Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers

B. Esteve-Zarzoso,^{1,2} C. Belloch,¹ F. Uruburu¹ and A. Querol²

Author for correspondence: A. Querol. Tel: +34 6 390 00 22. Fax: +34 6 363 63 01.
e-mail: aquerol@iata.csic.es

¹ CECT (Spanish Type Culture Collection), Universitat de València, Edificio de Investigación, Campus de Burjassot, 46100 Burjassot, València, Spain

² Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P. Box 73, 46100 Burjassot, València, Spain

The identification and classification of yeasts have traditionally been based on morphological, physiological and biochemical traits. Various kits have been developed as rapid systems for yeast identification, but mostly for clinical diagnosis. In recent years, different molecular biology techniques have been developed for yeast identification, but there is no available database to identify a large number of species. In the present study, the restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were used to identify a total of 132 yeast species belonging to 25 different genera, including teleomorphic and anamorphic ascomycetous and basidiomycetous yeasts. In many cases, the size of the PCR products and the restriction patterns obtained with endonucleases *CfoI*, *HaeIII* and *HinfI* yielded a unique profile for each species. Accordingly, the use of this molecular approach is proposed as a new rapid and easy method of routine yeast identification.

Keywords: yeast identification, RFLP, 5.8S–ITS region

INTRODUCTION

The beneficial activities of yeasts are of great economic significance and they have been used for millennia in the production of fermented foods and alcoholic beverages (Deák & Beuchat, 1996). In the present century, as the fermentation industry has developed, yeasts have been used in the production of valuable products, including antibiotics, vitamins and enzymes. However, they also cause immense economic losses, and their harmful activities as saprotrophs include damage to food and manufactured goods. Consequently, the reliable and rapid identification of those species involved in food processing and the detection of yeasts causing food spoilage are desirable (Carlie & Watkinson, 1994).

Yeasts are unicellular fungi and can be classified into two phylogenetic groups, teleomorphic and anamorphic ascomycetous yeasts and teleomorphic and anamorphic basidiomycetous yeasts (Kurtzman & Fell, 1998). The differentiation of taxa is usually

achieved by comparison of morphological traits and physiological features (Barnett *et al.*, 1990; Krejer-van Rij, 1984), which in some cases can lead to an incorrect classification of species or a false identification of strains.

The conventional methodology for yeast identification requires evaluation of some 60–90 tests, and the process is complex, laborious and time consuming (Deák, 1995; Deák & Beuchat, 1996). In recent years, to improve the conventional methods, rapid kits for yeast identification have been developed. However, they were initially designed for clinical diagnosis and their application is restricted to 40–60 yeast species of medical interest (Deák, 1993). In general, it is necessary to do around 100 tests to obtain a reliable identification of yeasts at the species level, and 1–3 weeks are often necessary to obtain a final result (Barnett *et al.*, 1990; Krejer-van Rij, 1984; Lin & Fung, 1987). Some methods based on the analysis of total cell proteins (Guillamón *et al.*, 1993; van Vuuren & van Der Meer, 1987; Vacanneyt *et al.*, 1991) and long-chain fatty acids using gas chromatography (Augustyn *et al.*, 1991; Cottrell *et al.*, 1986; Moreira da Silva *et al.*, 1994; Tredoux *et al.*, 1987) have also been developed. However, the reproducibility of these techniques is

Abbreviation: ITS, internal transcribed spacer.

questionable due to the fact that they depend on the physiological state of the yeast cells (Golden *et al.*, 1994).

Recent progress in molecular biology has led to the development of new techniques for yeast identification based on similarity or dissimilarity of DNA, RNA or proteins. These include allozyme patterns (Naumov *et al.*, 1997), DNA–DNA hybridization (Török *et al.*, 1993; Vaughan Martini & Martini, 1985, 1987), electrophoretic karyotyping (Guillamón *et al.*, 1996; Nadal *et al.*, 1996; Perez *et al.*, 1995; Querol *et al.*, 1992b, Schütz & Gafner, 1993; Török *et al.*, 1993), microsatellite analysis (Baleiras Couto *et al.*, 1996), nested-PCR (Ibeas *et al.*, 1996), random amplified polymorphic DNA (RAPD) analysis (Baleiras Couto *et al.*, 1994; Lopandic *et al.*, 1996; Quesada & Cenis, 1995), RFLP of chromosomal DNA (Versavaud & Hallet, 1995) or RFLP of mitochondrial DNA (Belloch *et al.*, 1997; Guillamón *et al.*, 1994, 1997; Ibeas *et al.*, 1997; Nadal *et al.*, 1996; Pérez *et al.*, 1995; Querol *et al.*, 1992a, Romano *et al.*, 1996). However, these techniques are impractical for the routine identification of a large number of species since they were developed for species characterization and there is no available database which would permit the analysis of new results.

Previous results have demonstrated that the complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than the 18S and 25S rRNA genes (Cai *et al.*, 1996; James *et al.*, 1996; Kurtzman, 1992, 1993). Because ribosomal regions evolve in a concerted fashion, they show a low intraspecific polymorphism, and a high interspecific variability (Li, 1997) has been proved very useful for the classification of *Saccharomyces* species (Huffman *et al.*, 1992; Molina *et al.*, 1992; Valente *et al.*, 1996; Wyder & Puhán, 1997), *Kluyveromyces* species (Belloch *et al.*, 1998) and, recently, for the identification of a small collection of wine yeast species (Guillamón *et al.*, 1998).

In the present study, we have tested the application of the restriction analysis of the rRNA region spanning the 5.8 rRNA gene and the two ITSs (here referred to as the 5.8S–ITS region) as a rapid and easy method for yeast species identification. For this purpose, we have obtained restriction patterns of PCR products for the identification of 132 species from 25 different genera, including teleomorphic and anamorphic ascomycetous and basidiomycetous yeasts.

METHODS

Yeast strains. We have analysed 243 different strains belonging to 132 different yeast species of 25 genera, obtained from the Spanish Type Culture Collection (CECT). To keep this study to a manageable size, we have mainly relied on

type strains representing the 132 yeast species most frequently isolated from food. For some species, when substantial heterogeneity was suspected, or due to their biotechnological importance, additional strains were also characterized. The designations of the strains are listed in Tables 1–3.

