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Strain typing of *Zygosaccharomyces* yeast species using a single molecular method based on polymorphism of the intergenic spacer region (IGS)

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ABSTRACT

Unlike previously reported methods that need a combination of several typing techniques, we have developed a single method for strain typing of the *Zygosaccharomyces bailii*, *Z. mellis* and *Z. rouxii* spoilage species. Strains belonging to other species have also been included for comparison. We have demonstrated that the IGS-PCR RFLP method has a high discriminative power. Considering the three endonucleases used in this work, we have obtained a variability of 100% for *Z. mellis* and *Z. rouxii* strains and up to 70% for *Z.bailii*. We have also detected two misidentified *Z. mellis* strains (CBS 711 and CBS 7412) which have RFLP patterns with a set of bands characteristic of *Z. rouxii* strains. Sequencing of 26S rDNA D1/D2 domains and the 5.8-ITS rDNA region confirmed these strains as *Z. rouxii*. The method also groups three certified hybrid strains of *Zygosaccharomyces* in a separate cluster.

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1. Introduction

It has been shown that yeasts are involved in the spoilage of an extensive range of foods according to their metabolic and physiological capabilities (Stratford, 2006). However, in order to establish contamination sources during food processing and thus avoid economic losses, it is essential not only to identify which species are present but also to discriminate them at strain level.

In terms of spoilage ability some of the most dangerous yeasts are found in the *Zygosaccharomyces* genus. This genus includes osmotolerant, strongly fermentative yeasts that are able to resist weak-acid preservatives such as benzoic and sorbic acids (Casas et al., 2004). These physiological characteristics are responsible for their well-known ability to cause spoilage (Stratford, 2006). Among these yeasts are *Z. mellis*, isolated from honey, syrups and from low a_w products in general (Stratford, 2006), and the *Z. rouxii* and *Z. bailii* species, commonly found in the food and drinks industries. Although the *Z. rouxii* species is involved in production of food such as miso and traditional balsamic vinegar (Solieri and Giudici, 2008), it is also frequently isolated from low a_w spoiled foods like marzipan or nougat (Casas et al., 2004; Martorell et al., 2005). According to the zymological indicators defined by Sancho et al. (2000), they are considered to be some of the most dangerous yeasts for product stability in fruit pulps and concentrates.

In order for some of the *Zygosaccharomyces* species to be strain-typed, several molecular techniques have been studied. Török et al. (1993) proposed an electrophoretic karyotyping, and Esteve-Zarzoso et al. (2003) the RFLP of mtDNA. Martorell et al. (2005) demonstrated that if the objective is to differentiate species belonging to the same genus, the best result is obtained by electrophoretic analysis. If, on the other hand, it is to characterize *Z. bailli* and *Z. rouxii* at strain level, they suggested the combination of RFLP and RAPD analysis. A combination of several typing techniques was therefore required (Maqueda et al., 2010).

The intergenic region (IGS) of rDNA has the advantage that its locus is more variable than other existing loci investigated so far (Sugita et al., 2001). Several studies have exploited this region. Sequence analysis of the IGS region permitted the separation of clinical isolates of *Cryptococcus neoformans* into two varieties (Diaz et al., 2005; Diaz and Fell, 2000; Fan et al., 1995). Strain typing of *Pichia anomala* has also been achieved by analysing the sequence of the intergenic region 1(IGS1) (Bhardway et al., 2007). Other studies have focused on discriminating strains belonging to different yeast species, such as *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* (Fell and Blatt, 1999). The Restriction Fragment Length Polymorphism (RFLP) of the IGS2 region of rDNA made it possible to differentiate between the physiologically similar dairy yeast species *Kluyveromyces marxianus* and *K. lactis* (Naumova et al., 2005). We were previously able to differentiate the *Debaryomyces hansenii* yeast species in foods through PCR-RFLP of the IGS region (rDNA) (Romero et al., 2005). This also allowed us to separate the *Debaryomyces* genus into species and varieties (Quirós et al., 2006).

The aim of this investigation is to evaluate the usefulness of PCR-RFLP analysis of the IGS region of rDNA as a single typing method for

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Table 1
Strains studied, their origin, source of isolation, size of the amplified IGS region and code of the RFLP pattern obtained with *Hap*II, *Hha*I and *Mbo*I endonucleases.

