strains after digestion with the seven restriction endonucleases indicated above, that differentiate both groups of *S. cerevisiae* strains. These results suggest that this strain should not be considered as a flor *S. cerevisiae*, a result in line with physiological tests (lactose, galactose, maltose, raffinose, sucrose, dextrose, melibiose fermentation) carried out according to Martínez et al. (1995).

The entire sequence of the 5.8S-ITS region was obtained for one of the *S. cerevisiae* flor strains (CECT11757), and compared with sequences available in databases for other *Saccharomyces sensu stricto* strains. The alignment of the sequences confirmed the length difference of the 5.8S-ITS region. This difference is due to a 24-bp deletion between nucleotides 131 and 154 in the ITS1 of the flor strain CECT11757. The sequence alignment was used to construct an unrooted MP tree (Figure 1). This tree minimises the number of nucleotide substitutions required to connect the different 5.8S-ITS sequences. Insertions or deletions of a few nucleotides are not considered in the tree, because some of them could be due to sequencing errors. As an example, there are several nucleotide sequences of this region from the same strain obtained by different authors that differ in several insertions or deletions of single nucleotides or, even, in a few nucleotide substitutions. As examples, the 5.8S-ITS region from the type strain of *S. paradoxus* CBS432 has been sequenced by three different authors (accession numbers AJ229059, Z75729-Z75730, and Z95933) and their sequences show one nucleotide difference and three single-nucleotide deletions. The same region from the type strain of *S. bayanus* (CBS380), has been sequenced three times (AJ229058, Z75717-Z75718, and Z95945) and the sequences show 4 single-nucleotide deletion differences. Strain CBS1538, the neotype strain of *S. pastorianus*, has been sequenced twice (AJ229060 and Z95949) and the sequences show 1 nucleotide and 3 single-nucleotide deletion differences. To avoid this problem, only those insertions/deletions longer than 20 bp were considered as single mutational steps in the tree.

The sequence analysis clearly showed two major clusters: *S. bayanus*/*S pastorianus* and *S. cerevisiae*. Within the first cluster, the 5.8S-ITS sequences of the species *S. bayanus* and *S. pastorianus* appear to be highly similar with few or no nucleotide differences, as was indicated in the original study by Montrocher et al. (1998). This result is congruent with those obtained from the RFLP analysis of this region, both species exhibited the same restriction patterns with the nine endonucleases tested. The most interesting result regarding the sequence analysis is that flor *S. cerevisiae* CECT11757 (this study) and

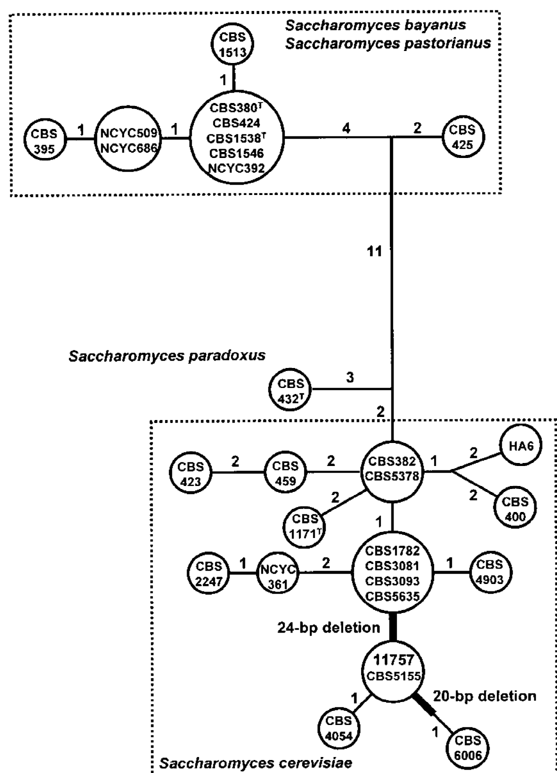

Figure 1 Unrooted maximum parsimony tree for the 5.8S-ITS region sequences from 29 *Saccharomyces sensu stricto* strains. The sequence from strain CECT11757 was obtained in the present study, the other sequences were available in the EMBL nucleotide database and their accession numbers are given in the main text. The numbers along the branches represent the estimated minimum number of nucleotide substitutions required to connect the different sequences. Only two deletions longer than 20 bp have been considered as single mutational steps to obtain the tree. The branches where these deletion events occurred are indicated by thicker lines.

three other strains, *Saccharomyces aceti* CBS4054, *Saccharomyces gaditensis* CBS6006 and *Saccharomyces prostoserdovii* CBS5155 (Montrocher et al. 1998) shared the same 24-bp deletion responsible of the restriction patterns characteristic of the flor yeast strains.

A subsequent restriction analysis of the 5.8S-ITS region from more than 150 *Saccharomyces* flor yeast strains isolated from the yeast film growing on the surface of sherry wines from wineries located in Jerez, Spain, showed that 100% of the flor yeasts have the restriction pattern using the endonuclease *Cfo*I described in the present study, indicating the presence of the 24-bp deletion in all of them (data not shown).