PCR reaction and DNA digestions. Cells were directly collected from a fresh yeast colony using a yellow tip and suspended in 100 µl PCR reaction mix containing 0.5 µM primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3'), 0.5 µM primer ITS4 (5' TCCTCCGCTTATTGATATGC 3'), 10 µM deoxynucleotides, 1.5 mM MgCl₂ and 1 × buffer (MAD-GEN). The suspension was heated at 95 °C for 15 min in a Progene (Techne) thermocycler. One unit of DNA Polymerase SuperTherm (MAD-GEN) was then added to each tube. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing 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. PCR products (10 µl or approximately 0.5–1.0 µg) were digested without further purification with the restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I (Boehringer Mannheim), although endonucleases *Alu*I, *Dde*I, *Scr*FI or *Taq*I were additionally used in some particular cases. The PCR products and their restriction fragments were separated on 1.4% and 3% agarose gels, respectively, with 1 × TAE buffer. After electrophoresis, gels were stained with ethidium bromide, visualized under UV light and photographed (Image Master, Pharmacia). Sizes were estimated by comparison against a DNA length standard (100 bp ladder, Gibco-BRL).

RESULTS AND DISCUSSION

ITS1 and ITS4 primers were used to amplify the region of the rDNA repeat unit that includes the 5.8S rRNA gene and the two non-coding regions designated the internal transcribed spacers (ITS1 and ITS2) (White *et al.*, 1990) of 243 strains belonging to 132 species. Tables 1, 2 and 3 show the sizes of the PCR products and the restriction fragments obtained using the restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I. Fragments smaller than 50 bp could not be reproducibly visualized and were not included in these tables.

The PCR products showed a high degree of length variation, ranging from 380 bp for *Yarrowia lipolytica* (CECT 1240) and *Pichia pastoris* (CECT 11078) to 1050 bp for the type strain of *Schizosaccharomyces pombe* var. *pombe*. In the majority of cases, the PCR products from strains of the same species had identical molecular sizes, and species of the same genus had similar sizes for the amplified fragment. In the following sections results are discussed for each yeast class, genus by genus.

Teleomorphic and anamorphic ascomycetous yeasts

The genus *Candida* includes all yeast species that cannot be classified in other asexual ascomycetous yeast genera. As a result it is a very heterogeneous genus and the perfect state of most *Candida* species is

still unknown (Krejer-van Rij, 1984). Different molecular techniques have been used to identify and characterize *Candida* species. However, except in the case of the clinical species (Botelho & Planta, 1994; Iwaguchi *et al.*, 1990; Jordan, 1994), a standard method of classification has not been developed. In this study, we have analysed by RFLP of the 5.8S-ITS region 56 strains belonging to 30 species. These strains showed a unique restriction pattern for each species with the three endonucleases used (see Table 1). The size of the amplified fragment was very variable, ranging from 400 bp for *Candida agrestis* (CECT 11179^T) to 800 bp for *Candida glabrata* (CECT 1448), showing the heterogeneity of this genus. Due to the high diversity and complexity of this genus we recommend use of the information obtained with the three restriction enzymes *Cfo*I, *Hae*III and *Hinf*I to achieve a reliable classification of the 31 species included in this study.

Debaryomyces is a complex genus in which species previously identified as *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, *Issatchenkia* and *Pichia* have been included. In our study, three species, *Debaromyces hansenii*, *Debaromyces polymorphus* and *Debaromyces pseudopolymorphus*, were analysed. Because some of the morphological and physiological tests produce variable or delayed responses, it is difficult to achieve a correct identification of the species of this genus by classical methods (Barnett *et al.*, 1990; Krejer-van Rij, 1984). In most cases, restriction analysis of the 5.8S-ITS region of *Debaryomyces* species exhibited the same patterns with different endonucleases, not only with the three general restriction enzymes included in Table 1, but also with *Alu*I, *Dde*I, *Scr*FI and *Taq*I (results not shown). Some strains of *Debaromyces polymorphus* showed a different pattern, indicating that this taxon includes two different types of strains. One type had the same pattern as the other two species of the genus. The second type exhibited a clearly different pattern, and therefore their assignation to this species could be reconsidered in further studies. In the light of these results and due to the heterogeneity of this genus, more strains representing all the species from this genus should be used to verify our results.

As representatives of the genus *Dekkera*/*Brettanomyces*, six strains of *Dekkera anomala* and *Dekkera bruxellensis* were analysed. Both species are important spoilage yeasts present in soft drinks and alcoholic beverages. The restriction patterns obtained with the three restriction enzymes are species-specific. A striking result is the large difference in the PCR product length, namely 800 bp for *Dekkera anomala*, which is in the range of the largest fragments, and 485 bp for *Dekkera bruxellensis*, in the range of the shortest sizes. Another very interesting result is the fact that both anamorph (*Brettanomyces bruxellensis*, CECT 1009^{LT}) and teleomorph forms (*Dekkera bruxellensis*, CECT 1451^T) exhibited the same PCR lengths and restriction patterns, indicating that this

technique is also useful to detect perfect-imperfect pairs.

Only six species are currently accepted in the genus *Hanseniaspora* (anamorph = *Kloeckera*) according to DNA reassociation studies (Meyer *et al.*, 1978) and partial 18S and 26S rRNA nucleotide sequence analysis (Yamada *et al.*, 1992). According to our results, *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* are closely related species, both showing the same pattern using *Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Scr*FI and *Taq*I restriction endonucleases. However, their differentiation can be obtained with the use of *Dde*I (Table 2). For the remaining species the use of only one enzyme (*Cfo*I, *Hae*III or *Hinf*I) is sufficient to obtain a pattern representative of each species.

In the genus *Kluyveromyces*, the approximate lengths of the amplified products were enough to identify the majority of the species. However, to differentiate among the species *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Kluyveromyces wickerhamii*, the digestion of the PCR product with restriction enzymes *Hae*III and *Hinf*I was required. The pair *Kluyveromyces thermotolerans* and *Kluyveromyces waltii* presented the same pattern with all the restriction enzymes used. All the strains belonging to the same species showed the same pattern with the different endonucleases, with the exception of the strains of *Kluyveromyces lactis* formerly classified as *Kluyveromyces marxianus* var. *drosophilae* (CECT 10390 and 11337) and *Kluyveromyces marxianus* var. *phaseolusporus* (CECT 11340), which exhibited different patterns with *Hinf*I.

