ELSEVIER

International Journal of Food Microbiology 69 (2001) 11–24

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

DNA typing methods for differentiation of *Debaryomyces hansenii* strains and other yeasts related to surface ripened cheeses

Kamilla Munk Petersen*, Peter Lange Møller, Lene Jespersen

*Department of Dairy and Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Roligheds*v*ej 30, DK-1958 Frederiksberg, Copenhagen, Denmark*

Abstract

The discriminative power of ITS-PCR, ITS-PCR RFLP and mitochondrial (mt)-DNA RFLP were evaluated for differentiation of yeasts of importance for surface ripened cheeses. In total 60 isolates were included. Of these, 40 strains of the following species, *Debaryomyces hansenii* var. *hansenii*, *D. hansenii* var. *fabryi*, *Saccharomyces cerevisiae*, *Candida zeylanoides*, *Kluyveromyces lactis* and *Yarrowia lipolytica*, were obtained from culture collections and 20 isolates of *D. hansenii* representing six different phenotypes were collected from seven Danish producers of surface ripened cheeses.

ITS-PCR was evaluated for differentiation at species level on the 40 strains obtained from culture collections. Ten strains of each variety of *D. hansenii* and five strains of each of the above mentioned species were analysed. For each of the investigated species, a specific ITS1-5.8S rDNA-ITS2 region size was observed. Accordingly ITS-PCR was found valuable for differentiation at species level of yeasts of importance for surface ripened cheeses.

ITS-PCR RFLP was investigated for the purpose of strain typing of *D. hansenii*. Ten CBS strains of each variety of *D. hansenii* were analysed. Only one enzyme (*Taq*I) out of several investigated (*Bam*HI, *Dpn*I, *Fnu*4HI, *Hae*III, *Hin*dIII, *Hpa*II, *Nla*II, *Sau*3AI, *Taq*I) demonstrated genetic diversity within the strains. This enzyme divided the 20 strains in three groups. Sequence analysis of the ITS1-5.8S rDNA-ITS2 region for the type strains of each variety of *D. hansenii* showed an identity of 99.84%, corresponding to a difference in one basepair. Based on these results, ITS-PCR RFLP was found ineffective for strain typing of *D. hansenii*.

MtDNA RFLP using *Hae*III and *Hpa*II was evaluated for strain typing of *D. hansenii* on the 20 CBS strains of *D. hansenii*. The CBS strains were divided into 16 groups according to their restriction profiles, which proved the method useful for typing of *D. hansenii* at subspecies level. The 20 dairy isolates showed a lower genetic variability than the CBS strains as they were divided into eight groups. Cluster analysis of the 20 CBS strains and the 20 dairy isolates based on their mtDNA restriction profiles showed (max. similarity level = 52%) that the dairy isolates only clustered with the CBS strains of *D. hansenii* var. *hansenii*. For some of the dairies more than one strain of *D. hansenii* were found to be involved in the ripening process, indicating that the method could be useful for subspecies typing and investigation of the microbial

* Corresponding author. Tel.: +45-35-28-33-48, fax: +45-35-28-32-14.
E-mail address: kams@kvl.dk (K.M. Petersen).

0168-1605/01/$ - see front matter
PII: S0168-1605(01)00568-2

succession between strains of *D. hansenii* during the ripening process of surface ripened cheeses.

Keywords: *Debaryomyces hansenii*; Yeasts; Surface ripened cheese

1. Introduction

Cheeses such as Limburger, Tilsitter, Port Salut, Trappist, Brick and the Danish Danbo belong among others to the group of surface ripened cheeses. These cheeses are characterised by an additional ripening from the cheese surface to the interior, mainly through the activity of microorganisms established on the cheese surface. The surface smear has been shown to consist of a complex mixed microflora including both yeasts and bacteria (Reps, 1993). The yeast flora includes species such as *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Candida zeylanoides* (Fleet, 1990; Eliskases-Lechner and Ginizinger, 1995a), while the bacterial flora mainly includes *Brevibacterium linens*, *Arthrobacter* spp., *Corynebacterium* spp. and *Micrococcus* spp. (Seiler, 1986; Eliskases-Lechner and Ginizinger, 1995b; Valdés-Stauber et al., 1997).

Yeasts are predominant during the initial period of ripening, where they initiate the ripening process by raising the pH at the cheese surface, thereby allowing the growth of a more acid sensitive bacterial flora (Fleet, 1990; Reps, 1993; Eliskases-Lechner and Ginizinger, 1995a). Strains of *D. hansenii* are supposed to be responsible for the increase of pH at the cheese surface by their degradation of lactic acid (Eliskases-Lechner and Ginizinger, 1995a; Lerlercq-Perlat et al., 1999). In addition, yeasts might produce growth factors of importance for the bacteria as well as aroma components and lipolytic and proteolytic enzymes that might contribute to the ripening process (Lenoir, 1984; Fleet, 1990; Jakobsen and Narvhus, 1996).