Species and strains studied	Source of isolation	IGS (bp)	Patterns		
Zygosaccharomyces bailii (B)					
CECT 1898[T]	Apple juice	6300	BA1	BB1	BC1
CYC 1226	Culture contaminant	6300	BA1	BB2	BC2
CECT 1924	Unknown	6300	BA1	BB1	BC1
CECT 11042	Grape	6300	BA2	BB3	BC5
CECT 11043	Turbid wine	6300	BA3	BB4	BC3
MAY(12)	Mayonnaise	6300	BA4	BB5	BC4
MAY(13)	Mayonnaise	6300	BA4	BB5	BC4
Zygosaccharomyces bisporus (Bi)					
CECT 11055[T]	Beer	5500	BiA1	BiB1	BiC1
CECT 11348	Beer	5500	BiA2	BiB2	BiC2
Zygosaccharomyces cidrii (C)[a]					
CECT 10657[T]	Cider	2900	CA1	CB1	CC1
CECT 11349	Cider	2900	CA1	CB1	CC1
Zygosaccharomyces fermentati (F)[a]					
CECT 11056[T]	Sediment of pepermint	2900	FA1	FB1	FC1
CECT 10382	Alpechin	2900	FA3	FB2	FC2
CECT 10678	*Drosophila* sp.	2900	FA2	FB2	FC2
Zygosaccharomyces kombuchaensis (K)					
CBS 8849[T]	Kombucha tea	7000	KA1	KB1	KC1
Zygosaccharomyces lentus (L)					
CECT 11040	Swiss wine yeast	6700	LA1	LB1	LC1
CECT 11041	Wine	6700	LA2	LB1	LC1
Zygosaccharomyces mellis (M)					
CECT 11057 [T]	Honey	4200	MA4	MB3	MC3
CBS 684	Honey	4200	MA1	MB1	MC1
CBS 711[b]	Strawberry juice	4200	RA19[a]	RB12	RC24
CBS 735	Fermenting honey	4200	MA5	MB5	MC1
CBS 738	Fermenting honey	4200	MA1	MB4	MC1
CBS 1091	Honey	4200	MA2	MB2	MC2
CBS7277	Alpechin	4200	MA3	MB3	MC3
CBS 7412[b]	Honey	4200	RA20[a]	RB13	RC25
Zygosaccharomyces microellipsiodes (Mi)[a]					
CBS 427 [T]	Apple juice	3000	MiA1	MiB1	MiC1
Zygosaccharomyces rouxii (R)					
CECT 1232 [T]	Grape juice	4200	RA1	RB1	RC1
CECT 1231	Bombon	4200	RA9	RB1	RC8
CECT 10132	Unknown	4200	RA4	RB7	RC16
CECT 10137	Raisin	4200	RA2	RB6	RC14
CECT 10312	Fig cake	4200	RA1	RB7	RC5
CECT 10313	Fig cake	4200	RA1	RB1	RC5
CECT 10350	Dried fig	4200	RA3	RB4	RC7
CECT 10377	Phoenix dactilifera	4200	RA8	RB2	RC3
CECT 10381	Molasses	4200	RA4	RB7	RC15
CECT 10425	Honey	4200	RA7	RB10	RC22
CECT 10427	Honey	4200	RA3	RB9	RC9
CECT 10445	Plum jam	4200	RA10	RB8	RC6
CECT 10633	Honey	4200	RA17	RB7	RC20
CECT 11121	Grape juice	4200	RA2	RB7	RC14
CECT 11136	Grapes	4200	RA6	RB3	RC10
CECT 11189	White wine	4200	RA11	RB7	RC2
CECT 11923	Soy sauce	4200	RA18	RB11	RC23
CECT 11929	Orange and lemon juice	4200	RA14	RB8	RC17
CECT 12003	Cherry	4200	RA4	RB7	RC21
CECT 12004	Cherry	4200	RA12	RB7	RC11
CYC 1484	Unknown	4200	RA3	RB5	RC4
CYC 1486	Honey	4200	RA16	RB5	RC10
CYC 1487	Nougat	4200	RA13	RB7	RC12
CYC 1488	Honey	4200	RA4	RB7	RC9
NCYC 1522	Salty bean	4200	RA5	RB6	RC15
NCYC 1682	Miso	4200	RA21	RB12	RC24
NCYC 3060	Soy sauce	4200	RA21	RB12	RC25
NCYC 3061	Soy sauce	4200	RA22	RB12	RC26
T2R	Nougat fruit	4200	RA4	RB7	RC18
Bch	Chocolate bun	4200	RA5	RB6	RC18

Table 1 (*continued*)

Species and strains studied	Source of isolation	IGS (bp)	Patterns		
Zygosaccharomyces rouxii (R)					
MAY(1)	Liquid sugar	4200	RA15	RB3	RC13
MAY(15)	Liquid sugar	4200	RA15	RB5	RC15
Es 14	Marzipan	4200	RA4	RB6	RC19

The first letter corresponds to the species: *Zygosaccharomyces bailii* (B), *Z. bisporus* (Bi), *Z. cidri* (C), *Z. fermentati* (F), *Z. kombuchaensis* (K), *Z. lentus* (L), *Z. mellis* (M), *Z. microellipsoides* (Mi), *Z. rouxii* (R), followed by the letter corresponding to the endonucleases: *Hap*II (A), *Hha*I (B), *Mbo*I (C) and finally a number corresponding to the pattern. CBS, Centraalbureau voor Schimmelcultures, The Netherlands; CECT, Colección Española de Cultivos Tipo, Spain. The remaining strains were isolated and identified in our laboratory.

[a] *Z. cidri* and *Z. fermentati* are proposed as *Lachancea* species and *Z.microellipsiodes* as *Torulaspora species* (Kurtzman, 2003, FEMS Yeast 24,403–417).

[b] Strains identified in this study as belonging to *Zygosaccharomyces rouxii* species.

rapid discrimination at strain level for the *Z. bailii*, *Z. mellis* and *Z. rouxii* species.

2. Materials and methods

2.1. Strain and culture conditions

Fifty-nine strains belonging to the *Zygosaccharomyces* genus and one strain of *Saccharomyces cerevisiae* were used in this research. They were obtained from different Type Culture Collections or isolated in our laboratory from contaminated or spoiled products. The sources of isolation, obtained from information provided by collections or in our laboratory, are shown in Table 1. The strains were grown in Yeast Morphology Broth at 28 °C and routinely maintained on Yeast Morphology Agar (YMA) containing 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, Mitch, USA), 0.3% (w/v) proteose-peptone No.3 (Difco), 0.3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain), and 2% (w/v) agar.

2.2. DNA isolation, amplification protocols and RFLP analysis

DNA was isolated following the standard protocol of Querol et al. (1992), which includes a first enzymatic step to obtain spheroplasted cells, followed by the isolation and purification of DNA. Alternatively, the DNeasy plant minikit (Qiagen, Hilden, Germany) was used after the spheroplast step. The IGS region of the rDNA was amplified by PCR in a Mastercycler gradient device (Eppendorf, Germany) using CNL12 (*5′*CTGAACGCCTCTAAGTCAG*3′*) and CNS1 (*5′*GAGACAAGCATATGAC-TACTG*3´*) forward and reverse primers respectively (Appel and Gordon, 1995). The region defined by these primers spans from base position 3046–3064 on the 26SrDNA (GenBank Accession no. AY048154) to base position 37-17 on the 18S rDNA (GenBank Accession no. J01353). A set of PCR reactions were carried out in microtubes containing a master mix with a final volume of: (i) 25 μl, containing target DNA (50 ng), 2 mM Mg Cl_2, 1 mM dNTPs (Ecogen, Madrid, Spain), 1 μM of each primer (Sygma–Genosys, Cambridge, UK), 1U *Taq* polymerase (Ecogen) and sterilized distilled water (MO Laboratories, Inc., USA) up to final volume. The thermal cycling parameters were as follows: an initial denaturation step at 94 °C for 85 s, followed by 35 cycles of 35 s at 95 °C (denaturation), 55 s at 58 °C (annealing) and a final extension at 72 °C for 10 min as previously developed in our laboratory (Romero et al., 2005; Quirós et al., 2006). In order to improve the results the following protocol was evaluated: (ii) 25 μl containing target DNA (100 ng), 12.5 μl 2xGC buffer I or II (TaKaRa bio IncShiga, Japan), 4 μl dNTP mixture (2.5 mM each) (TaKaRa), 1,25 μl of each primer (20 mM) (Sygma–Genosys, Cambridge, UK), 0.25 μl *La Taq*GC (TaKaRa) and sterilized distilled water (MO Laboratories, Inc) up to 25 μl. PCR conditions were an initial denaturation at 94 °C for 85 s and 35 cycles of denaturation at 95 °C