According to morphological and physiological tests, ten species are recognized in the genus *Metschnikowia*. Four of them have been isolated from aquatic sources and the others from terrestrial environments. *Metschnikowia pulcherrima* is one of the most important species involved in food processing. We analysed eight strains of this species and all of them showed identical patterns.

The genus *Pichia*, in the description given by Kurtzman (1984) is, with 57 species, the largest ascomycetous yeast genus. A wide variation exists in their morphological and physiological responses, and some have been transferred to the genera *Issatchenkia* and *Debaryomyces* (Krejer-van Rij, 1987). In this study we analysed 20 species recovered from diverse habitats (insects, soils and drinks). The complexity of this genus is reflected in the heterogeneity in the sizes of their PCR fragment lengths, ranging from 380 bp in *Pichia pastoris* (CECT 11078) to 750 in *Pichia angusta* (CECT 10220). We obtained unique species patterns with the exception of the pair *Pichia segobiensis* (CECT 10210) and *Pichia stipitis* (CECT 1922), which were impossible to differentiate with all the endonucleases tested (*Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hinf*I, *Scr*FI and *Taq*I). We paid special attention to the species isolated mainly from fruit, soft drinks and alcoholic beverages, such as

Table 1. Size in bp of the PCR products and the restriction fragments of teleomorphic and anamorphic ascomycetous yeasts

Species*	CECT strain no.	AP†	Restriction fragments		
			<i>CfoI</i>	<i>HaeIII</i>	<i>HinfI</i>
<i>Candida agrestis</i>	11179 ^T	400	210 + 190	290 + 110	220 + 180
<i>Candida albicans</i>	1002 ^T , 1392, 1439, 11181, 11182	550	290 + 260	460 + 90	280 + 270
<i>Candida apicola</i>	11167	750	340 + 310 + 100	730	390 + 195 + 160
<i>Candida aurangiensis</i>	10623 ^T	450	240 + 210	440	340 + 100
<i>Candida beechii</i>	11165 ^T	650	300 + 300	400 + 140 + 90	330 + 330
<i>Candida boidinii</i>	1014 ^T , 11153	750	350 + 310 + 90	700	390 + 190 + 160
<i>Candida cacaoi</i>	11059 ^T	700	310 + 310 + 80	430 + 180 + 90	360 + 175 + 165
<i>Candida cantarellii</i>	11170	700	300 + 300 + 85	435 + 180 + 90	360 + 175 + 165
<i>Candida diversa</i>	11159, 11178 ^T	450	160 + 100 + 100 + 90	410	220 + 200
<i>Candida ergatensis</i>	10261 ^T	650	300 + 275	500 + 150	325 + 190 + 130
<i>Candida glabrata</i>	1448	800	395	675	360 + 270
<i>Candida incommunis</i>	11166 ^T	425	400	425	225 + 200
<i>Candida intermedia</i> var. <i>intermedia</i>	10118, 11054, 11154	425	220 + 190	410	225 + 200
<i>Candida magnoliae</i>	10664	425	200 + 190	285 + 140	225 + 200
<i>Candida maltosa</i>	1936 ^T	550	300 + 250	550	280 + 270
<i>Candida mesenterica</i>	1025	650	600	500 + 150	320 + 220 + 100
<i>Candida montana</i>	11180 ^T	525	250 + 230	400 + 125	270 + 250
<i>Candida norvegica</i>	10310	580	510	370 + 190	290 + 260
<i>Candida parapsilosis</i>	1449, 10434, 10437	550	300 + 240	400 + 115	290 + 260
<i>Candida sake</i>	1044, 10034, 10276	450	250 + 200	450	230 + 220
<i>Candida salmanticensis</i>	10487 ^T	450	240 + 200	440	410
<i>Candida sorbosa</i>	11204	500	215 + 100 + 80	300 + 90 + 90	270 + 120 + 110
<i>Candida stellata</i>	11046, 11109, 11110	475	215 + 110 + 80 + 60	475	235 + 235
<i>Candida terebra</i>	11015	650	300 + 250 + 90	400 + 150 + 100	325 + 325
<i>Candida tropicalis</i>	1440	550	280 + 250	450 + 90	270 + 270
<i>Candida wandervallii</i>	11169	480	225 + 150 + 100	480	240 + 240
<i>Candida vinaria</i>	11177 ^T	560	290 + 160 + 110	460 + 100	250 + 200 + 100
<i>Candida vini</i>	10053, 11137, 11174	500	230 + 180 + 90	500	220 + 220 + 60
<i>Candida wickerhamii</i>	10231 ^T	660	600	560 + 100	325 + 325
<i>Candida zeylanoides</i>	1441	620	295 + 295	415 + 140	310 + 310
<i>Debaromyces hansenii</i>	10386	650	300 + 300 + 50	420 + 150 + 90	325 + 325
<i>Debaromyces polymorphus</i>	1132	730	300 + 200 + 180 + 100	650 + 80	310 + 200 + 140 + 100
	10043	650	300 + 300 + 50	420 + 150 + 90	325 + 325
<i>Debaromyces pseudopolymorphus</i>	10018, 10286	650	300 + 300 + 50	420 + 150 + 90	325 + 325
<i>Dekkera anomala</i>	11162 ^T	800	340 + 340 + 120	800	360 + 190 + 160 + 80
<i>Dekkera bruxellensis</i>	1009, 1010, 1451 ^T , 1452, 11045	485	255 + 140 + 90	375 + 95	270 + 215
<i>Hansenula mrakii</i>	11031	610	580	330 + 140 + 85	305 + 305
<i>Issatchenkia terricola</i>	11139, 11176	450	130 + 100 + 90 + 85 + 45	290 + 125	240 + 105 + 105
<i>Kluyveromyces aestuarii</i>	1949, 1955	750	285 + 165 + 125 + 100 + 65	640 + 95	365 + 180 + 165 + 40
<i>Kluyveromyces africanus</i>	1963 ^T	780	350 + 320 + 105	680 + 110	395 + 385
<i>Kluyveromyces bacillisporus</i>	1979 ^T	820	330 + 305 + 105 + 105	550 + 280	310 + 285 + 115 + 110
<i>Kluyveromyces blattae</i>	1964 ^T	530	280 + 250	380 + 150	295 + 235
<i>Kluyveromyces delphensis</i>	1954 ^T	765	350 + 320 + 90	420 + 240 + 100	380 + 245 + 140
<i>Kluyveromyces dobzhanskii</i>	1952 ^T , 10147, 10177, 10194	740	285 + 190 + 165 + 90	655 + 80	240 + 185 + 120 + 115 + 80
<i>Kluyveromyces lactis</i>	1121, 1961 ST , 10356, 10669	740	285 + 190 + 165 + 90	655 + 80	290 + 180 + 120 + 80 + 65
<i>(Kluyveromyces marxianus</i> var. <i>drasophilum)</i>	10390, 11337	740	285 + 190 + 165 + 90	655 + 80	240 + 185 + 120 + 80 + 65 + 50
<i>(Kluyveromyces marxianus</i> var. <i>phaseolusporus)</i>	11340	740	285 + 190 + 165 + 90	655 + 80	240 + 185 + 185 + 65 + 50 + 15
<i>Kluyveromyces lodderae</i>	1126 ^T	740	320 + 320 + 100	640 + 100	350 + 240 + 110 + 55
<i>Kluyveromyces marxianus</i>	1123, 10368, 10585 ^T , 10668	740	285 + 185 + 140 + 100	655 + 80	240 + 185 + 120 + 80 + 65 + 50
<i>Kluyveromyces phaffii</i>	10646 ^T	575	190 + 150 + 100 + 95 + 60	520 + 50	315 + 250
<i>Kluyveromyces polysporus</i>	1960 ^T	755	330 + 320 + 110	650 + 100	390 + 365
<i>Kluyveromyces thermotolerans</i>	1962, 10141, 10183, 10559	700	315 + 285 + 95	310 + 215 + 90 + 90	355 + 345
<i>Kluyveromyces waltii</i>	1950 ^T	700	315 + 285 + 95	310 + 215 + 90 + 90	350 + 350
<i>Kluyveromyces wickerhamii</i>	1953, 1966 ^T	740	285 + 190 + 165 + 100	665 + 80	240 + 185 + 120 + 80 + 65 + 50
<i>Kluyveromyces yarrowii</i>	1958 ^T	780	320 + 240 + 110 + 100	680 + 100	410 + 370
<i>Lodderomyces elongisporus</i>	10645	600	330 + 250	550	300 + 270
<i>Metschnikowia pulcherrima</i>	1691, 10408, 10546, 11202 ^T	400	205 + 100 + 95	280 + 100	200 + 190
<i>Pichia angophorae</i>	1899 ^T	750	350 + 320 + 80	750	410 + 340
<i>Pichia angusta</i>	10220	750	370 + 300 + 80	500 + 150 + 100	425 + 315
<i>Pichia anomala</i>	1110, 1114, 10320, 10410, 10590, 10591, 10667	650	575	600 + 50	310 + 310
<i>Pichia capsulata</i>	11013	650	290 + 290	490 + 125 + 35	230 + 175 + 150 + 95
<i>Pichia carsonii</i>	10227 ^T , 10230, 10541	650	290 + 290	410 + 145 + 90	310 + 310
<i>Pichia castillae</i>	10017 ^T	650	300 + 290 + 60	410 + 145 + 90	310 + 175 + 140
<i>Pichia delftensis</i>	10238 ^T	500	260 + 180 + 60	500	270 + 230
<i>Pichia fabianii</i>	1921	650	290 + 200 + 90	560 + 90	310 + 310
<i>Pichia farinosa</i>	1447, 10348	700	300 + 300 + 80	440 + 175 + 80	350 + 185 + 160
<i>Pichia fermentans</i>	1455 ^T , 10064, 10078, 10413, 10454	450	170 + 100 + 100 + 80	340 + 80 + 30	250 + 200
<i>Pichia fluxuum</i>	10659	500	230 + 180 + 90	500	240 + 240

