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International Journal of Food Microbiology 107 (2006) 48 – 58
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

DNA typing methods for differentiation of yeasts related to dry-cured meat products

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Received 10 January 2005; received in revised form 3 August 2005; accepted 7 August 2005

Abstract

RFLP analysis of the ITS and 18S rDNA, RAPD-PCR using mini- and microsatellite primers and RFLP analysis of mitochondrial DNA were examined to discriminate yeasts related to dry-cured meat products at species and strain level. Seven species and 35 strains of yeasts usually found in dry-cured meat products were tested. RFLP analysis of the ITS1-5.8S rDNA-ITS2 and 18S rDNA did not allow the separation at species level of all of the species tested. RAPD with a M13 primer was found to be useful for differentiation of *Rhodotorula mucilaginosa*, *Candida zeylanoides*, *Yarrowia lipolytica*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae*. However, no differences were observed between *Debaryomyces polymorphus* and *Pichia carsonii*. RAPD analysis with microsatellite primers $(GACA)_4$, $(GTG)_5$ and $(GAC)_5$ enabled discrimination at species and strain level. However, the degree of discrimination by means of RAPD-PCR depends highly on the primers used. Thus, the PCR fingerprinting with primer $(GACA)_4$ enabled a higher level of discrimination than primers $(GAC)_5$ and $(GTG)_5$. The RFLP analysis of mtDNA allowed the discrimination at the species and strain level except for *R. mucilaginosa*, where no polymorphisms were observed in the strains tested. RAPD analysis with primer $(GACA)_4$ and the restriction analysis of mtDNA used in the present work are useful for the differentiation at species and strain level of yeasts related to dry-cured meat products.

Keywords: Yeast; RAPD-PCR; mtDNA RFLP; ITS; Dry-cured meat products

1. Introduction

Yeasts are predominant microorganisms during ripening period of different products of intermediate moisture such as dry-cured meat products (Núñez et al., 1996). *Debaryomyces hansenii* is the most common species in the above products (Fleet, 1990; Núñez et al., 1996). Unlike other yeasts species such as *Candida zeylanoides*, *Yarrowia lipolytica* and *Rhodotorula* spp. it can be isolated in these products. Some of these species yield a beneficial effect to dry-cured meat products, since they provide proteolytic and lipolytic effects and could be involved in the generation of volatile compounds. However, some of the above species such as *Y. lipolytica* and *C. zeylanoides* and even some strains of *D. hansenii* have been reported to be involved in spoilage of meat products (Diriye et al., 1993; Ismail et al., 2000; Martínez et al., 2004). To avoid the presence of spoilage species or strains of yeasts, technologically tested yeasts as starter cultures could be used in dry-cured meat products. To differentiate starter cultures from spoilage yeasts rapid and simple methods should be applied to quality control systems.

Traditionally, yeasts have been characterized by morphological and physiological traits (Barnett et al., 1990; Núñez et al., 1996). These methods are laborious and time-consuming, and are, therefore, not suitable for routine analysis in quality control systems. Moreover, these characteristics are influenced by culture conditions and can provide uncertain results (Yamamoto et al., 1991). The introduction of molecular methods provided new approaches to industrial yeasts differentiation (Querol and Ramón, 1996; Loureiro and Querol, 1999).

Several DNA based methods such as RFLP analysis of the 5.8S and 18S rDNA, RAPD-PCR and RFLP analysis of mitochondrial DNA have been used to discriminate wine yeasts at strain level (Capece et al., 2003; Schuller et al., 2004). In ripened cheese the above and related methods have been

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doi:10.1016/j.ijfoodmicro.2005.08.011

Table 1
Yeasts strains used in this study

Species designation	Strain reference
Candida zeylanoides	CECT 10048
Candida zeylanoides	CECT 10051
Candida zeylanoides	CECT 1441
Candida zeylanoides	CECT 10125
Candida zeylanoides	CECT 10128
Debaryomyces polymorphus	CECT 10282
Debaryomyces polymorphus	CECT 10251
Debaryomyces polymorphus	CECT 10099
Debaryomyces polymorphus	CECT 10135
Debaryomyces polymorphus	CECT 10280
Debaryomyces hansenii	CECT 10352
Debaryomyces hansenii	CECT 10026
Debaryomyces hansenii	CECT 10360
Debaryomyces hansenii	CECT 10202
Debaryomyces hansenii	CECT 10353
Pichia carsonii	CECT 10230
Pichia carsonii	CECT 10229
Pichia carsonii	CECT 1129
Pichia carsonii	CECT 10307
Pichia carsonii	CECT 10542
Rhodotorula mucilaginosa	CECT 10291
Rhodotorula mucilaginosa	CECT 10359
Rhodotorula mucilaginosa	CECT 10044
Rhodotorula mucilaginosa	CECT 10087
Rhodotorula mucilaginosa	CECT 11016
Saccharomyces cerevisiae	CECT 1414
Saccharomyces cerevisiae	CECT 1387
Saccharomyces cerevisiae	CECT 1443
Saccharomyces cerevisiae	CECT 1477
Saccharomyces cerevisiae	CECT 1485
Yarrowia lipolytica	CECT 1468
Yarrowia lipolytica	CECT 1469
Yarrowia lipolytica	CECT 10358
Yarrowia lipolytica	CECT 10363
Yarrowia lipolytica	CECT 10448

CECT: Spanish type culture collection.

assayed to discriminate *D. hansenii* and *C. zeylanoides* at strain level (Romano et al., 1996; Petersen et al., 2001, 2002). However, there are few DNA-based methods to differentiate spoilage yeasts species such as *Y. lipolytica* and *C. zeylanoides* from normal yeasts of dry-cured meat products. Chromosome length polymorphism by pulsed-field gel electrophoresis has been proven to be useful for strains typing of *Y. lipolytica* and *C. zeylanoides* (Deák et al., 2000). However, this DNA technique is laborious and time-consuming. Thus, it is not adequate for rapid routine differentiation of yeasts of dry-cured meat products.

In the present work the RFLP analysis of the ITS and 18S rDNA, RAPD-PCR using mini- and microsatellite primers and RFLP analysis of mitochondrial DNA were examined to discriminate yeasts species usually found in dry-cured meat products. In addition, the ability of the above methods to discriminate yeasts at strain level was also tested.