Traditionally, yeasts are introduced in the production by process equipment, the brine or by inoculation of the cheese surface with a slurry containing smear from previously produced cheeses. However, the use of well defined starter cultures of *D. hansenii* for surface ripening of cheeses is paid increased attention (Eliskases-Lechner and Ginizinger, 1995a). Little is known about the microbial succession between the *D. hansenii* starter culture and the strains of *D. hansenii* and other yeasts introduced either from the environment or by inoculation. In order to follow and control the starter culture during the production, fast and simple methods are required for identification of *D. hansenii* at subspecies level.

Various methods using DNA typing techniques for yeast differentiation have been developed. The main part of these methods have been introduced for differentiation of *Saccharomyces cerevisiae*. In the recent years many of these methods have been adapted to other yeast species, e.g. RAPD-PCR for identification of yeast species from cheese (Prillinger et al., 1999), PFGE for differentiation of *C. krusei* (Hayford and Jakobsen, 1999), mitochondrial DNA RFLP for differentiation of *Kluyveromyces* species (Belloch et al., 1997) and ITS-PCR RFLP for differentiation of yeasts isolated during wine production (Guillamón et al., 1998).

Amplification by PCR of the region spanning the two intergenic transcribed spacers (ITS) and the 5.8S ribosomal gene (ITS1-5.8S rDNA-ITS2) followed either by restriction or sequence analysis has been investigated for typing of several yeast species. Baleiras Couto et al. (1996) analysed 15 *S. cerevisiae* isolates from wine and beer by restriction analysis with *Mse*I and found that the isolates could be divided into four groups. Accordingly they concluded the method valuable for subspecies typing of *S. cerevisiae*. Guillamón et al. (1998) reported restriction analysis useful for differentiation at species level of yeasts related to wine; they analysed 33 yeast species and obtained a specific restriction profile for each species. Sequence analysis of the ITS1-5.8S rDNA-ITS2 region has been reported to be useful for differentiation of closely related species by James et al. (1996) who reported this method useful for differentiation of closely related species in the genera *Zygosaccharomyces* and *Torulaspora*, whereas Oda et al. (1997) used this method for differentiation of yeast species within the genus *Saccharomyces*.

Mitochondrial (mt)-DNA RFLP was originally developed for differentiation of wine and beer strains of *Saccharomyces* spp. (Aiglé et al., 1984; Lee and Knudsen, 1985; Vezinhet et al., 1990). The recent improvement of the method by implementation of a simple method for mtDNA restriction analysis (Querol et al., 1992) has increased its usefulness. This rapid method has been reported useful for strain differentiation of *S. cerevisiae* (Querol et al. 1992; Guillamón et al., 1994). Recently, Romano et al. (1996) used this rapid method of mtDNA RFLP for evaluation of the genetic diversity of *D. hansenii* and *C. zeylanoides* isolates.

It has been the objective of the present study to evaluate the use of ITS-PCR for differentiation of yeast species of importance for surface ripened cheeses and further to examine the use of ITS-PCR RFLP and mtDNA RFLP for strain typing of *D. hansenii*. Isolates of both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi* were included. Isolates were obtained either from culture collections or collected from seven Danish producers of surface ripened cheeses.

2. Materials and methods

2.1. Yeast isolates

All isolates used in this study are listed in Table 1. CBS strains were obtained from Centraalbureau voor Schimmelcultures (CBS, Baarn and Delft, The Netherlands). The isolates denoted DI were collected from seven different Danish dairies, all producers of red smear cheeses of the Danbo type. The isolates were either collected from the smear or from the brine. All isolates were predominant and supposed to play a major role in cheese ripening. The commercial starter cultures for cheese ripening, DH-com (*D. hansenii*) and SC-com (*S. cerevisiae*), were obtained from Laboratorium Visby (Tønder, Denmark).