Table 1 (cont.)

Species*	CECT strain no.	AP†	Restriction fragments		
			<i>CfoI</i>	<i>HaeIII</i>	<i>HinII</i>
<i>Pichia guilliermondii</i>	1019, 1021, 1438, 1456 ^T	625	300+265+60	400+115+90	320+300
<i>Pichia jadinii</i>	1060	575	280+230	390+145+40	300+275
<i>Pichia kluyveri</i>	11023 ^T	450	175+115+80+80	370+80	250+200
<i>Pichia membranaefaciens</i>	1115, 10570, 10568	500	175+110+90+75	330+90+50	275+200
	10037, 10113	500	260+110+75	330+90+50	275+200
<i>Pichia pastoris</i>	11078	380	360	380	250+130
<i>Pichia pijperi</i>	10662 ^T	650	300+300	425+140+85	310+190+120
<i>Pichia scolymii</i>	10661	650	300+300	425+140+85	310+310
<i>Pichia segobiensis</i>	10210 ^T	650	300+285	490+140	310+310
<i>Pichia stiptis</i>	1922 ^T	650	300+285	490+140	310+310
<i>Saccharomyces bayanus</i>	1941 ^T , 1969	880	385+365	500+220+145	365+155
<i>Saccharomyces cerevisiae</i>	1942 ^T , 1971	880	385+365	320+230+180+150	365+155
<i>Saccharomyces exiguus</i>	11192 ^T	675	320+200+90	400+200+75	400+275
<i>Saccharomyces paradoxus</i>	1939 ^T , 11143	880	385+365	320+230+180+150	440+440
<i>Saccharomyces pastorianus</i>	1940 ^T , 1320	880	385+365	500+220+145	365+155
<i>Saccharomycodes ludwigii</i>	10450 ^T , 11141, 11191	750	360+350	700	450+275
<i>Saccharomycopsis capsularis</i>	10653 ^{NT}	675	600	500+95+80	335+335
<i>Schizosaccharomyces pombe</i> var. <i>pombe</i>	1379, 11197	1050	600+400	1050	600+450
	1378, 10685	950	525+375	950	500+425
<i>Torulaspora delbrueckii</i>	1880, 10558, 10589, 10651, 10676, 10683, 10693, 10694, 11146, 11199	800	330+220+150+100	800	410+380
<i>Torulaspora globosa</i>	10655	650	300+300	425+150+90	325+325
<i>Torulaspora pretoriensis</i>	10679, 10680	825	375+330+110	800	380+290+125
<i>Wickerhamiella domerquia</i>	11173 ^T	825	300+250	525	275+275
<i>Yarrowia lipolytica</i>	1240	380	210+170	380	190+190
<i>Zygoascus hellenicus</i>	11163	650	325+325	625	350+170+130
<i>Zygosaccharomyces bailii</i>	10674, 11042, 11043	790	320+270+95+95	690+90	340+225+160+55
<i>Zygosaccharomyces bisporus</i>	11055 ^T	790	300+275+110+90	690+100	390+225+150
<i>Zygosaccharomyces cidri</i>	10657	700	310+280+90	300+210+95+95	340+340
<i>Zygosaccharomyces fermentati</i>	10382, 10478, 11056 ^T	700	310+280+90	300+210+95+95	340+340
<i>Zygosaccharomyces florentinus</i>	11200 ^T	600	275+185+80	590	295+295
<i>Zygosaccharomyces mellis</i>	11057 ^{LT}	850	350+250+210	560+200+90	400+270+180
<i>Zygosaccharomyces microellipsoides</i>	11198 ^T	825	350+285+100+90	725+100	425+400
	10039	825	330+220+150+100	800	425+400
<i>Zygosaccharomyces mrakii</i>	10656 ^T	650	290+280+80	390+115+80	300+190+130
<i>Zygosaccharomyces rouxii</i>	1230, 1232, 10137, 11136, 11189	750	290+200+170+90	400+210+90	350+260+140