2. Materials and methods

2.1. Yeast strains

Representative spoilage and normal yeasts species usually found in ripened products were used in this study (Table 1). All the strains tested were obtained from the Spanish Type Culture Collection (CECT) (López-Coronado, 2002). All the yeasts used were purified on repeated cultivation on malt extract agar (2% malt extract, 2% glucose, 0.1% peptone, 2% agar) before analysis. Characterization of these strains was confirmed by API ID 20 C kit (Biomerieux, Lyon, France) and the tests proposed by Deák and Beuchat (1987): cellular morphology in Yeast Morphology Agar and assimilation of D-lactose, D-maltose, D-raffinose, D-ellobiose, D-sucrose and D-xylose in Yeast Nitrogen Base (BD Difco, New Jersey, USA).

2.2. DNA isolation

Pure cultures of each yeast strain were grown in 10 ml of YPD (1% yeast extract, 2% peptone, 2% glucose) at 25 °C for 48 h at 250 rpm on an orbital shaker. DNA was isolated according to the method of Querol et al. (1992). The DNA concentration was spectrophotometrically quantified and brought to a final value of 100 ng/μl.

2.3. RFLP analysis of the ribososomal internal transcribed spacers (ITS) and the 5.8S rDNA region

The amplification of the ITS1-5.8S rDNA-ITS2 region was carried out under the following conditions: each 50 μl reaction mixture containing 200 ng template DNA, 10 mM Tris–HCl, pH 9.0, 2.8 mM $MgCl_2$ 0.2 mM each of dATP, dCTP, dGTP and dTTP; 200 ng of each primer; and 1 U Taq DNA polymerase (Finnzymes, Espoo, Finland). The primers were ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) as described by White et al. (1990). Amplification was performed in a Thermal cycler of Bio Rad (mod. iCycler 170-8731) using an initial denaturation during 5 min at 94 °C followed by 40 cycles consisting of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. A final step of 5 min at 72 °C was carried out.

Table 2
RAPD-PCR conditions with the M13 minisatellite primer and the microsatellite primers $(GACA)_4$, $(GAC)_5$ and $(GTG)_5$

Primer	Initial denaturation	Cycles	Cycles conditions		
			Denaturation	Primer annealing	Extension
M13	94 °C, 3 min	30	94 °C, 45 s	50 °C, 1 min	60 °C, 3 min
$(GACA)_4$	–	30	94 °C, 1 min	36 °C, 1 min	55 °C, 5 min
$(GAC)_5$	–	30	94 °C, 1 min	36 °C, 1 min	55 °C, 5 min
$(GTG)_5$	94 °C, 5 min	30	94 °C, 30 s	45 °C, 1 min	60 °C, 5 min

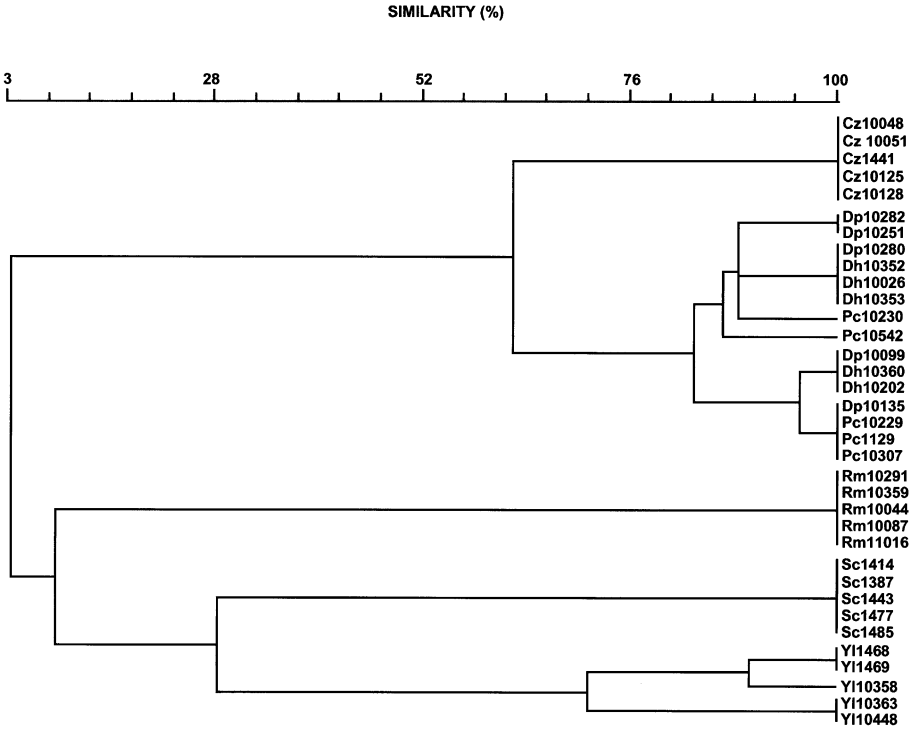

Fig. 1. Dendrogram based on the ITS1-5.8S rDNA-ITS2 restriction profiles obtained by digestion with *Taq*I, *Sau*3AI and *Hae*III of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

Amplification products were separated by electrophoresis in 1% agarose gels and detected by staining with ethidium bromide (0.5 μg/ml).

For RFLP analysis, 11.5 μl PCR products were digested with 2 U of restriction enzymes *Taq*I, *Sau*3AI and *Hae*III (Amersham Biosciences, Uppsala, Sweden) in 15 μl reaction volume, using