for 35 s. For annealing, a gradient of 54.9 °C to 69.5 °C for 55 s was probed, followed by an extension for 10 min at 72 °C. PCR-amplified DNA fragments were separated in 1% (w/v) agarose gels (Bio-Rad), stained with 0.05% (v/v) ethidium bromide (Bio-Rad) and visualized under UV light. The 1 kb DNA ladder (MBI Fermentas) was used as a molecular size marker.

PCR amplification products from the IGS region of DNA (20 µl) were digested without further purification using *Hap*II, *Hha*I and *Mbo*I endonucleases (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Romero et al., 2005; Quirós et al., 2006). Restriction fragments were separated on 2.5% (w/v) agarose gels, stained with 0.05% (v/v) ethidium bromide and visualized under UV light. The 100 bp DNA ladder (MBI Fermentas) was used as a molecular size marker.

2.3. Sequence determination

An analysis of sequences was carried out only on those strains which had a doubtful species classification. The 5.8-ITS rDNA region sequences were amplified by PCR using its1 (5′-TCCGTAGGTGAACCTGCGG-3′) and its 4 (5′TCCTCCGCTTATTGATATGC3′) primers (White et al., 1990). The D1/D2 domains of the 26S rDNA were amplified using NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Kurtzman and Robnett, 1998). PCR products were cleaned using the Ultraclean™ PCR clean-up Kit (MO-BIO, Larsband, USA) and 5 µM directly sequenced using the ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Foster City, USA). Each set of sequences was aligned using the MegAlign program of DNASTAR software (Lasergene, Wisconsin, USA), with the most similar sequences obtained from the GenBank Nucleotide sequence Database by BLAST. Alignments were manually revised and edited in the insertion-deletion regions for which alignment was uncertain.

2.4. Data analysis

Distance analysis was performed with TREECOM v1.3b software (Van de Peer and De Wachter, 1993). Distance estimations were calculated following the method described by Nei and Li (1979). The data on RFLP patterns were coded as binary tables, with 1 representing the presence of a fragment and 0 representing the absence of a fragment. The tree was inferred by the Neighbor-Joining algorithm. The robustness of the tree was estimated with bootstrap values on 1000 replicates. *S. cerevisiae* CECT 1942^{NT} was the designated outgroup in the analysis.

3. Results

The IGS region (rDNA) with a size lower than 4000 bp was amplified using the protocol (see method 2.2. (i) in Materials and methods). Those with a size higher than 4000 bp had to be amplified with *La Taq GC* polimerase for DNA fragments rich in G + C (GC buffer I). The annealing temperature was set at 55 °C for 55 s (see method 2.2. (ii) in Materials and methods). The PCR yielded a single fragment that ranged from 2900 bp for the *Z. cidri* (now *Lachancea cidri*, Kurtzman, 2003) species to 7000 bp for the *Z. kombuchaensis* species. As can be observed in Table 1, the size of the amplification products is identical when the strains belong to the same species.

The digestion of the IGS amplification product reveals 37 different patterns for *Hap*II, 32 for *Hha*I, and 43 for *Mbo*I enzymes (Tables 1 and 2). In general, high polymorphism was obtained for each species with the three enzymes assayed (Tables 1 and 2, Figs. 1 and 2). None of the endonuclease could be used independently to discriminate strains and species. The number of patterns varies depending on the endonuclease used. For example, for *Z. bailii* we obtained five patterns with the *Hha*I endonuclease and four with the *Mbo*I and *Hap*II endonucleases (Tables 1 and 2, Fig. 2). However, in *Z. rouxii* the best discrimination between strains was obtained with the *Mbo*I endonuclease (Tables 1 and 2, Fig. 1).

The restriction patterns obtained with all three endonucleases, as illustrated in Table 1, enable us to differentiate all the *Z. mellis* and *Z. rouxii* strains examined in this study. For example, strains that show the RA1 pattern with *Hap*II (*Z.rouxii* CECT 1232^{T}, CECT 10312 and CECT 10313) can be differentiated if the other two enzymes are considered, namely, RA1/RB1/RC1, RA1/RB7/RC5 and RA1/RB1/RC5 respectively.

When the strain patterns belonging to different species were analysed, together with the dendrogram (Fig. 3), some conflicting results were observed. Two strains, CBS 711 and CBS 7412, identified as *Z. mellis,* appeared inside the main cluster of *Z. rouxii*. The restriction profiles for the strains included in this cluster showed common bands with whichever endonucleases were assayed. However, the *Z. rouxii* CECT 11923 and CECT 10425 strains, together with *Z. rouxii* hybrids, appeared in a second cluster because of the lack of these common bands (Table 2). To solve this conflict, sequence analyses of ITS rDNA and the D1/D2 domains of 26S rDNA were performed (Table 3). The sequences studied showed that *Z. mellis* CBS 711 and CBS 7412 have 100% similarity with strains belonging to *Z. rouxii* species (see Table 3). Two patterns were therefore added to *Z. rouxii* strains (Table 1). The CECT 11923 and CECT 10425 strains were also confirmed as *Z. rouxii*.

4. Discussion

In food industries, strain typing can be considered from two perspectives: one for discriminating between different biotypes with specific properties, such as the production of volatile compounds for improving taste, ripening etc. (Andrade et al., 2009), as well as for monitoring the behaviour of a strain, for example, during the fermentation process (Suezawa et al., 2008), and the other for following one strain to determine the source of contamination, for example, to solve spoilage problems along the production chain in a specific industrial process (Martorell et al., 2005). Traceability is becoming a concept for providing safer food supplies and connecting the producer with the consumer (Regattiere et al., 2007). EC regulation 178/2002 (European Parliament, 2002) defines traceability as “the ability to trace and follow a food, feed, food-producing animals or ingredients through all stages of production and distribution”. In terms of food safety, it can tell us the history of the product. In the food spoilage context a method that attains a high grade of discrimination between strains allows us to follow the “traceable spoilage strain” along the production line. This provides the industrialist with a useful tool which may be required for legal demands.