* Original epithets are indicated in parentheses.

† PCR-amplified product.

Table 2. Size in bp of PCR products and restriction fragments of ascomycetous yeasts in the genus *Hanseniaspora*To differentiate these species, restriction analysis with *DdeI* was required.

Species	CECT strain no.	AP*	Restriction fragments			
			<i>CfoI</i>	<i>DdeI</i>	<i>HaeIII</i>	<i>HinII</i>
<i>Hanseniaspora guilliermondii</i>	11027, 11029 ^T , 11102, 11104, 11111, 11155, 11205	750	320+310+105	380+180+95+80	750	350+200+180
<i>Hanseniaspora osmophila</i>	11206	750	275+150+135+95+75	650+100	460+120+90+80	390+360
	11207	750	275+185+150+95	650+100	460+120+90+80	390+360
<i>Hanseniaspora uvarum</i>	1444, 10389, 11105, 11106, 11107, 11156, 11172	750	320+310+105	300+180+95+90+85	750	350+200+180
<i>Hanseniaspora valbyensis</i>	1445 ^T , 10122	750	630+120	290+150+95+95	750	250+220+170+105

* PCR-amplified product.

Table 3. Size in bp of PCR products and restriction fragments of teleomorphic and anamorphic basidiomycetous yeasts

Species*	CECT strain no.	AP†	Restriction fragments		
			<i>CfoI</i>	<i>HaeIII</i>	<i>HinfI</i>
<i>Cryptococcus albidus</i>	1069, 1072, 1073	630	330 + 300	500 + 70 + 60	350 + 160 + 120
(<i>Cryptococcus diffluens</i> var. <i>uruguayensis</i>)	1086 ^T , 11138	630	330 + 300	500 + 70 + 60	350 + 280
<i>Cryptococcus ater</i>	1084 ^T	650	350 + 300	500 + 90 + 60	275 + 245 + 130
<i>Cryptococcus bhutanensis</i>	1085 ^T	630	330 + 300	500 + 70 + 60	350 + 280
<i>Cryptococcus curvatus</i>	1992	700	305 + 300 + 50 + 45	460 + 160 + 80	320 + 320 + 60
<i>Cryptococcus dimennae</i>	1087 ^T	500	255 + 245	420 + 40 + 40	280 + 220
<i>Cryptococcus flavus</i>	1074	540	190 + 180 + 90	460 + 80	230 + 170 + 70 + 60
<i>Cryptococcus gastricus</i>	1089 ^T	630	630	505 + 65 + 60	235 + 150 + 135 + 110
<i>Cryptococcus himalayensis</i>	1090 ^T	660	345 + 315	400 + 70 + 70 + 60 + 60	370 + 290
<i>Cryptococcus humicola</i>	1251	520	265 + 255	425 + 55 + 40	230 + 230 + 60
<i>Cryptococcus hungaricus</i>	1091	500	260 + 240	500	260 + 240
<i>Cryptococcus kuetzingii</i>	1093 ^T	630	330 + 300	500 + 70 + 60	350 + 280
<i>Cryptococcus laurentii</i>	1095	600	575	600	340 + 260
	11171	550	290 + 260	375 + 100	270 + 270
<i>Cryptococcus luteolus</i>	1099	490	260 + 230	490	250 + 240
<i>Cryptococcus macerans</i>	1101 ^T	650	260 + 200 + 95 + 95	440 + 80 + 70 + 60	270 + 200 + 175
<i>Cryptococcus magnus</i>	1103	650	355 + 295	495 + 95 + 60	270 + 240 + 140
<i>Cryptococcus marinus</i>	1975	500	260 + 140 + 90	500	220 + 210 + 70
<i>Cryptococcus skinneri</i>	1105 ^T	565	260 + 200	400 + 110	220 + 150 + 120 + 60
<i>Cryptococcus terreus</i>	1106 ^T	900	400 + 180 + 165 + 155	670 + 230	360 + 270 + 270
<i>Cryptococcus uniguttulatus</i>	1108	650	355 + 295	500 + 150	270 + 240 + 140
<i>Filobasidium capsuligenum</i>	11190	650	340 + 300	550 + 100	360 + 280
<i>Rhodotorula acuta</i>	11175	675	355 + 315	650	250 + 190 + 185
<i>Rhodotorula glutinis</i>	1137	640	320 + 240 + 80	430 + 210	340 + 225 + 75
<i>Rhodotorula graminis</i>	10097	660	320 + 290	400 + 215	230 + 215 + 150
<i>Rhodotorula minuta</i>	10033	660	300 + 300	400 + 215	345 + 215
<i>Rhodotorula mucilaginosa</i>	11010	640	320 + 240 + 80	425 + 215	340 + 225 + 75
<i>Sporidiobolus pararoseus</i>	10153, 1270	630	320 + 310	410 + 220	250 + 230 + 120
<i>Sporidiobolus salmonicolor</i>	1274, 11115	600	400 + 240	410 + 140 + 95	300 + 250 + 125
<i>Sporobolomyces roseus</i>	1930	610	320 + 250	600	280 + 120 + 105 + 105
<i>Trichosporon beigelii</i>	1304	550	280 + 90 + 90 + 90	525	300 + 250

* Original epithets are indicated in parentheses.