All isolates were purified on repeated cultivation on MYGP agar (3.0 g malt extract (Difco, Detroit, MI, USA), 3.0 g yeast extract (Difco), 10.0 g glucose (Merck, Darmstadt, Germany), 5.0 g bactopeptone (Difco) and 20.0 g agar (Difco) per litre distilled water), pH = 5.6, at 25 °C and maintained at −80 °C in Yeast extract Peptone Glucose broth (5.0 g yeast extract (Difco), 10.0 g bactopeptone (Difco),

Table 1
List of yeast isolates used in the present study

Isolate	Species	Origin
TT56	*S. cerevisiae*	Cheese, Danablu
SC-com	*S. cerevisiae*	Commercial isolate Laboratorium Visby
CBS400, CBS420, CBS1171[a]	*S. cerevisiae*	CBS[b]
CBS619[a], CBS5446, CBS5447, CBS6391, CBS6408	*C. zeylanoides*	CBS[b]
CBS599, CBS2073, CBS2075, CBS6124[a], CBS6317	*Y. lipolytica*	CBS[b]
CBS683[a], CBS739, CBS743, CBS845, CBS1065	*K. lactis*	CBS[b]
CBS117, CBS164, CBS766, CBS767[a], CBS772, CBS796, CBS1102, CBS1792, CBS1800, CBS8416	*D. hansenii* var. *hansenii*	CBS[b]
CBS789[a], CBS1796, CBS4373, CBS5230, CBS5572, CBS6066, CBS7254, CBS7761, CBS7784, CBS8417	*D. hansenii* var. *fabryi*	CBS[b]
DI1, DI2, DI3, DI5, DI17	*D. hansenii* var. *hansenii*	Dairy A (smear)
DI4	*D. hansenii* var. *hansenii*	Dairy B (smear)
DI6, DI7, DI22, DI25	*D. hansenii* var. *hansenii*	Dairy C (smear)
DI8, DI9	*D. hansenii* var. *hansenii*	Dairy D (brine)
DI10, DI11	*D. hansenii* var. *hansenii*	Dairy D (smear)
DI12, DI13	*D. hansenii* var. *hansenii*	Dairy E (smear)
DI14	*D. hansenii* var. *hansenii*	Dairy F (smear)
DI15, DI16	*D. hansenii* var. *hansenii*	Dairy G (smear)
DH-com	*D. hansenii* var. *hansenii*	Commercial isolate Laboratorium Visby

[a] Type strains.
[b] Centraalbureau voor Schimmelcultures.

10.0 g glucose (Merck)) containing 20% (v/v) glycerol.

2.2. Phenotypic characterisation of dairy isolates

The isolates obtained from Danish dairies were identified to the species level by description of their micro- and macromorphological characteristics according to Nakase et al. (1998). The isolates were further characterised by use of the API ID 32 C kit (Bio Merieux SA, Marcy-LÈtoile, France). The *D. hansenii* var. *hansenii* isolates were distinguished from the *D. hansenii* var. *fabryi* isolates by their inability to grow at 36 °C (Nakase et al., 1998). The isolates were pregrown in 10 ml MYGP broth at 25 °C for 48 h and then inoculated on MYGP slants at 36 ± 0.1 °C for 3–4 weeks. *D. hansenii var. hansenii* CBS767 was used as a positive control and *D. hansenii* var. *fabryi* CBS6066 as a negative control.

2.3. Determination of ITS1-5.8S rDNA-ITS2 region size

The yeast cultures were pregrown on MYGP agar at 25 °C for 5 days. For each yeast culture a loop full was incubated in 500 μl lysis buffer (2 mM Tris–HCl (Sigma, St. Louis, MO, USA), 10 mM KCl (Merck), 0.3 mM $MgCl_2$ (Merck), 0.02% (v/v) Triton X-100 (Sigma), 10.0 mg/l Proteinase K (Sigma), 0.617 g/l 1,4-Dithiothreitol (Amersham Pharmacia Biotech, Uppsala, Sweden)) at 37 °C for 1 h, boiled for 15 min and centrifuged at 14,000 × *g* for 2 min. The supernatant was used as template. Primers for amplification of the ITS1-5.8S rDNA-ITS2 region were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990); primer ITS1 were 5′-Cy5 labelled. DNA amplification was performed in a 100-μl volume containing 10 μl template, 1 μM of each primer, 200 μM of each nucleotide (dATP, dCTP, dGTP, dTTP (Promega, Madison, WI, USA)), 2.5 U *Taq* polymerase (Amersham Pharmacia Biotech), 10 μl 10 × PCR buffer (Amersham Pharmacia Biotech) and added up to 100 μl with milliQ water. The reactions were performed in an automatic thermal cycler (GeneAmp® PCR System 9700, Perkin Elmer, Norwalk, CT, USA). PCR conditions were as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 2 min, 60 °C for 1 min, 72 °C for 2.5 min; final extension at 72 °C for 7 min. The amplified ITS1-5.8S rDNA-ITS2 regions were then analysed on an Automatic Laser Fluorescence (ALF)-*express* sequencer (Amersham Pharmacia Biotech) using a 6% denaturing polyacrylamide gel (6.6 ml Hydrolink Long Ranger (FMC BioProducts, Philadelphia, PA, USA), 21.8 g urea (ICN Biomedicals, Aurora, OH, USA), 7.2 ml 10 × TBE, and added up to 60 ml with milliQ water) in 0.6 × TBE buffer (53.4 mM Tris-base (Sigma), 53.4 mM boric acid (Sigma), 1.2 mM EDTA (Sigma)), under the following conditions: 300 min, at 60 mA, 700 V and 55 °C. As marker ALF express™Sizer™ 50–500 (Amersham Pharmacia Biotech) was used together with a marker based on amplification of previously sequenced ITS regions from *Bacillus subtilis* (441–849 bp).