Our results show that the PCR-RFLP-IGS analysis presents a variability of 70% for *Z. bailii* with both *Hha*I and *Mbo*I endonucleases. Meanwhile, 100% variability was obtained for *Z. mellis* and *Z. rouxii* strains when the three enzymes were analysed together. This variability is higher than what has previously been reported for some of these species using other methods (Martorell et al., 2005; Suezawa et al., 2008). Moreover, if we analyse some of the strains with the same patterns some considerations should be made. For example, in *Z. bailii* the May (12) and May (13) strains were isolated in our laboratory from the same mayonnaise sample. It is therefore possible that they come from two colonies of the same strain. The CECT 1924 and CECT 1898 strains show the same RFLP pattern. Martorell et al. (2005) had previously studied them using RFLP mtDNA and RAPDs and found that they could not be distinguished by those methods. Although both come from Japan, the origin of only one of them is known (Table 1). Based on these results we propose that both isolates belong to the same strain.

Other examples of identical RFLP pattern were found in the CECT 10657^{T} and CECT 11349 strains of *Z. cidri* (now *Lachancea cidri*, Kurtzman, 2003). Both were deposited by the same author in the same year (1955) and were from the same origin: cider. Once again, we propose that both isolates belong to the same strain.

Table 2
Restriction fragments (bp) obtained after the digestion of the IGS region of rDNA of species belonging to the *Zygosaccharomyces* genus with *Hap*II, *Hha*I and *Mbo*I endonucleases.

Species/No. (Code of Species)	Restriction fragments[a] (bp)		
	Patterns Hap II (A)	Patterns Hha I (B)	Patterns Mbo I (C)
Zygosaccharomyces bailii/7. (B)			
		BB1 (1750 + 610 + 450 + 430 + 360 + 340 + 300 + 200 + 160)	BC1 (2600 + 950 + 610 + 410 + 320 + 270)
	BA1 (1000 + 900 + 700 + 530 + 290 + 270 + 200 + 170 + 140)	BB2 (1900 + 1700 + 610 + 450 + 430 + 360 + 300 + 200 + 160)	BC2 (2700 + 2600 + 950 + 610 + 410 + 320 + 270)
	BA2 (1000 + 700 + 530 + 420 + 220 + 180 + 170 + 140)	BB3 (2300 + 2000 + 610 + 450 + 430 + 350 + 330 + 300 + 200 + 160)	BC3 (3000 + 950 + 610 + 410 + 320 + 270)
	BA3 (1000 + 700 + 530 + 350 + 290 + 200 + 170 + 140)	BB4 (2800 + 2500 + 610 + 450 + 430 + 360 + 300 + 200 + 160)	BC4 (1300 + 1200 + 950 + 610 + 410 + 370 + 350 + 320 + 270)
	BA4 (900 + 620 + 530 + 480 + 430 + 390 + 350 + 220 + 180 + 170 + 140)	BB5 (2500 + 610 + 480 + 430 + 360 + 340 + 300 + 160)	BC5 (2700 + 950 + 610 + 410 + 400 + 320 + 270)
Zygosaccharomyces bisporus/2. (Bi)			
	BiA1 (1150 + 1100 + 630 + 510 + 430 + 370 + 300 + 220 + 170 + 120 + 90)	BiB1 (1150 + 870 + 580 + 390 + 360 + 290 + 240 + 200 + 190 + 140 + 100)	BiC1 (1500 + 1150 + 680 + 630 + 400 + 330 + 270 + 230 + 150)
	BiA2 (1500 + 1200 + 630 + 600 + 510 + 430 + 370 + 300 + 170 + 120 + 90)	BiB2 (1150 + 870 + 580 + 480 + 390 + 360 + 290 + 240 + 200 + 190 + 140 + 100)	BiC2 (1500 + 1150 + 710 + 630 + 400 + 330 + 270 + 230 + 150)
Zygosaccharomyces cidrii/2. (C)			
	CA1 (2000 + 320 + 270 + 210 + 150)	CB1 (1180 + 760 + 730 + 300)	CC1 (1100 + 460 + 330 + 320 + 250 + 210 + 170 + 110)
Zygosaccharomyces fermentati/3. (F)			
	FA1 (1150 + 800 + 390 + 170)	FB1 (1100 + 820 + 380 + 380 + 170)	FC1 (750 + 690 + 390 + 320 + 250 + 210 + 110)
	FA2 (1100 + 900 + 550 + 300 + 250)	FB2 (1100 + 850 + 550 + 300 + 250)	FC2 (1000 + 570 + 390 + 320 + 250 + 210 + 110)
	FA3 (1500 + 600 + 320 + 270 + 210 + 150)		
Zygosaccharomyces kombuchaensis/1. (K)			
	KA1 (1600 + 1150 + 600 + 510 + 430 + 410 + 300 + 250 + 190 + 170 + 120)	KB1 (1900 + 600 + 600 + 500 + 450 + 450 + 400 + 250 + 210 + 120)	KC1 (1750 + 1250 + 1150 + 950 + 550 + 500 + 450)
Zygosaccharomyces lentus/2. (L)	LA1 (680 + 580 + 530 + 500 + 430 + 330 + 300 + 280 + 240 + 200 + 180 + 150)	LB1 (1500 + 1300 + 900 + 600 + 580 + 500 + 420 + 300 + 270 + 200 + 150 + 90)	LC1 (1100 + 850 + 610 + 590 + 550 + 450 + 320 + 310 + 270 + 250 + 150 + 110)
	LA2 (730 + 580 + 530 + 500 + 430 + 330 + 300 + 280 + 240 + 200 + 180 + 150)		
Zygosaccharomyces mellis/10. (M)	MA1 (1000 + 930 + 300 + 280 + 190 + 160 + 120 + 90)	MB1 (1750 + 900 + 800 + 400 + 250)	MC1 (1300 + 900 + 720 + 430 + 350 + 270 + 150 + 120 + 100)
	MA2 (1000 + 930 + 480 + 300)	MB2 (1750 + 850 + 500 + 400 + 390)	MC2 (1300 + 900 + 750 + 450)
	MA3 (1000 + 800 + 750 + 300 + 250)	MB3 (1300 + 680 + 530 + 450 + 380 + 310 + 210)	MC3 (1250 + 1000 + 450 + 410 + 260)
	MA4 (1000 + 750 + 300 + 250	MB4 (1750 + 900 + 800 + 530 + 400 + 250)	RC24 (760 + 670 + 520 + 500 + 410 + 350 + 260 + 170)
	MA5 (1100 + 1000 + 930 + 300 + 280 + 190 + 160 + 120 + 90)	MB5 (1750 + 1050 + 900 + 400 + 250)	RC25 (760 + 650 + 500 + 500 + 410 + 350 + 310 + 260 + 170)
	RA19 (970 + 700 + 500 + 400 + 380 + 300 + 290 + 160)	RB12 (960 + 700 + 630 + 450 + 420 + 210 + 190)	
	RA20 (970 + 700 + 480 + 400 + 310 + 300 + 290 + 160)	RB13 (960 + 700 + 680 + 630 + 450 + 420 + 210 + 190)	
Zygosaccharomyces microellipsiodes/1. (Mi)	MiA1 (1100 + 650 + 370 + 180 + 160)	MiB1 (800 + 670 + 610 + 320 + 350)	MiC1 (800 + 550 + 370 + 270)
Zygosaccharomyces rouxii/33. (R)	RA1 (970 + 700 + 500 + 400 + 300 + 290 + 160)	RB1 (960 + 700 + 630 + 450 + 420)	RC1 (760 + 670 + 520 + 500 + 410 + 350 + 260 + 200 + 170)
	RA2 (970 + 700 + 480 + 400 + 300 + 270 + 250 + 160)	RB2 (960 + 700 + 600 + 450 + 420)	RC2 (760 + 520 + 410 + 350 + 260 + 170)
	RA3 (970 + 700 + 480 + 400 + 300 + 270 + 160)	RB3 (960 + 700 + 630 + 450)	RC3 (760 + 520 + 410 + 350 + 300 + 260 + 170)
	RA4 (970 + 700 + 480 + 400 + 300 + 290 + 160)	RB4 (960 + 720 + 700 + 600 + 450 + 420)	RC4 (760 + 520 + 410 + 350 + 310 + 260 + 170)
	RA5 (970 + 700 + 480 + 400 + 300 + 290 + 270 + 160)	RB5 (960 + 720 + 700 + 630 + 450)	RC5 (760 + 520 + 500 + 410 + 350 + 260 + 170)
	RA6 (970 + 700 + 480 + 400 + 330 + 300 + 270 + 160)	RB6 (960 + 720 + 700 + 630 + 450 + 420 + 210)	RC6 (760 + 650 + 500 + 410 + 350 + 260 + 170)
	RA7 (1200 + 700 + 410 + 380 + 320 + 210 + 180 + 90)	RB7 (960 + 740 + 700 + 630 + 450 + 420 + 210)	RC7 (760 + 650 + 500 + 410 + 350 + 290 + 260 + 170)
	RA8 (970 + 700 + 480 + 400 + 350 + 300 + 270 + 160)	RB8 (960 + 790 + 700 + 600 + 450 + 420)	RC8 (760 + 650 + 520 + 410 + 350 + 260 + 210 + 170)
	RA9 (970 + 700 + 480 + 400 + 350 + 300 + 290 + 160)	RB9 (960 + 700 + 630 + 450 + 420 + 180)	RC9 (760 + 650 + 520 + 410 + 350 + 260 + 170)
	RA10 (970 + 700 + 480 + 420 + 400 + 300 + 270 + 160)	RB10 (1500 + 800 + 700 + 400 + 350 + 310 + 190 + 120 + 80)	RC10 (760 + 650 + 520 + 410 + 350 + 310 + 260 + 170)