Discussion

The RFLP analysis of the 5.8S RNA gene and the two ribosomal internal transcribed spacers has been shown to be a fast and simple method for species identification (Esteve-Zarzoso et al. 1999). In the present study, we have applied this method to the differentiation of 15 species belonging to the genus *Saccharomyces*. In addition, this method has proved to be a simple technique for differentiating *Saccharomyces* flor strains from other *S. cerevisiae* wine yeasts that do not survive under high conditions of alcohol content.

The restriction analysis of the 5.8S-ITS region has been applied to *Saccharomyces* species in previous studies (Huffman et al. 1992; Molina et al. 1992; Valente et al. 1996; Guillamón et al. 1998; Esteve-Zarzoso et al. 1999). However, identification studies undertaken with *Saccharomyces sensu lato* species are limited and is complete since new species have recently been established within this group (Vaughan-Martini & Martini 1998). Sequence analyses of the 5.8S-ITS region (Oda et al. 1999), as well as of the 18S and 28S rRNA gene regions (James et al. 1996; Kurtzman & Robnett 1998) of *Saccharomyces sensu lato* species, have shown that this group of species is not monophyletic and is formed by closely related species as with the *Saccharomyces sensu stricto* species. This fact is reflected in the length polymorphism of the 5.8S-ITS region PCR product as well in the restriction patterns obtained for these species. In fact, the size of the PCR amplification products combined with the restriction analysis of the 5.8S-ITS region using endonucleases *Alu*I, *Cfo*I or *Scr*FI can be used as a rapid method to identify the species included in the *sensu lato* group.

However, there is a correspondence between the length of the 5.8S-ITS region and the phylogenetic relationships among *Saccharomyces sensu lato* species (James et al. 1997; Kurtzman & Robnett 1998). Thus, *sensu lato* species can be divided into five groups according to PCR product length: a 5.8S-ITS region of 700 bp for *S. kunashirensis*; 750 bp for the species *S. exiguus*, *S. rosinii* and *S. spencerorum*; 775 bp for the closely related species *S. martiniae*, *S. servazzii* and *S. unisporus*; 800 bp for the related species pair *S. castellii* and *S. dairenensis*; and 875 bp for *S. transvaalensis.*

S. kluyveri has been reported to have ecological, physiological and genetic differences which distinguish it from the other *Saccharomyces* species (Vaughan-Martini & Martini 1993; Vaughan-Martini

et al. 1993). In the present study, *S. kluyveri* exhibited a PCR amplification product of the same length (700 bp) as *S. kunashirensis*. However, restriction patterns specific for *S. kluyveri* were obtained with most of the endonucleases.

The majority of the studies based on RFLP analysis of the 5.8S-ITS region have been focused, within the genus *Saccharomyces*, on the species of the *sensu stricto* group (Huffman et al. 1992; Molina et al. 1992; Montrocher et al. 1998). The 5.8S-ITS amplification products from species of the *Saccharomyces sensu stricto* complex are larger than those from the species in the other groups, with the exception of *S. transvaalensis*. The lengths of the PCR products are within the same range as those reported in previous studies (Huffman et al. 1992; Molina et al. 1992; Valente et al. 1996; McCullough et al. 1998; Oda et al., 1999).

Our analysis of the restriction polymorphisms in the amplified 5.8S-ITS region support the evidence that the species *S. bayanus* and *S. pastorianus* are a very closely related pair. None of the restriction patterns yielded by the nine enzymes assayed allowed differentiation between strains of *S. bayanus* and *S. pastorianus*. These results are consistent with the hybrid origin (*S. bayanus* × *S. cerevisiae*) of *S. pastorianus* (Vaughan-Martini & Martini 1987; Tamai et al. 1998, Yamagishi & Ogata 1999).

Molina et al. (1992) reported that RFLP analysis of the 5.8S-ITS region provided adequate resolution to distinguish *S. pastorianus* from *S. bayanus* by using the restriction enzyme *Scr*FI. We cannot support this finding since the restriction pattern obtained with *Scr*FI for the strains of both species in this study are identical. RAPD analysis has shown that these two *S. pastorianus* strains (CECT1940, CECT1970) are different from the two *S. bayanus* strains (CECT1369, CECT1969) and are clustered within the *S. pastorianus* and the *S. bayanus* groups, respectively (Molnár et al. 1995a, b, Fernández-Espinar et al. in preparation). Surprisingly, the restriction patterns obtained for the two strains of *S. paradoxus* with *Scr*FI (two bands of approximately 520 and 320 bp) seem to be identical to that reported by Molina et al. (1992) for the *S. pastorianus* strain CBS1513 which was probably a misidentified strain. Therefore, we propose the use of *Scr*FI for identification of *S. paradoxus* strains and their differentiation from *S. cerevisiae* and the two indistinguishable species *S. bayanus* and *S. pastorianus*. In addition, *Hpa*II can also be used to differentiate *S. paradoxus* from the other three sibling species of the *sensu stricto* group.