The sizes of the amplified ITS1-5.8S rDNA-ITS2 regions were determined by use of the computer program Fragment Manager (Amersham Pharmacia Biotech).

2.4. Genotyping by ITS-PCR RFLP

Amplification of the ITS1-5.8S rDNA-ITS2 region was done as described in Section 2.3, with the exception that none of the primers were 5′-Cy5 labelled. For RFLP analysis 10 μl of the PCR products (0.2–0.5 μg) was digested with 5 U of one of the following restriction enzymes: *Bam*HI, *Dpn*I, *Fnu*4HI, *Hae*III, *Hind*III, *Hpa*II, *Nla*II, *Sau*3AI, *Taq*I (New England BioLabs, Beverly, MA, USA) overnight at 37 °C except for *Taq*I, which was incubated at 65 °C according to the supplier's manual. The restriction fragments were analysed by electrophoresis through a 3% (w/v) gel Nusieve agarose (FMC BioProducts) in 1 × TBE buffer (89 mM Tris-base, 89 mM boric acid (Merck), 2 mM EDTA) at 80 V for 2 h. As marker ΦX174/*Hae*III (Promega) was used. The restriction fragments were visualised by ethidium bromide staining and UV transillumination.

2.5. Sequence analysis of the ITS1-5.8S rDNA-ITS2 region

The external primers used for amplification of the ITS1-5.8S rDNA-ITS2 region were ITS5 (5′-GGA

Table 2
ITS1/5.8S rDNA/ITS2 region size for yeast species of importance for cheese production

Species	Isolate number	Size (bp)
C. zeylanoides	CBS619[a], CBS5446, CBS5447, CBS6391, CBS6408	620
D. hansenii var. *hansenii*	CBS164, CBS766, CBS767[a], CBS772, CBS1800	639
D. hansenii var. *fabryi*	CBS789[a], CBS4373, CBS7761, CBS7784, CBS8417[b]	639 (607)
K. lactis	CBS683[a], CBS739, CBS743, CBS845, CBS1065	740
S. cerevisiae	CBS400, CBS420, CBS1171[a], TT56, SC-com	850
Y. lipolytica	CBS599, CBS2073, CBS2075, CBS6124[a], CBS6317	360

[a] Type strains.
[b] ITS1-5.8S rDNA-ITS2 region size = 607 bp.

AGT AAA AGT CGT AAC AAG G-3′) (White et al., 1990) and ITS6 (5′-GCC GCT TCA CTC GCC GTT ACT-3′). The design of primer ITS6 was based on information (accession number U45771) from GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank/). The reactions were performed in an automatic thermal cycler (GeneAmp® PCR System 9700, Perkin Elmer) as described previously. The amplified products were purified using the QIAGEN PCR purification kit (QIAGEN, Dorking, UK). Direct sequencing of the purified PCR product was performed by the dideoxy chain-terminating method of Sanger et al. (1977) using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (RPN 2436, Amersham Pharmacia Biotech) and an automated DNA sequencer (ALF*express*, Amersham Pharmacia Biotech). The internal primers used for cycle sequencing were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′), ITS 2 (5′-GCT GCG TTC TTC ATC GAT GC-3′), ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). All primers used in the sequence reactions were 5′-Cy5 labelled. The sequence reactions were performed in an automatic thermal cycler (GeneAmp® PCR System 9700, Perkin Elmer). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min; 25 cycles of 95 °C for 30 s, 60 °C for 40 s; final extension at 72 °C for 5 min. The sequences were then analysed on the ALF-*express* using a 6% denaturing polyacrylamide gel in 0.6 × TBE buffer under the following conditions: 1000 min at 60 mA, 1500 V and 55 °C. Fluorescent signals were determined using a sampling interval of 2 s. The sequences were computer analysed by use of the programs Editseq (DNASTAR, Madison, WI, USA) and Mapdraw (DNASTAR).