Table 2 (*continued*)

Species/No. (Code of Species)	Restriction fragments[a] (bp)		
	Patterns Hap II (A)	Patterns Hha I (B)	Patterns Mbo I (C)
Zygosaccharomyces rouxii/33. (R)	RA11 (970 + 700 + 500 + 400 + 300 + 270 + 160) RA12 (970 + 700 + 500 + 400 + 300 + 290 + 200 + 160) RA13 (970 + 700 + 500 + 400 + 350 + 300 + 290 + 270 + 200 + 160) RA14 (970 + 700 + 500 + 450 + 400 + 300 + 290 + 160) RA15 (970 + 700 + 500 + 480 + 400 + 300 + 290 + 160) RA16 (970 + 700 + 500 + 480 + 400 + 370 + 300 + 290 + 270 + 160) RA17 (970 + 700 + 480 + 400 + 300 + 290 + 200 + 160) RA18 (1300 + 800 + 700 + 410 + 380 + 320 + 210 + 180) RA21 (1300 + 730 + 700 + 410 + 380 + 350 + 320 + 210 + 180 + 160 + 90) RA22 (1300 + 730 + 700 + 410 + 380 + 320 + 210 + 180 + 160 + 90)	RB11 (1500 + 850 + 700 + 400 + 350 + 290 + 120 + 80) RB12 (1500 + 850 + 680 + 400 + 360 + 350 + 190)	RC11 (760 + 650 + 520 + 500 + 410 + 350 + 310 + 260 + 170) RC12 (760 + 670 + 520 + 500 + 410 + 350 + 310 + 260 + 170) RC13 (760 + 700 + 500 + 410 + 350 + 260 + 170) RC14 (760 + 700 + 520 + 410 + 350 + 290 + 260 + 170) RC15 (760 + 700 + 520 + 500 + 410 + 350 + 260 + 170) RC16 (760 + 700 + 520 + 500 + 410 + 350 + 300 + 260 + 170) RC17 (760 + 700 + 650 + 520 + 500 + 410 + 350 + 260 + 170) RC18 (760 + 700 + 670 + 520 + 500 + 410 + 350 + 310 + 260 + 170) RC19 (760 + 700 + 690 + 650 + 520 + 500 + 410 + 350 + 310 + 260 + 170) RC20 (760 + 650 + 520 + 500 + 410 + 350 + 260 + 170) RC21 (760 + 700 + 650 + 520 + 500 + 410 + 350 + 310 + 290 + 260 + 170) RC22 (1700 + 700 + 630 + 500 + 400 + 250 + 170) RC23 (1700 + 800 + 700 + 500 + 400 + 250) RC24 (1700 + 800 + 750 + 700 + 650 + 500 + 400 + 270 + 170 + 160 + 130) RC25 (1700 + 800 + 650 + 500 + 400 + 270 + 170 + 160 + 130) RC26 (1700 + 800 + 650 + 500 + 400 + 270 + 160 + 130)

Z.cidri and *Z. fermentati* are proposed as *Lachancea* species and *Z.microellipsiodes* as *Torulaspora* species (Kurtzman, 2003, FEMS Yeast 24,403–417).
[a] Some fragments could be duplicated.