† PCR-amplified product.

Pichia anomala, *Pichia fermentans*, *Pichia guilliermondii* and *Pichia membranaefaciens*. Among the strains of *Pichia membranaefaciens* we found two different patterns with *CfoI* (CECT 10037 and CECT 10113). This could be a way recognizing varieties within this heterogeneous species.

Schizosaccharomyces pombe, characterized by its special mode of vegetative reproduction and a certain degree of osmophily, is probably the only one to cause food spoilage of the three species of the genus. This species presents a great variability in the size of the 5-8S region, as described by Schaak *et al.* (1982). In the four strains analysed, we found two fragment lengths (950 bp and 1050 bp) that agree with the two different restriction patterns, one of them for the strains CECT

1378 and CECT 10685, and the other for CECT 1379 and CECT 11197 (see Table 1).

In the genus *Torulaspora* three species are accepted according to Barnett *et al.* (1990), although the most frequently isolated from foods is *Torulaspora delbrueckii*. We observed the same pattern for ten strains of this species, and species-specific patterns for the other two species of the genus, *Torulaspora globosa* and *Torulaspora pretoriensis*.

The species of the genus *Zygosaccharomyces* are important osmotolerant food spoilage ascomycetous yeasts. In this study, we analysed 18 strains from the 9 species of this genus, and we could associate each species with a particular pattern, with the exception of

Zygosaccharomyces cidri and *Zygosaccharomyces fermentati*, which yielded the same pattern with all the endonucleases used (*AluI*, *CfoI*, *Dde I*, *HaeIII*, *HinfI*, *ScrFI* and *TaqI*). The two strains of *Zygosaccharomyces microellipsoides* showed the same pattern using only the endonuclease *HinfI*, but were polymorphic for the other two enzymes. For this reason we recommend the use of only *HinfI* to identify this species.

Teleomorphic and anamorphic basidiomycetous yeasts

Species belonging to the genus *Cryptococcus* are defined by a combination of morphological and physiological characters, the absence of pseudomycelium, colony pigmentation and ultrastructure of the septal pore (Guého *et al.*, 1989). Due to the lack of fermentation and sexual reproduction their identification by conventional approaches is rather difficult, so we propose this new method also for the identification of the species of this genus. The amplified fragments present a high degree of variation in size, ranging from 490 bp for *Cryptococcus luteolus* (CECT 1099) to 900 bp for *Cryptococcus terreus* (CECT 1106). Using the endonucleases *HaeIII* and *CfoI*, all the strains of *Cryptococcus albidus* var. *albidus* yielded the same restriction fragments, but with *HinfI* the strains CECT 11138 and CECT 1086 (type strain of *Cryptococcus diffluens* var. *uruguiensis*) showed a different restriction pattern, which could be an indication of the existence of a different variety or species. The species *Cryptococcus magnus* and *Cryptococcus uniguttulatus* have the same PCR product size and restriction patterns with *HinfI* and *CfoI*. They can be distinguished by using the endonuclease *HaeIII* (see Table 3).

The genera *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces* include basidiomycetous yeasts producing red- or orange-pigmented colonies. An important characteristic of *Rhodotorula* species is the inability to assimilate inositol, which distinguishes *Rhodotorula* from *Cryptococcus* (Krejer-van Rij, 1987). We could differentiate the five species analysed in the present study. The species *Rhodotorula graminis* and *Rhodotorula minuta* had the same pattern with *HaeIII*, but different patterns with *CfoI* and *HinfI*. With these endonucleases it is possible to obtain a species-specific pattern.

Conclusions

The restriction patterns of the 5.8S-ITS region have been used to differentiate 132 yeast species belonging to 25 different yeast genera, the majority isolated from food and some additional species representing related genera. Analysis of Tables 1, 2 and 3 confirmed that this method can be used to differentiate all the species with the exceptions of *Cr. albidus* (strains previously identified as *Cr. diffluens* var. *uruguiensis*)/*Cr.*

bhutanensis/*Cr. kuetzingii*; *Deb. polymorphus*/*Deb. pseudopolymorphus*; *P. segobiensis*/*P. stipitis*; *Sacch. bayanus*/*Sacch. pastorianus*; *Zygosacch. cidri*/*Zygosacch. fermentati*. In all cases, the method proved to be reproducible and very useful to easily and rapidly identify and classify all the species included in the present work. This special feature has been used for identification of wine yeast species (Guillamón *et al.*, 1998) and also for strains isolated from cheese and fermented sausages (unpublished).

Using this method, the anamorph and teleomorph forms yielded the same pattern, as exemplified by the pairs *Br. bruxellensis*/*D. bruxellensis*, *H'spora uvarum*/*Kl. apiculata* and *T'spora delbrueckii*/*C. colliculosa*. In addition, the majority of the strains of the same species showed the same restriction pattern with all the enzymes analysed, with the exception of *K. lactis* (Belloch *et al.*, 1998) and *Cr. albidus*, where strains previously identified as synonymous could be recognized as varieties.

Finally, the results presented in this work constitute an initial database, and should be supplemented with further species not used in the present work. To prevent errors we recommend the use of the same conditions that we have applied in this study, namely to separate the PCR products on 1.4% and the restriction fragments on 3% agarose gels. In the case of doubt, we suggest including the type strains in the same gel and, finally, it is worth noting that fragments smaller than 50 bp could not be clearly visualized and were not considered.

ACKNOWLEDGEMENTS

This work was supported by CICYT grants to A. Q. (ALI96-0457-CO2-01) and to F. U. (ALI96-0457-CO2-02). B. E. was the recipient a FPI fellowship from the Spanish Government. We express our gratitude to J. M. Guillamón for participating in the application of this method to wine yeast identification. These preliminary results showed us the potentiality of the technique. Thanks are due to D. Ramón and E. Barrio for the critical discussion of the manuscript.