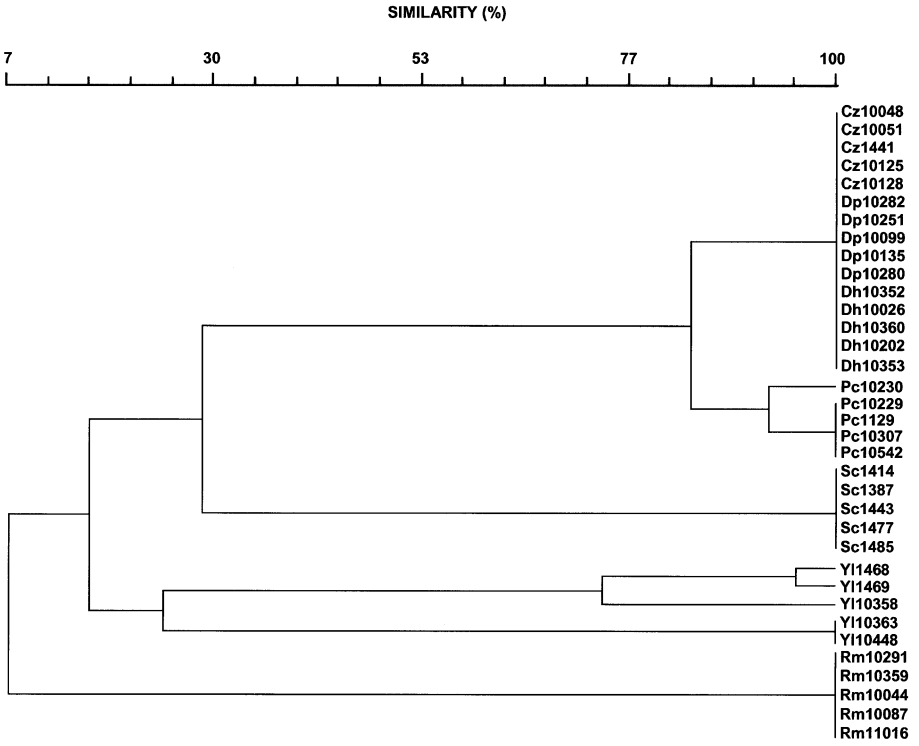

Fig. 2. Dendrogram based on the 18S rDNA restriction profiles obtained by digestion with *Hae*III, *Rsa*I and *Taq*I of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

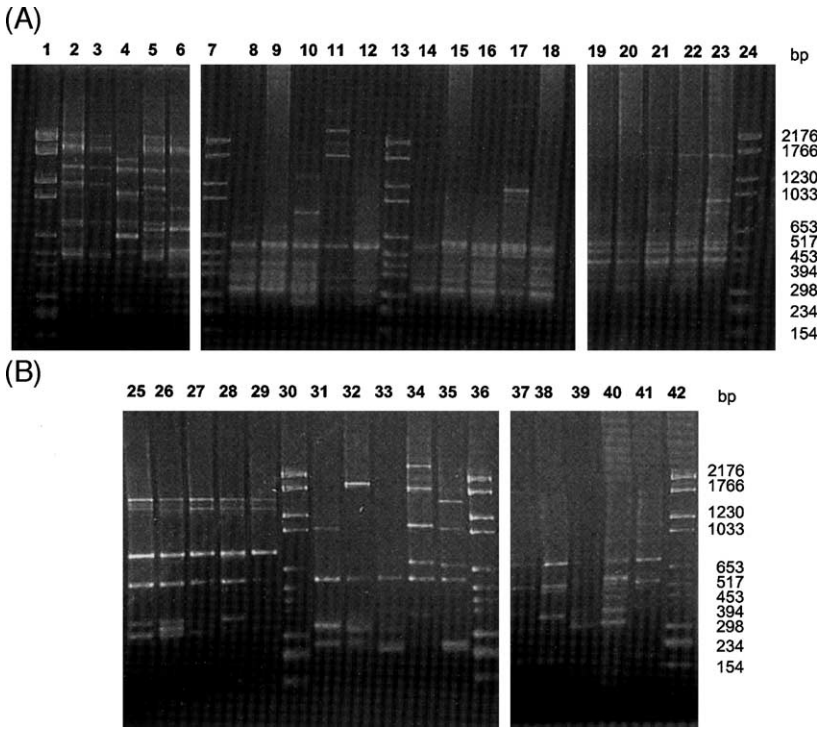

Fig. 3. PCR-fingerprinting patterns obtained with primer M13 from *Y. lipolytica*, *D. polymorphus*, *P. carsonii* and *R. mucilaginosa* (A) and *C. zeylanoides*, *D. hansenii* and *S. cerevisiae* (B). Lanes 1, 7, 13, 24, 30, 36 and 42: DNA molecular size marker of 2.1–0.15 kbp; lane 2: CECT 1468; lane 3: CECT 1469; lane 4: CECT 10358; lane 5: CECT 10363; lane 6: CECT 10448; lane 8: CECT 10282; lane 9: CECT 10251; lane 10: CECT 10099; lane 11: CECT 10135; lane 12: CECT 10280; lane 14: CECT 10230; lane 15: CECT 10229; lane 16: CECT 1129; lane 17: CECT 10307; lane 18: CECT 10542; lane 19: CECT 10291; lane 20: CECT 10359; lane 21: CECT 10044; lane 22: CECT 10087; lane 23: CECT 11016; lane 25: CECT 10048; lane 26: CECT 10051; lane 27: CECT 1441; lane 28: CECT 10125; lane 29: CECT 10128; lane 31: CECT 10352; lane 32: CECT 10026; lane 33: CECT 10360; lane 34: CECT 10202; lane 35: CECT 10353; lane 37: CECT 1414; lane 38: CECT 1387; lane 39: CECT 1443; lane 40: CECT 1477; lane 41: CECT 1485.