Studies of other regions of the repeated ribosomal unit have been conducted to distinguish species within the *Saccharomyces sensu stricto* complex. These studies also failed to differentiate between *S. bayanus*-*S. pastorianus* (Molina et al. 1992, 1993; Messner & Prillinger 1995; 1993; Smole Mozina et al. 1997), at least with the wide range of restriction endonucleases tested. However, it seems obvious that other molecular markers should be used to decipher relationships between these two closely related species, such as RAPDs (Molnár et al. 1995a, b; Fernández-Espinar et al. in preparation) and PCR-fingerprinting based in the intron splice sites (de Barros Lopes et al. 1998).

The *S. cerevisiae* strains analysed by RFLP analysis of the 5.8S-ITS region can be divided into two groups according to their restriction patterns. The first group includes the neotype strain of *S. cerevisiae* (CECT1942^{NT}) and wine strains (CECT1485, CECT1883) and the second is formed by the strains isolated from the film of sherry wines (formerly considered as *S. beticus*, *S. cheresiensis* or *S. montuliensis*). The *Saccharomyces* strains responsible for sherry wine ageing constitute a special group of wine yeasts poorly studied at the molecular level. Only one attempt has been made to characterise these yeast strains using molecular techniques (Martínez et al. 1995). These authors characterised several *Saccharomyces* strains isolated from the surface of sherry wine using two molecular methods: mitochondrial DNA restriction analysis and electrophoretic karyotyping. They were able to distinguish between different flor yeast strains according to their rich mtDNA restriction polymorphism. However, the high level of polymorphism of mtDNA did not allow them to describe a specific pattern for flor strains and, as a consequence, this technique was only useful for studies of population dynamics of specific flor yeast strains (Ibeas et al. 1997; Martínez et al. 1997). On the contrary, according to electrophoretic karyotyping, flor strains showed a specific pattern different from other *S. cerevisiae* strains isolated from fermenting wines and other sources. Although electrophoretic karyotyping proved to be a good technique for differentiating flor strains, it is expensive and time-consuming.

The analysis of 5.8S-ITS region sequences has highlighted a 24-bp deletion in the ITS1 region from flor strain CECT11757 that explains why the restriction patterns of this region can be used as a simple method for the differentiation of *Saccharomyces* flor yeasts from other *S. cerevisiae* strains. This same deletion is present in the sequences of three other *Sacchar-*

omyces strains obtained by Montrocher et al. (1998): *S. aceti* CBS4054, *S. gaditensis* CBS6006 and *S. prostoserdovii* CBS5155. Interestingly, *S. aceti* and *S. gaditensis* were isolated from 'flor' of sherry wines in Spain (Santa María 1959, 1970) and *S. prostoserdovii* is usually isolated from 'flor' of Vernaccia di Oristano, a sherry-like wine from Italy (Guido & Carnacini 1996).

The presence of the 24-bp deletion affecting the ITS1 region has been demonstrated from sequences obtained by two different laboratories, and its wide distribution in flor *Saccharomyces* yeasts has been confirmed by restriction analysis of the PCR-amplified 5.8S-ITS region from more than 150 strains isolated from sherry wine vela. Repeatability and specificity support the non-artifactual origin of the deletion.

The deleted region of the *Saccharomyces* ITS1 does not correspond to a stable hairpin loop structure according to the secondary structural model of the ITS1 region of *Saccharomyces cerevisiae* (Yeh & Lee 1991), but the presence of flanking direct repeats of 8 nucleotides (one within and the others outside the deleted region) suggests that the origin of the 24-bp deletion may be due to a slipped-strand mispairing during replication (replication slippage) or to unequal crossing-over. The subsequent expansion across the hundreds of copies of the 5.8S-ITS region within the yeast genome could have occurred by any of the mechanisms proposed to explain the concerted evolution of the ribosomal units (Li 1997).

An interesting question is how this 24-bp deletion became fixed in flor yeast populations. *Saccharomyces* flor yeasts proliferate at the surface of sherry and sherry-like wines which contain over 15% (vol.) ethanol. A possible explanation could be that the 24-bp deletion is linked to a nuclear gene involved in ethanol tolerance. However, Ibeas & Jiménez (1997) demonstrated that the tolerance of flor yeasts to high ethanol concentrations is conferred by their mitochondrial genomes. The most plausible explanation is that positive selection favoured a mitochondrial variant conferred a higher fitness to flor yeasts in these extreme ethanol concentrations. During the colonisation of this new habitat, as flor yeasts are homothallic and exhibit a low percentage of sporulation (Martínez et al., 1995; Guijo et al. 1997), the 24-bp deletion may have become fixed in flor yeasts by hitchhiking with the selected mitochondrial genome.

In conclusion, restriction of 5.8S-ITS region analysis offers a convenient tool for the fast identification of yeast species, especially now that new data about the restriction patterns of type and reference strains of many yeast species are becoming available (Esteve-Zarzoso et al. 1999). In addition, the method allows the rapid and easy identification of *Saccharomyces* flor yeasts considered to be synonyms or physiological races of *S. cerevisiae*.

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