One interesting result, although it was not part of our objective, was that the method enabled us to detect two misidentified strains of *Z. mellis* (CBS 711 and CBS 7412). These strains presented a restriction profile of the IGS region of rDNA which included the common bands described above for the greater part of *Z. rouxii* examined (Table 2). The D1/D2 domain 26S rDNA and ITS sequence analysis showed that

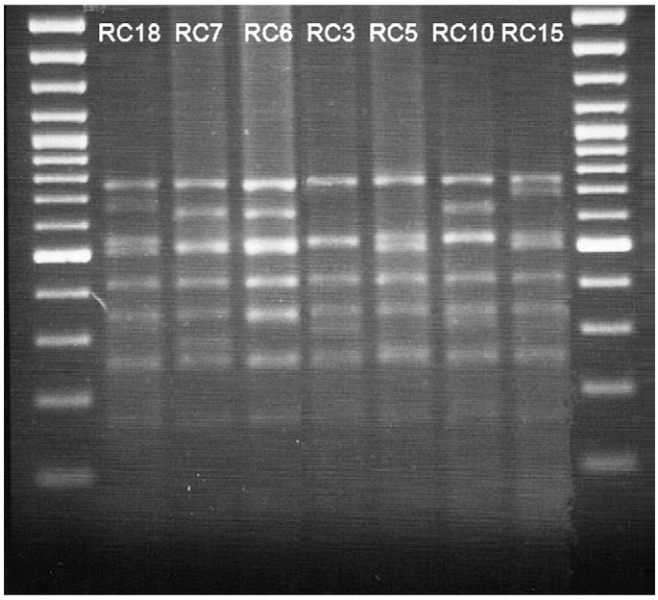

Fig. 1. *Zygosaccharomyces rouxii* strain typing. Some PCR-RFPLs patterns of the IGS region of rDNA with *Mbo*I endonuclease. Lanes 1 and 9 correspond to the 100 bp DNA ladder (MBI Fermentas).

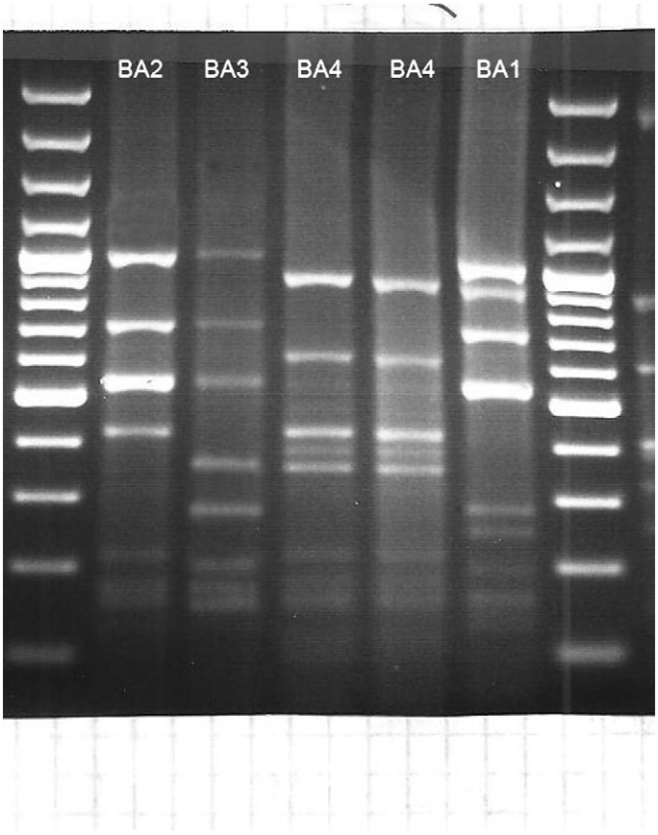

Fig. 2. *Zygosaccharomyces bailii* strain typing. Some PCR-RFPLs patterns of the IGS region of rDNA with *Hap*II endonuclease. Lanes 1 and 7 correspond to the 100 bp DNA ladder (MBI Fermentas).