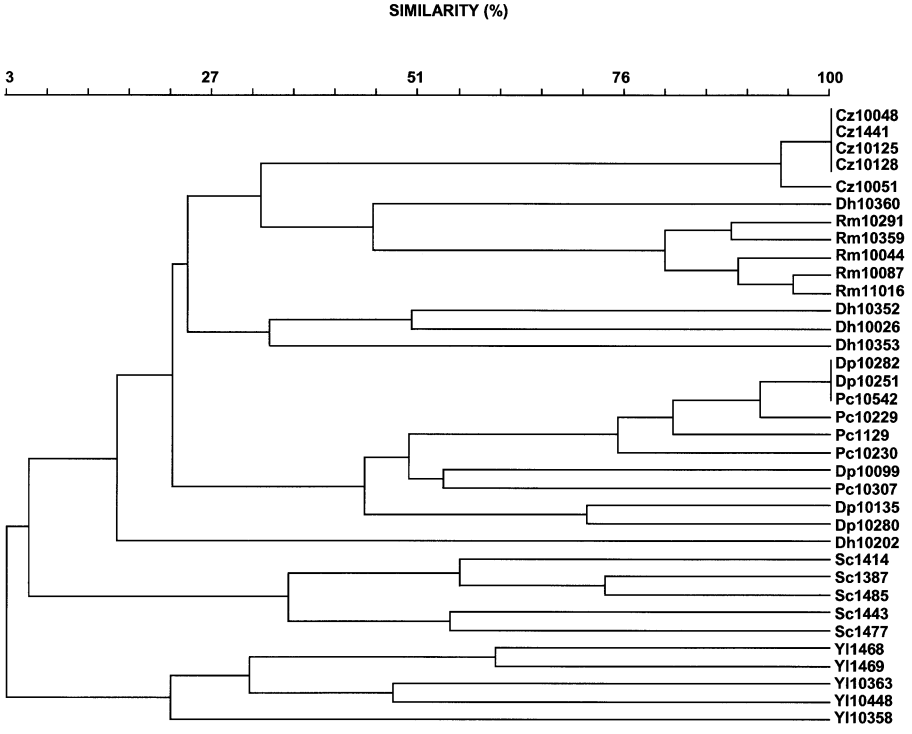

Fig. 4. Dendrogram based on the PCR-fingerprinting patterns obtained with primer M13 of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

the manufacturer's instructions and conditions. The resulting fragments were separated on 2.5% agarose gels in 1XTAE buffer at 70 V for 1.5 h. The gels were stained with ethidium bromide (0.5 μg/ml), the products were visualized by U.V. transillumination and photographed. A DNA molecular size marker of 2.1–0.15 kbp from Roche (Roche Farma, IN, USA) was used to determine the size of the PCR products. Electrophoretic patterns were compared using ID Image Analysis Software (Kodak Digital Science, Rochester, NY, USA).

Similarities among isolates were estimated using the DICE coefficient and clustering was based on the UPGMA method (NTSYS-Pc version 2.0).

2.4. RFLP analysis of the 18S rDNA

The 18S rDNA was amplified using the primers P108 (5′ACCTGGTTGATCCTGCCAGT3′) and M3989 (5′CTACGGAAACCTCTACGGAAACCTTGTTACGACT3′) described by James et al. (1994).

The reaction was performed in a total volume of 50 μl, containing 50 ng of DNA, 10 mM Tris–HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 1 U of Taq polymerase (Finnzymes) and 100 ng of the above primers. The reactions were incubated in a Thermal Cycler of Bio Rad (mod. iCycler 170-8731), using an initial denaturation during 3 min at 94 °C followed by 29 cycles consisting of 1 min at 92 °C, 1 min at 47 °C and 5 min at 58 °C. A final step of 5 min at 58 °C was carried out. Amplification products were separated by electrophoresis in 1% agarose gels and detected by staining with ethidium bromide (0.5 μg/ml).

The PCR products were digested with the restriction enzymes *Hae*III, *Rsa*I and *Taq*I following the supplier's instructions. The resulting fragments were separated on 2.5% agarose gels in 1XTAE buffer at 70 V for 1.5 h. Gel analysis was done as described above.

2.5. RAPD-PCR

For RAPD-PCR the M13 minisatellite primer (5′GAGGGTGGCGGTTCT3′) (Huey and Hall, 1989) and the microsatellite primers $(GACA)_4$, $(GAC)_5$ and $(GTG)_5$ were used. Amplification reaction was performed in a total volume of 50 μl, containing 200 ng of DNA, 10 mM Tris–HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 1 U of Taq polymerase (Finnzymes) and 100 ng of primer. The thermal cycler was programmed for the above primers as it is indicated in Table 2. PCR products were separated on 1% agarose gels using 1XTAE buffer at 70 V for 1.5 h. The gels were stained with ethidium bromide (0.5 μg/ml) and analyzed as described above. The size of the amplification products were determined