REFERENCES

- Augustyn, O. P. H., Ferreira, D. & Kock, J. L. F. (1991). Differentiation between yeast species, and strains within species by cellular fatty acid analysis. 4. *Saccharomyces sensu stricto*, *Hanseniaspora*, *Saccharomycodes* and *Wickerhamiella*. *Syst Appl Microbiol* **14**, 324-334.
- Baleiras Couto, M. M., van der Vossen, J. M., Hofstra, H. & in 't Veld, J. H. (1994). RAPD analysis: a rapid technique for differentiation of spoilage yeasts. *Int J Food Microbiol* **24**, 249-260.
- Baleiras Couto, M. M., Eijlsma, B., Hofstra, H., in 't Veld, J. H. & van der Vossen, J. M. (1996). Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol* **62**, 41-46.
- Barnett, J. A., Payne, R. W. & Yarrow, D. (1990). *Yeasts: Characteristics and Identification*, 2nd edn. Cambridge: Cambridge University Press.

- Belloch, C., Barrio, E., Uruburu, F., García, M. D. & Querol, A. (1997). Characterization of four species of the genus *Kluyveromyces* by mitochondrial DNA restriction analysis. *Syst Appl Microbiol* **20**, 397–408.
- Belloch, C., Barrio, E., García, M. D. & Querol, A. (1998). Phylogenetic reconstruction of the genus *Kluyveromyces*: restriction map analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Syst Appl Microbiol* **21**, 266–273.
- Botelho, A. R. & Planta, R. J. (1994). Specific identification of *Candida albicans* by hybridization with oligonucleotides derived from ribosomal DNA internal spacers. *Yeast* **10**, 709–717.
- Cai, J., Roberts, I. N. & Collins, M. D. (1996). Phylogenetic relationships among members of the ascomycetous yeast genera *Brettanomyces*, *Debaryomyces*, *Dekkera* and *Kluyveromyces* deduced by small-subunit rRNA gene sequences. *Int J Syst Bacteriol* **46**, 542–549.
- Carlie, M. J. & Watkinson, S. C. (1994). *The Fungi*. London: Academic Press.
- Cottrell, M., Kock, J. L. F., Lategan, P. M. & Britz, T. J. (1986). Long-chain fatty acid composition as an aid in the classification of the genus *Saccharomyces*. *Syst Appl Microbiol* **8**, 166–168.
- Deák, T. (1993). Simplified techniques for identifying foodborne yeasts. *Int J Food Microbiol* **19**, 15–26.
- Deák, T. (1995). Methods for the rapid detection and identification of yeasts in foods. *Trends Food Sci Tech* **6**, 287–292.
- Deák, T. & Beuchat, L. R. (1996). *Handbook of Food Spoilage Yeasts*. Boca Raton, FL: CRC Press.
- Golden, D. A., Beuchat, L. R. & Hitchcock, H. L. (1994). Changes in fatty acid composition of *Zygosaccharomyces rouxii* as influenced by solutes, potassium sorbate and incubation temperature. *Int J Food Microbiol* **21**, 293–303.
- Guého, E., Kurtzman, C. P. & Peterson, S. W. (1989). Evolutionary affinities of heterobasidiomycetous yeasts estimated from 18S and 25S ribosomal RNA sequence divergence. *Syst Appl Microbiol* **12**, 230–236.
- Guillamón, J. M., Querol, A., Jimenez, M. & Huerta, T. (1993). Phylogenetic relationships among wine yeast strains based on electrophoretic whole-cell protein patterns. *Int J Food Microbiol* **18**, 115–125.
- Guillamón, J. M., Barrio, E., Huerta, T. & Querol, A. (1994). Rapid characterization of four species of the *Saccharomyces sensu stricto* complex according to mitochondrial DNA patterns. *Int J Syst Bacteriol* **44**, 708–714.
- Guillamón, J. M., Barrio, E. & Querol, A. (1996). Characterization of wine yeast strains of the *Saccharomyces* genus on the basis of molecular markers. Relationships between genetic distance and geographic origin. *Syst Appl Microbiol* **19**, 122–132.
- Guillamón, J. M., Sanchez, I. & Huerta, T. (1997). Rapid characterization of wild and collection strains of the genus *Zygosaccharomyces* according to mitochondrial DNA patterns. *FEMS Microbiol Lett* **147**, 267–272.
- Guillamón, J. M., Sabaté, J., Barrio, E., Cano, J. & Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal ITS regions. *Arch Microbiol* **169**, 387–392.
- Huffman, J. L., Molina, F. I. & Jong, S. C. (1992). Authentication of ATCC strains in the *Saccharomyces cerevisiae* complex by PCR fingerprinting. *Exp Mycol* **16**, 316–319.
- Ibeas, J. I., Lozano, I., Perdígones, L. & Jimenez, J. (1996). Detection of *Dekkera/Brettanomyces* strains in sherry by a nested PCR method. *Appl Environ Microbiol* **62**, 998–1003.
- Ibeas, J. I., Lozano, I., Perdígones, F. & Jimenez, J. (1997). Dynamics of flor yeast populations during the biological aging of sherry wines. *Am J Enol Vitic* **48**, 75–79.
- Iwaguchi, S.-I., Homma, M. & Tanaka, K. (1990). Variation in the electrophoretic karyotype analysed by the assignment of DNA probes in *Candida albicans*. *J Gen Microbiol* **136**, 2433–2442.
- James, S. A., Collins, M. D. & Roberts, I. N. (1996). Use of an rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulaspora*. *Int J Syst Bacteriol* **46**, 189–194.
- Jordan, J. A. (1994). PCR identification of four medically important *Candida* species by using a single primer pair. *J Clin Microbiol* **32**, 2962–2967.
- Krejer-van Rij, N. J. W. (1984). *The Yeasts: a Taxonomic Study*, 3rd edn. Amsterdam: Elsevier.
- Krejer-van Rij, N. J. W. (1987). Classification of yeasts. In *The Yeasts*, vol 1, *Biology of the Yeasts*, 2nd edn, pp. 5–61. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Kurtzman, C. P. (1984). Synonymy of the yeast genera *Hansenula* and *Pichia* demonstrated through comparisons of deoxyribonucleic acid relatedness. *Antonie Leeuwenhoek* **50**, 209–217.
- Kurtzman, C. P. (1992). rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *Int J Syst Bacteriol* **42**, 1–6.
- Kurtzman, C. P. (1993). Systematics of the ascomycetous yeasts assessed from ribosomal RNA sequence divergence. *Antonie Leeuwenhoek* **63**, 165–174.
- Kurtzman, C. P. & Fell, J. W. (1998). *The Yeasts – a Taxonomic Study*, 4th edn. Amsterdam: Elsevier.
- Li, W. H. (1997). *Molecular Evolution*. Sunderland, MA: Sinauer Associates.
- Lin, C. C. S. & Fung, Y. (1987). Conventional and rapid methods for yeast identification. *Crit Rev Microbiol* **14**, 273–289.
- Lopandic, K., Prillinger, H., Molnár, O. & Gimenez-Jurado, G. (1996). Molecular characterization and genotypic identification of *Metschnikowia* species. *Syst Appl Microbiol* **19**, 393–402.
- Meyer, S. A., Smith, M. Th. & Simone, F. P., Jr (1978). Systematics of *Hanseniaspora* Zikes and *Kloeckera* Janke. *Antonie Leeuwenhoek J Microbiol Serol* **44**, 79–96.
- Molina, F. I., Inoue, T. & Jong, S. C. (1992). Ribosomal DNA restriction analysis reveals genetic heterogeneity in *Saccharomyces cerevisiae* Meyen ex Hansen. *Int J Syst Bacteriol* **42**, 499–502.
- Moreira da Silva, M., Malfeito-Ferreira, M., Aubyn, St. & Loureiro, V. (1994). Long-chain fatty acid composition as a criterion for yeast distinction in the brewing industry. *J Inst Brew* **100**, 17–22.
- Nadal, D., Colomer, B. & Piña, B. (1996). Molecular polymorphism distribution in phenotypically distinct populations of wine yeast strains. *Appl Environ Microbiol* **62**, 1944–1950.
- Naumov, G. I., Naumova, E. S. & Sniegowski, P. D. (1997). Differentiation of European and far east Asian populations of *Saccharomyces paradoxus* by allozyme analysis. *Int J Syst Bacteriol* **47**, 341–344.
- Perez, L., Martinez, P., Codon, A. C. & Benítez, T. (1995). Physiological and molecular characterization of flor yeasts: polymorphisms of flor yeast populations. *Yeast* **11**, 1399–1411.
- Querol, A., Barrio, E., Huerta, T. & Ramón, D. (1992a). Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl Environ Microbiol* **58**, 2948–2953.
- Querol, A., Barrio, E. & Ramón, D. (1992b). A comparative study