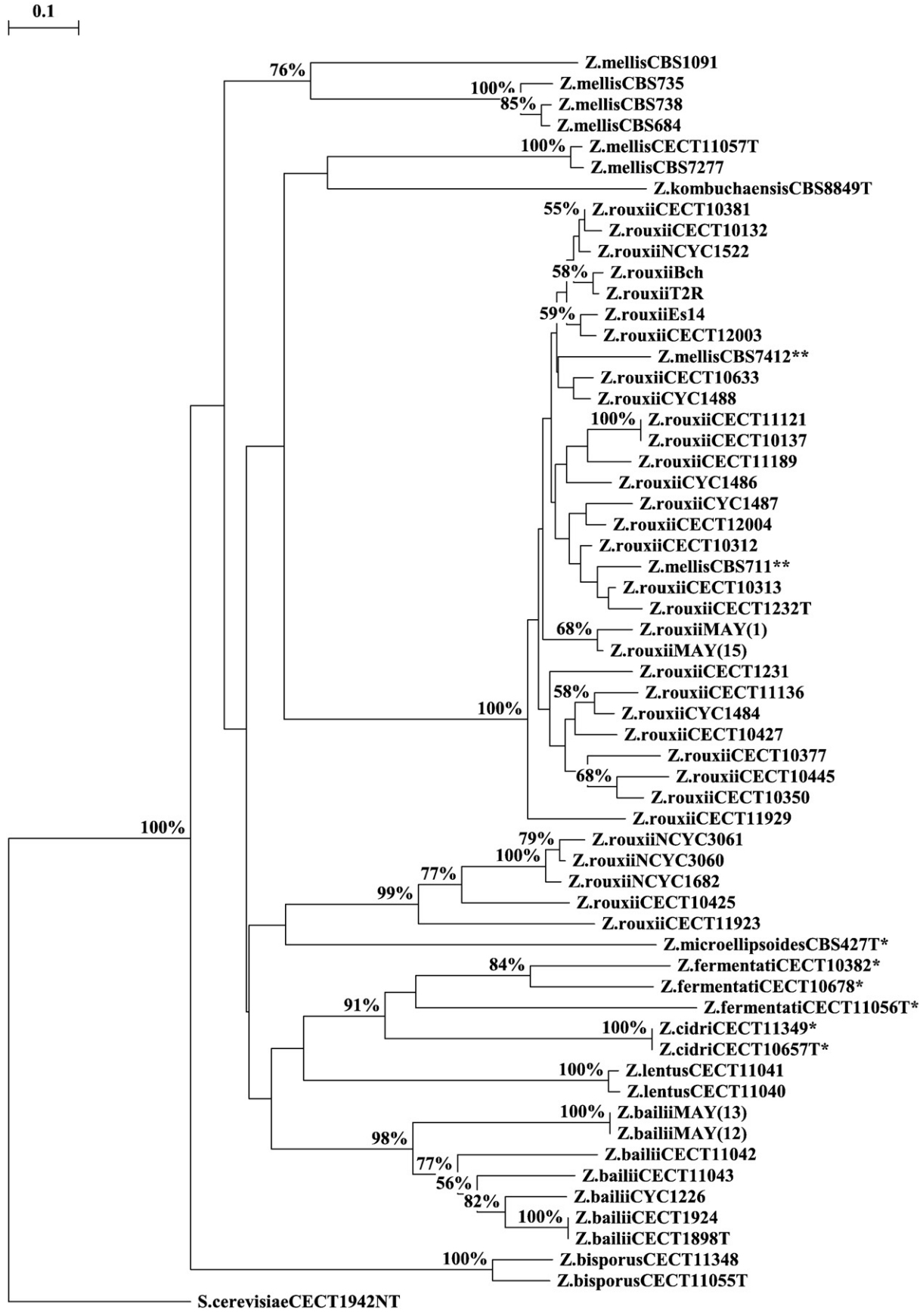

Fig. 3. Dendrogram based on the IGS-PCR restriction analysis of *Zygosaccharomyces* species isolated in our laboratory or from Type Culture Collections. Distance estimations were calculated following the method described by Nei and Li (1979). Robustness of the tree was estimated with bootstrap values on 1000 replicates indicated as a percentage. *Saccharomyces cerevisiae* CECT194n^{NT} was used as the outgroup species. *Z. cidri* and *fermentati* are proposed as *Lachancea* species and *Z. microellipsiodes* as *Torulaspora* species (Kurtzman, 2003, FEMS yeast 24, 403–417). ** Strains identified in this study as *Z.rouxii* are shown in Table 3.

Table 3
GenBank accession numbers of the strains included in the study, and strains that presents a 100% similarity with them.