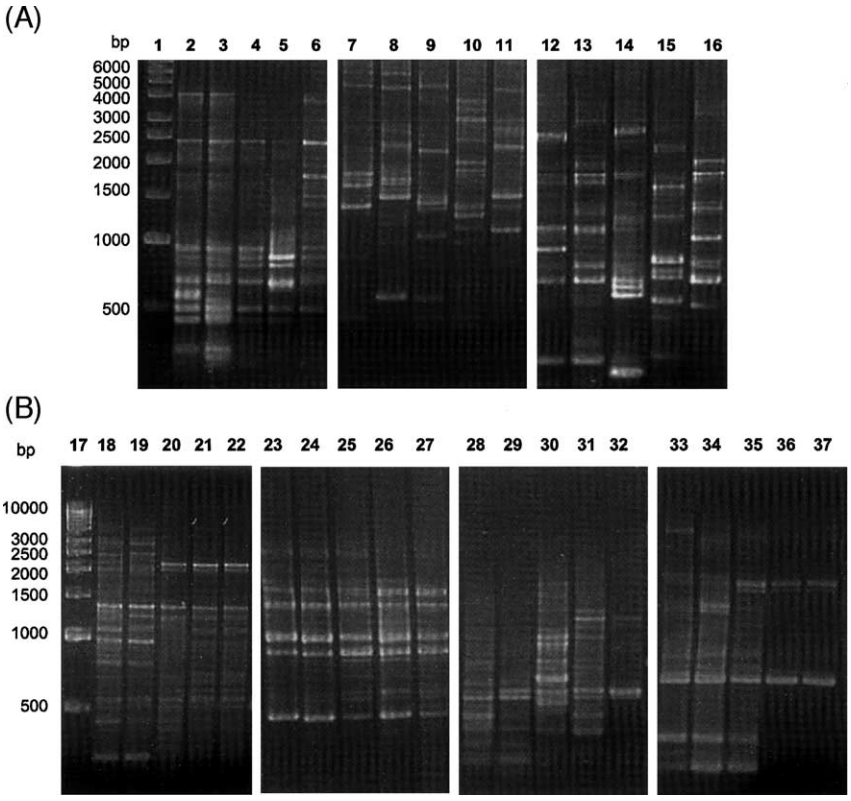

Fig. 5. PCR-fingerprinting patterns obtained with primer $(GACA)_4$ from *C. zeylanoides*, *D. polymorphus* and *D. hansenii* (A) and *R. mucilaginosa*, *P. carsonii*, *Y. lipolytica* and *S. cerevisiae* (B). Lanes 1 and 17: DNA molecular size marker of 10–0.5 kbp; lane 2: CECT 10048; lane 3: CECT 10051; lane 4: CECT 1441; lane 5: CECT 10125; lane 6: CECT 10128; lane 7: CECT 10282; lane 8: CECT 10251; lane 9: CECT 10099; lane 10: CECT 10135; lane 11: CECT 10280; lane 12: CECT 10352; lane 13: CECT 10026; lane 14: CECT 10360; lane 15: CECT 10202; lane 16: CECT 10353; lane 18: CECT 10291; lane 19: CECT 10359; lane 20: CECT 10044; lane 21: CECT 10087; lane 22: CECT 11016; lane 23: CECT 10230; lane 24: CECT 10229; lane 25: CECT 1129; lane 26: CECT 10307; lane 27: CECT 10542; lane 28: CECT 10363; lane 29: CECT 1469; lane 30: CECT 10358; lane 31: CECT 10363; lane 32: CECT 10448; lane 33: CECT 1414; lane 34: CECT 1387; lane 35: CECT 1443; lane 36: CECT 1477; lane 37: CECT 1485.

with a DNA molecular size marker of 10.0–0.5 kbp from Amersham Biosciences.

2.6. Mitochondrial DNA restriction patterns

The reactions were performed overnight at 37 °C and prepared for a final volume of 15 μl as follows: 5 μl of DNA obtained according to Querol et al. (1992), 20 U of the restriction enzyme *Hae*III and 1.5 μl of 10× buffer. The restriction fragments were separated on a 0.7% agarose gel in 1XTAE buffer at 45 V for 3 h. Gel analysis was done as described above.

3. Results

3.1. Identification by RFLP of the ITS1-5.8S rDNA-ITS2

Amplification of the ITS1-5.8S rDNA-ITS2 region resulted for all strains tested in a single fragment with a molecular size of approximately 650 bp, except for *S. cerevisiae* that the only amplified fragment was of approximately 850 bp (results not included). After digestion with the restriction enzymes the strains tested showed different fragment sizes, ranging from approximately 415 to 100 bp with enzyme *Hae*III, 445 to 166 bp with *Sau*3AI and 315 to 90 bp with *Taq*I. Fig. 1 shows the dendrogram constructed for the 35 yeast strains tested based on their RFLP analysis of the restriction profiles obtained from the ITS1-5.8S rDNA-ITS2 with the above enzymes. A similarity higher than 80% was observed for species *D. hansenii*, *D. polymorphus* and *P. carsonii*. *C. zeylanoides* showed a similarity of about 60% with the former species. Only *S. cerevisiae*, *R. mucilaginosa* and *Y. lipolytica* showed very low similarity with any other species (always lower than 30%) (Fig. 1). No differences were observed among strains of the same species in *C. zeylanoides*, *R. mucilaginosa* and *S. cerevisiae*. Minimal differences at strain level were found in the species *P. carsonii*, *Y. lipolytica*, *D. polymorphus* and *D. hansenii*.

3.2. Identification by RFLP of the 18S rDNA

Amplification of the 18S rDNA resulted for all strains tested in a single fragment with a molecular size of approximately 1800 bp. The RFLP analysis yielded fragments ranging from 465 to 125 bp with enzyme *Hae*III, 1000 to 95 bp with enzyme *Rsa*I and 1260 to 170 bp with enzyme *Taq*I. Fig. 2 depicts the dendrogram derived from the combined RFLP analysis with the above three restriction enzymes. *S. cerevisiae*, *R. mucilaginosa* and *Y. lipolityca* showed low values of similarity (always lower than 30%). However, the remaining species showed a similarity higher than 80% and *D. polymorphus*, *D. hansenii* and *C. zeylanoides* cannot be distinguished (Fig. 2). At strain level, only differences among strains of *Y. lipolytica* and *P. carsonii* were found.

3.3. RAPD-PCR with minisatellite M13

The RAPD with M13 primer yield band profiles of 4 to 20 bands for the strains assayed ranging from approximately 2100

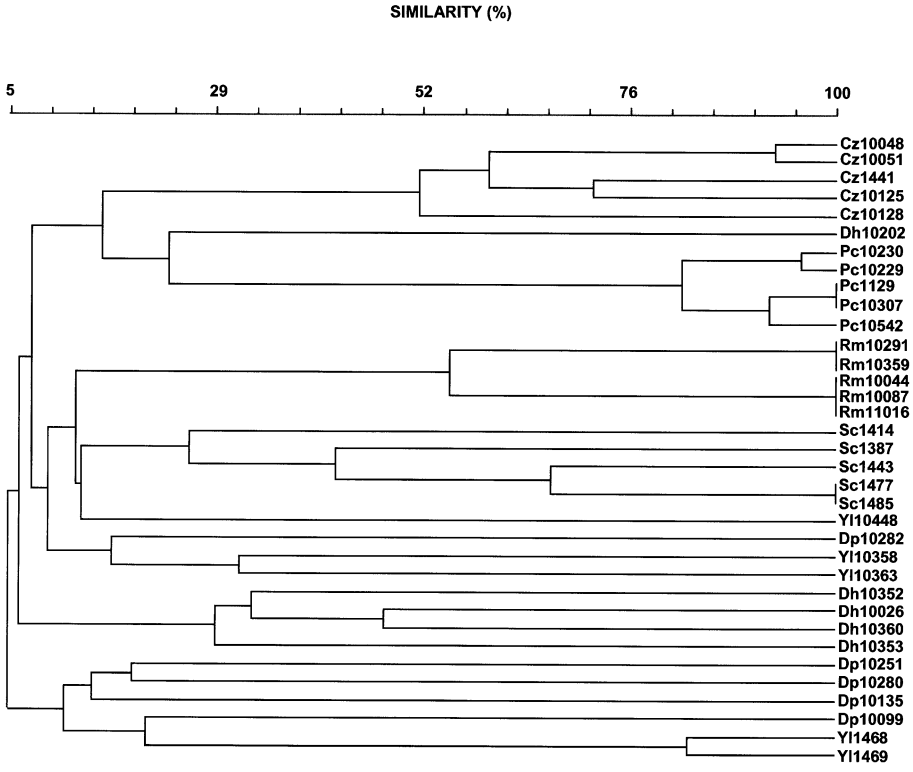

Fig. 6. Dendrogram based on the PCR-fingerprinting patterns obtained with primer $(GACA)_4$ of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