- of different methods of yeast strain characterization. *Syst Appl Microbiol* **15**, 439–446.
- Quesada, M. P. & Cenis, J. L. (1995).** Use of random amplified polymorphic DNA (RAPD)-PCR in the characterization of wine yeasts. *Am J Enol Vitic* **46**, 204–208.
- Romano, A., Casaregola, S., Torre, P. & Gaillardin, C. (1996).** Use of RAPD and mitochondrial DNA RFLP for typing of *Candida zeylanoides* and *Debaryomyces hansenii* yeast strains isolated from cheese. *Syst Appl Microbiol* **19**, 255–264.
- Schaak, J., Mao, J. I. & Soell, D. G. (1982).** The 5.8S RNA gene sequence and the ribosomal repeat of *Schizosaccharomyces pombe*. *Nucleic Acids Res* **10**, 2851–2864.
- Schüctz, M. & Gafner, J. (1993).** Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J Appl Bacteriol* **75**, 551–558.
- Török, T., Rockhold, D. & King, A. D. (1993).** Use of electrophoretic karyotyping and DNA-DNA hybridization in yeast identification. *Int J Food Microbiol* **19**, 63–80.
- Tredoux, H. G., Kock, J. L. F., Lategan, P. M. & Muller, H. B. (1987).** A rapid identification technique to differentiate between *Saccharomyces cerevisiae* strains and other yeasts species in the wine industry. *Am J Enol Vitic* **38**, 161–164.
- Valente, P., Gouveia, F. C., de Lemos, G. A., Pimentel, D., van Elsas, J. D., Mendonça-Hagler, L. C. & Hagler, A. N. (1996).** PCR amplification of the rDNA internal transcribed spacer region for differentiation of *Saccharomyces* cultures. *FEMS Microbiol Lett* **137**, 253–256.
- Vancanneyt, B. P., Hennebert, G. & Kersters, K. (1991).** Differentiation of yeast species based on electrophoretic whole-cell protein patterns. *Syst Appl Microbiol* **14**, 23–32.
- Vaughan Martini, A. & Martini, A. (1985).** Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces sensu stricto*. *Int J Syst Bacteriol* **35**, 508–511.
- Vaughan Martini, A. & Martini, A. (1987).** Taxonomic revision of the yeast genus *Kluyveromyces* by nuclear deoxyribonucleic acid reassociation. *Int J Syst Bacteriol* **37**, 380–385.
- Versavaud, A. & Hallet, J. N. (1995).** Pulsed-field gel electrophoresis combined with rare-cutting endonucleases for strain identification of *Candida famata*, *Kloeckera apiculata* and *Schizosaccharomyces pombe* with chromosome number and size estimation of the two former. *Syst Appl Microbiol* **18**, 303–309.
- van Vuuren, H. J. J. & van der Meer, L. (1987).** Fingerprinting of yeasts by protein electrophoresis. *Am J Enol Vitic* **38**, 49–53.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990).** PCR protocols. A guide to methods and applications. In *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.
- Wyder, M. T. & Puhán, Z. (1997).** A rapid method for identification of yeasts from kefir at species level. *Michwissenschaft* **52**, 327–330.
- Yamada, Y., Maeda, K. & Banno, I. (1992).** The phylogenetic relationships of the Q6-equipped species in the teleomorphic apiculate yeast genera *Hanseniaspora*, *Nadsonia* and *Saccharomyces* based on the partial sequences of 18S and 26S ribosomal ribonucleic acids. *J Gen Appl Microbiol* **38**, 585–596.