Accession number												
Strain	5.8S-ITS (strain)						D1/D2 26S rRNA gene (strain)					
Z. rouxii CECT 10425	FN431888						FN431893					
							AB363048.1	(ZR510)	AB363047.1	(ZR14)	AB363045.1	(TSY-5)
							AB363044.1	(TO)	AB363043.1	(TA10101)	AB363040.1	(No.3)
							AB363038.1	(KS02)	AB363035.1	(H14-8-1)	AB363033.1	(H14-5-1)
							AB363032.1	(H14-4-1)	AB363031.1	(H14-1-2)	AB363030.1	(-601)
							AB302811.1	(IFO 1877)	AB302810.1	(IFO 1876)	AB302807.1	(IFO 1812)
							AB302805.1	(IFO 0845)	AB302801.1	(IFO 0596)	AB302800.1	(IFO 0525)
							AB302799.1	(IFO 0523)	AB302798.1	(IFO 0521)	AB302794.1	(IFO 0510)
							AB302793.1	(IFO 0506)	AB302792.1	(IFO 0505)	AM947682.1	(ABT301)
							AM947680.1	(ATCC 42981)	AM943657.1	(ATCC 42981)	AJ966342.2	(ABT301)
							AY524006.1	AY524006.1 (NRRL Y-2547)	AJ555406.1	(NCYC3042)		
Z. rouxii CECT 11923	FN431886						FN431887					
	AB363062.1	(ZR510)	AB363061.1	(ZR14)	AB363060.1	(ZR-1)	CU928181.1	(CBS 732)	AB363046.1	(ZR-1)	AB363042.1	(SR-4)
	AB363057.1	(SR-4)	AB363056.1	(SR-2)	AB363055.1	(KS03)	AB363041.1	(SR-2)	AB363039.1	(KS03)	AB363037.1	(KS01)
	AB363054.1	(KS01)	AB363053.1	(KF-4)	AB363051.1	(H14-7-1)	AB363036.1	(KF-4)	AB363034.1	(H14-7-1)	AB302813.1	(IFO 1960)
	AB302834.1	(IFO 1960)	AB302827.1	(IFO 1130)	AB302826.1	(IFO 0845)	AB302812.1	(IFO 1914)	AB302809.1	(IFO 1814)	AB302808.1	(IFO 1813)
	AB302824.1	(IFO 0596)	AB302820.1	(IFO 0513)	AB302819.1	(IFO 0512)	AB302806.1	(IFO 1130)	AB302804.1	(IFO 0740)	AB302803.1	(IFO 0686)
	AB302818.1	(IFO 0511)	AM943656.1	(ATCC 42981)	AM943655.1	(CBS 732)	AB302797.1	(IFO 0513)	AB302796.1	(IFO 0512)	AB302795.1	(IFO 0511)
	AY046189.1	(Unknown)					AB302791.1	(IFO 0494)	AM943656.1	(ATCC 42981)	AM943655.1	(CBS 732)
							AM911009.1	(YSF21)	AM911008.1	(YSF34)	AJ783434.1	(ESAB21)
							AJ716118.1	(CBS 9714)	U72163.1	(Unknown)		
Z. mellis CBS 711	FN431889						FN431892					
	AB363062.1	(ZR510)	AB363061.1	(ZR14)	AB363060.1	(ZR-1)	CU928181.1	(CBS 732)	AB363046.1	(ZR-1)	AB363042.1	(SR-4)
	AB363057.1	(SR-4)	AB363056.1	(SR-2)	AB363055.1	(KS03)	AB363041.1	(SR-2)	AB303639.1	(KSO3)	AB393037.1	(KSO1)
	AB363054.1	(KS01)	AB363053.1	(KF-4)	AB363051.1	(H14-7-1)	AB363036.1	(KF-4)	AB363034.1	(H-14-7-1)	AB302813.1	(IFO1960)
	AB302834.1	(IFO 1960)	AB302827.1	(IFO 1130)	AB302826.1	(IFO 0845)	AB302812.1	(IFO1914)	AB302809.1	(IFO1814)	AB302808.1	(IFO1813)
	AB302825.1	(IFO 0740)	AB302824.1	(IFO 0596)	AB302820.1	(IFO 0513)	AB302806.1	(IFO1130)	AB302804.1	(IFO0740)	AB302803.1	(IFO0686)
	AB302819.1	(IFO 0512)	AB302818.1	(IFO 0511)	AM943656.1	(ATCC 42981)	AB302797.1	(IFO0513)	AB302796.1	(IFO0512)	AB302795.1	(IFO0511)
	AM943655.1	(CBS 732)	AM279465.1	(ABT301)			AB302791.1	(IFO0494)	AM943665.1	(ATCC42981)	AM943655.1	(CBS 732)
							AM911009.1	(YSF21)	AM911008.1	(YSF34)	AY524005.1	(PYCC3693)
							AJ783434.1	(ESAB21)	AJ716118.1	(CBS9714)	U72163.1	(Unknown)
Z. mellis CBS 7412	FN431890						FN431891					
	AB363062.1	(ZR510)	AB363061.1	(ZR14)	AB363060.1	(ZR-1)	AB363046.1	(ZR-1)	AB363042.1	(SR-4)	AB363041.1	(SR-2)
	AB363057.1	(SR-4)	AB363056.1	(SR-2)	AB363055.1	(KS03)	AB363039.1	(KS03)	AB363037.1	(KS01)	AB363036.1	(KF-4)
	AB363054.1	(KS01)	AB363053.1	(KF-4)	AB363051.1	(H14-7-1)	AB363034.1	(H14-7-1)	AB302813.1	(IFO 1960)	AB302812.1	IFO 1914
	AB302834.1	(IFO 1960)	AB302827.1	(IFO 1130)	AB302826.1	(IFO 0845)	AB302809.1	(IFO 1814)	AB302808.1	(IFO 1813)	AB302806.1	(IFO 1130)
	AB302825.1	(IFO 0740)	AB302824.1	(IFO 0596)	AB302820.1	(IFO 0513)	AB302804.1	(IFO 0740)	AB302803.1	(IFO 0686)	AB302797.1	IFO0513
	AB302819.1	(IFO 0512)	AB302818.1	(IFO 0511)	AM943656.1	(ATCC 42981)	AB302796.1	IFO0512	AB302795.1	IFO0511	AB302791.1	(IFO 0494)
	AM943655.1	(CBS 732)	AM279465.1	(ABT301)			AM943656.1	(ATCC 42981)	AM943655.1	(CBS 732)	AM911009.1	(YSF21)
							AM911008.1	(YSF34)	AY524005.1	(PYCC 3693)	AJ783434.1	(ESAB21)

Sequences retrieved from GenBank.
Accession number printed in bold corresponds to sequences obtained in the current study.

the phenotypic identification was not correct. We therefore concluded that these two strains belong to the *Z. rouxii* species (Fig. 3, Table 3).

Two *Z. rouxii* strains (CECT 11923 and CECT 10425) were confirmed as *Z. rouxii* in this study by sequencing. Although the undoubted value of using simple gene sequences (e.g. 26 S rDNA D1/D2) to identify yeasts is recognized, this method has some limitations for the identification of hybrids (James et al., 2005). Moreover, the fact that both strains appear together with the hybrids (NCYC1682, NCYC 3060 and NCYC 3061) identified by James et al. (2005) in a separate cluster (Fig. 3) may indicate that they could also be hybrids. These authors suggest that yeast hybrids may be more abundant than previously thought; in fact, Solieri et al. (2007) recently reported new *Z. rouxii* hybrids. For this reason, further studies are under way in our laboratory to clarify the nature of these strains. Note that this method also permits all the hybrid strains analysed so far to be distinguished, as shown in Tables 2 and 3.

On the other hand, during this study, we have developed a modification of the method previously described by Romero et al. (2005) and Quirós et al. (2006) (Materials and methods). After the spheroplasted treatment, the DNA extraction was done in half the time using DNeasy (see Materials and methods 2.2). Changing the DNA polymerase and the PCR conditions (see Material and methods 2.2 (ii)) made it possible to amplify IGS regions with a size as high as 7000, 6700 or 6000 bp. The amplicon size of the IGS is different in seven out of the nine species of *Zygosaccharomyces* tested (Table 1). For example, a size of 6300 bp corresponds to *Z. bailii* and 5500 bp to *Z. bisporus*. Although this technique is unreliable as a method of identification, in practice relatively few yeast species are responsible for the majority of food spoilage by yeasts (Stratford, 2006) and some specific associations are frequent and often predictable (Fleet, 2006). This generally narrows the candidates for yeast spoilage in a specific food. Typing methodologies are usually applied after the identification process. For example, *Z. mellis* and *Z. rouxii* may easily be identified from colonies by the size of the amplicon of the 5.8S-ITS region and the fragments obtained after digestion with three restriction endonucleases (Barata et al., 2008; Esteve-Zarzoso et al., 1999, 2003, yeast-id.com (CECT)).

In conclusion, the PCR-RFLP analysis of the IGS region of rDNA is a fast and single molecular typing method producing clear and reproducible restriction RFLP patterns. It constitutes a typing method for the *Z. bailii*, *Z. mellis* and *Z. rouxii* species as well as for other species belonging to the *Zygosaccharomyces* genus. The method also discriminates hybrid strains of *Zygosaccharomyces*. It does not require sequencing technologies and as a consequence is easier to implant in the routine of an industry laboratory.

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