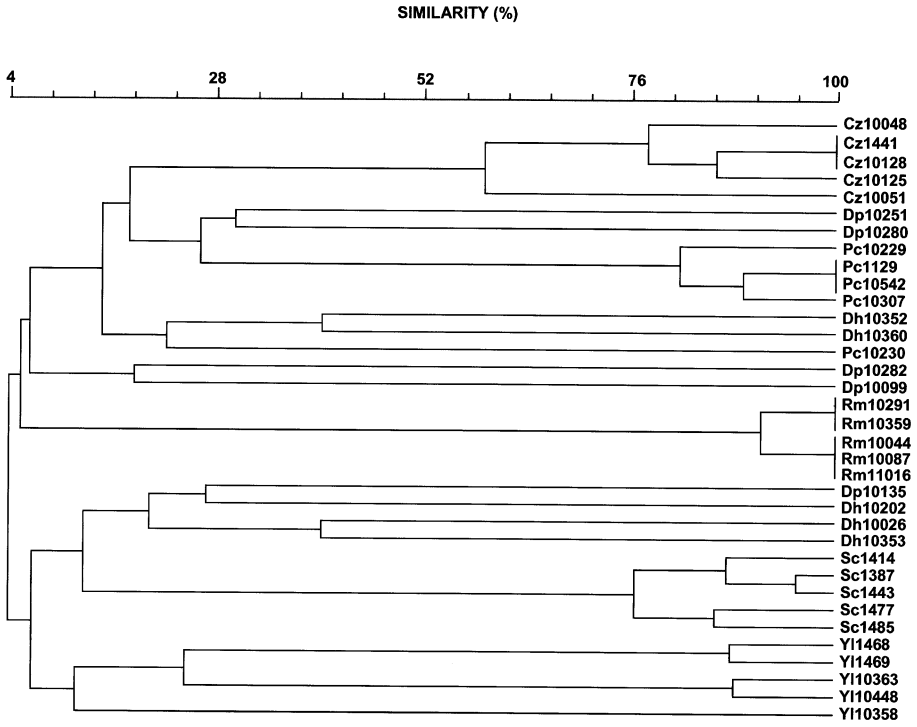

Fig. 7. Dendrogram based on the PCR-fingerprinting patterns obtained with primer $(GTG)_5$ of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

to 160 bp. (Fig. 3). Different patterns were generated for each of the 7 species tested, except for *D. polymorphus* and *P. carsonii*, which showed similar band profiles for some of their strains. Most of the strains tested for these two species showed a similarity higher than 75% (Fig. 4). At the strain level, different polymorphisms were observed in the different species

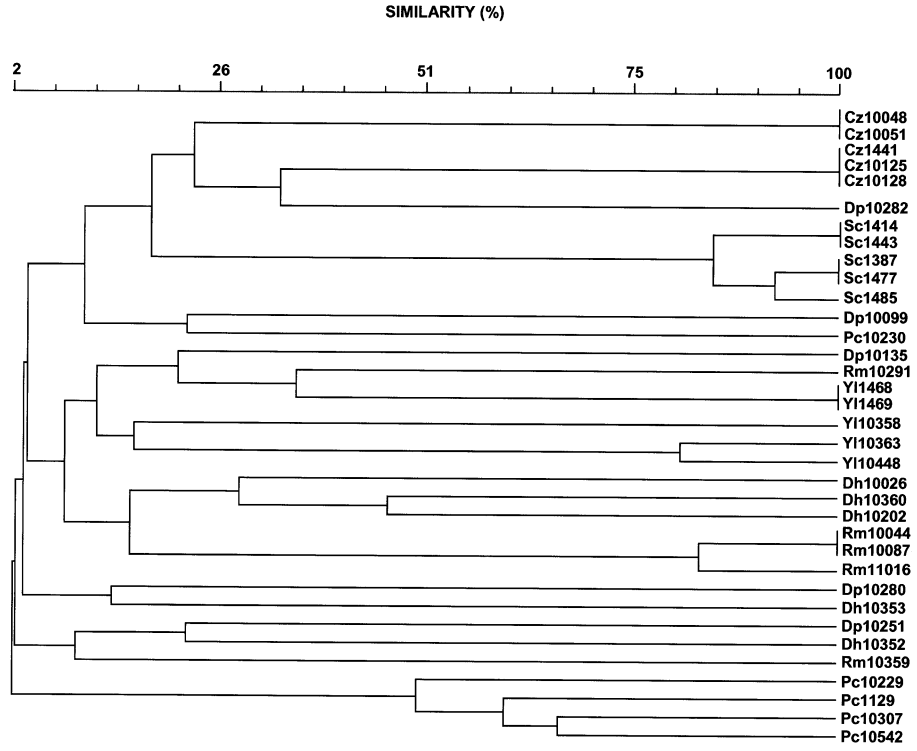

Fig. 8. Dendrogram based on the PCR-fingerprinting patterns obtained with primer $(GAC)_5$ of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

tested. However, these differences are very low for strains of *C. zeylanoides* and *R. mucilaginosa*, which showed a similarity higher than 80% (Fig. 4).

3.4. RAPD-PCR with microsatellites

The RAPD-PCR patterns of the 7 species tested using primer $(GACA)_4$ are shown in Fig. 5. Patterns obtained, ranging from 5900 to 150 bp, showed great differences at species level, with similarities always lower than 25% for all the species tested (Fig. 6). At strain level, a similarity lower than 50% was observed for strains tested of *D. hansenii* and *D. polymorphus*. The remaining species showed similarities higher than 50% for most of the strains tested. Very low polymorphisms were found in the strains tested for *R. mucilaginosa* and *P. carsonii*. In the last species all the strains showed a similarity higher than 80% (Fig. 6).

The RAPD analyses with primers $(GTG)_5$ and $(GAC)_5$ showed differences at species level for all species tested. As exhibited in the dendrograms obtained from the band patterns (Figs. 7 and 8), the similarity among strains of the different species was always lower than 50%. However, at strain level no relevant differences were observed within all species tested with primer $(GTG)_5$. Thus, a similarity higher than 75% was observed for most of the strains of *Y. lipolytica*, *S. cerevisiae*, *P. carsonii* and *C. zeylanoides* (Fig. 7). With primer $(GAC)_5$ a high similarity was observed for all of the strains of *S. cerevisiae* and for most of the strains of *R. mucilaginosa* and *C. zeylanoides* (Fig. 8).

3.5. Mitochondrial DNA RFLP

Fig. 9 shows the mtDNA restriction profiles obtained for the 35 strains tested by using the restriction enzyme *Hae*III. The band patterns obtained showed great differences at species level, with similarities always lower than 25% (Fig. 10). At strain level, similarities lower than 30% were found for the strains tested within the species *D. polymorphus*, *D. hansenii* and *S. cerevisiae* (Fig. 10). In every one of the species *P. carsonii*, *C. zeylanoides* and *Y. lipolytica* were found with two band profiles clearly differentiated, since the similarity between them was always lower than 30%. However, no differences were found among the strains of *R. mucilaginosa*.

4. Discussion

This study presents a contribution to the characterization of inter- and intraspecific variation of yeasts species belonging to

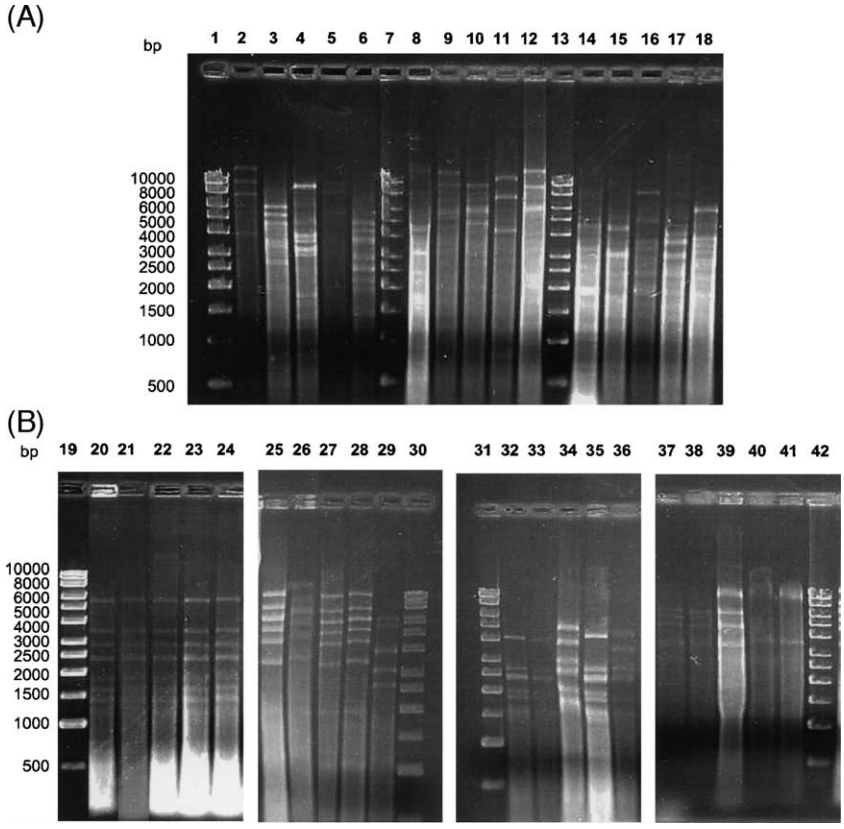

Fig. 9. Mitochondrial DNA restriction profiles with enzyme *Hae*III of *D. polymorphus*, *D. hansenii* and *S. cerevisiae* (A) and *R. mucilaginosa*, *Y. lipolytica*, *C. zeylanoides* and *P. carsonii* (B). Lanes 1, 7, 13, 19, 30, 31 and 42: DNA molecular size marker of 10–0.5 kbp; lane 2: CECT 10282; lane 3: CECT 10251; lane 4: CECT 10099; lane 5: CECT 10135; lane 6: CECT 10280; lane 8: CECT 10352; lane 9: CECT 10026; lane 10: CECT 10360; lane 11: CECT 10202; lane 12: CECT 10353; lane 13: DNA molecular size marker of 10–0.5 kbp; lane 14: CECT 1414; lane 15: CECT 1387; lane 16: CECT1443; lane 17: CECT 1477; lane 18: CECT 1485; lane 20: CECT 10291; lane 21: CECT 10359; lane 22: CECT 10044; lane 23: CECT 10087; lane 24: CECT 11016; lane 25: CECT 10363; lane 26: CECT 1469; lane 27: CECT 10358; lane 28: CECT 10363; lane 29: CECT 10448; lane 32: CECT 10048; lane 33: CECT 10051; lane 34: CECT 1441; lane 35 CECT 10125; lane 36: CECT 10128; lane 37: CECT 10230; lane 38: CECT 10229; lane 39: CECT 1129; lane 40: CECT 10307; lane 41: CECT 10542.

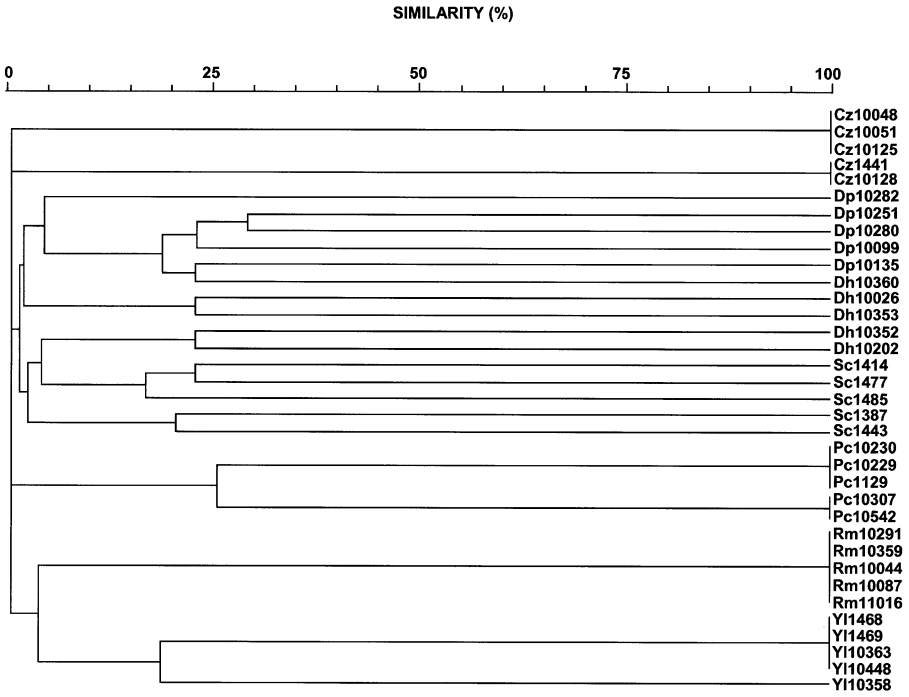

Fig. 10. Dendrogram based mitochondrial DNA restriction profiles obtained by digestion with *Hae*III of different strains of the yeasts *C. zeylanoides* (Cz), *D. polymorphus* (Dp), *D. hansenii* (Dh), *P. carsonii* (Pc), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl) and *R. mucilaginosa* (Rm). Distance values between branches in the dendrogram are reported as percentage of similarity (0–100%).

dry-cured meat products. RFLP analysis of the ITS-5.8S rDNA-ITS2 region did not allow the separation of *D. hansenii*, *D. polymorphus* and *P. carsonii*. Only *S. cerevisiae*, *R. mucilaginosa* and *Y. lipolytica* could be clearly differentiated from each other. In addition, this technique did not allow differentiation of strains within each species. RFLP of the ITS-5.8S rDNA-ITS2 has been reported as a rapid and easy method for the differentiation of several yeasts species, most of them related to *Saccharomyces* species (Guillamón et al., 1997; Dlauchy et al., 1999; Jespersen et al., 2000). However, Naumova et al. (2003) observed that this method could discriminate some yeasts species only when many restriction enzymes are used. In the present work, this method allowed differentiation of only some yeasts species. Thus, this method has not enough sensitivity to be used as a routine analysis to discriminate yeasts species related to dry-cured meat products. Probably it is necessary to combine this method with sequence analysis of the ITS-5.8S rDNA-ITS2 region as the best means of reliable identification of yeasts as has been proposed by Las Heras-Vazquez et al. (2003).

The RFLP of the 18S rDNA region allowed discrimination of the same species differentiated by RFLP of the ITS-5.8S rDNA-ITS2: *S. cerevisiae*, *R. mucilaginosa* and *Y. lipolytica*. However, no differences were found among *D. hansenii*, *D. polymorphus* and *C. zeylanoides*. In addition, minimal differences at the strain level were found for all of the species tested. Results found in the literature about suitability of RFLP of the 18S rDNA are contradictory. Thus, this method has been also reported as nonsuitable for the differentiation of yeasts from the genus *Saccharomyces* (Tornai-Lehoczki and Dlauchy, 2000) while it has been proven as useful technique for discriminating several yeasts species such as *Candida stellata*, *Metschnikowia pulcherrima*, *Kloeckera apiculata* and *Schizosaccharomyces pombe* (Capece et al., 2003). From our results, this method is not adequate as routine method analysis to discriminate yeasts species related to dry-cured meat products.

RAPD-PCR with M13 primer was found to be useful for the differentiation of *R. mucilaginosa*, *C. zeylanoides*, *Y. lipolytica*, *D. hansenii* and *S. cerevisiae*. However, no differences were observed between *D. polymorphus* and *P. carsonii.* Contrary to previous report (Prillinger et al., 1999; Andrighetto et al., 2000), we did not find RAPD analysis suitable for discrimination of all of the species tested. However, our results agree with the data found by Vasdinyei and Deák (2003) in that RAPD using a M13 primer did not discriminate the most frequently yeasts species found in dairy products.

RAPD analysis with microsatellite primers $(GACA)_4$, $(GTG)_5$ and $(GAC)_5$ enabled discrimination at species and strain level. However, the degree of discrimination by means of RAPD-PCR depends highly on the primers used. Thus, the PCR fingerprinting with primer $(GACA)_4$ enabled a higher level of discrimination than primers $(GAC)_5$ and $(GTG)_5$. Although primer $(GACA)_4$ is not usual in RAPD analysis of yeasts, a high discrimination level at species and strain level was reported for several yeasts species (Lieckfeldt et al., 1993; Casali et al., 2003). The only problem for primer $(GACA)_4$ was the high similarity for strains of *P. carsonii* tested that do not allow a reliable differentiation at strain level within this species.

The RFLP analysis of mtDNA allowed the discrimination at the species and strain level except for *R. mucilaginosa*, where no polymorphisms were observed in the strains tested. This

technique has been reported as a very good tool for the differentiation of yeasts species related to wine making (Esteve-Zarzoso et al., 2000; Fernández-Espinar et al., 2001) and spoilage yeasts of the genus *Zygosaccharomyces* (Guillamón et al., 1997). Since this method yielded polymorphisms in all species tested except *R. mucilaginosa* and RAPD analysis with primer $(GACA)_4$ gives different band patterns in all species except *P. carsonii*, both methods could be used as complementary techniques to differentiate yeasts related to dry-cured meat products. The results presented here highlighted how the molecular methods tested give different levels of discrimination, but this should be validated with a higher number of strains.

In conclusion RAPD analysis with primer $(GACA)_4$ and the restriction analysis of mtDNA used in the present work are useful for the differentiation at species and strain level of yeasts related to dry-cured meat products. For maximum discriminatory power of these types of yeast, both methods should be used. These techniques should be used to discriminate yeasts species usually found in dry-cured meat products. Furthermore, since differences at strain level were found, both methods are useful to differentiate yeasts used as starter cultures in dry-cured meat products.

Acknowledgements

This work is part of the project AGL01-0804 funded by the Spanish Comisión Interministerial de Ciencia y Tecnología. M. J. Andrade was the recipient of a Grant of the Regional Government of Junta de Extremadura (Consejería de Educación, Ciencia y Tecnología).

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