2.6. Genotyping by mtDNA RFLP

MtDNA profiles were obtained according to Querol et al. (1992) with the modifications made by

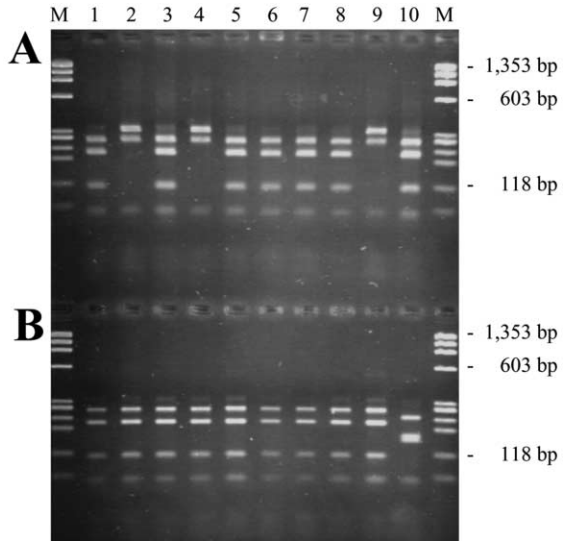

Fig. 1. *Taq*I restriction fragments of PCR amplified ITS1-5.8S rDNA-ITS2 regions of *D. hansenii* CBS strains. (A) *D. hansenii* var. *hansenii*. M: marker ΦX174/*Hae*III, lane 1: CBS117, lane 2: CBS164, lane 3: CBS766, lane 4: CBS767, lane 5: CBS772 , lane 6: CBS796, lane 7:CBS1102 , lane 8: CBS1792, lane 9: CBS1800, lane 10: CBS8416. (B) *D. hansenii* var. *fabryi*. M: marker ΦX174/*Hae*III, lane 1: CBS789, lane 2: CBS1796, lane 3: CBS4373, Lane 4: CBS5230, lane 5: CBS5572, lane 6: CBS6066, lane 7: CBS7254, lane 8: CBS7761, lane 9: CBS7784, lane 10: CBS8417.

Romano et al. (1996). MtDNA restriction profiles were obtained from total DNA by use of restriction enzymes with recognition sites rich in GC, which resulted in an overdigestion of the nuclear DNA and thereby gave specific bands for mtDNA. The restriction enzymes *Hae*III and *Hpa*II with the recognition sites GG/CC and C/CGG, respectively, were used in this study. DNA purified from 5 ml overnight culture was resuspended in 50 μl TE-buffer (10 mM Tris-Base (Sigma), 1 mM EDTA (Merck)). Of the purified DNA 10 μl was digested with 5 U of restriction enzyme *Hae*III or *Hpa*II (New England BioLabs) overnight at 37 °C. The restriction fragments were analysed by electrophoresis through a 1% (w/v) gel NA-agarose (Pharmacia Biotech) in 1 × TBE buffer (89 mM Tris-base, 89 mM boric acid (Merck), 2 mM EDTA) at 100 V for 3 h. As marker λDNA/*Hind*III (Promega) was used. The restriction fragments were visualised by ethidium bromide staining and UV transillumination.

2.7. Cluster analysis

The cluster analyses were carried out by use of the computer program BioNumerics (Applied Maths, Kortrijk, Belgium). The similarities between restriction profiles were determined by the fraction of shared bands (Dice coefficient). Clustering was calculated by the unweighted pair group method using arithmetic average linkage (UPGMA method).

3. Results

3.1. Differentiation of yeast genera by ITS-PCR

Initially ITS-PCR was used for differentiation of yeast species of importance for surface ripened cheeses. ITS-PCR was performed on five isolates of each of the following yeasts: *C. zeylanoides*, *K. lactis*, *S. cerevisiae*, *Y. lipolytica* and 10 strains of the yeasts *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. The type strain of each species was included. Amplification of the ITS1-5.8S rDNA-ITS2 region resulted for all isolates in a single fragment with a molecular size ranging from 360 to 850 bp. The size of the ITS1-5.8S rDNA-ITS2 regions for the analysed yeast isolates are shown in Table 2. Within each species no difference in the size of the ITS1-5.8S rDNA-ITS2 regions was found, with the exception that 1 of the 10 strains of *D. hansenii* var. *fabryi* (CBS8417) had a ITS1-5.8S rDNA-ITS2 re-

Fig. 2. ITS1-5.8S rDNA-ITS2 sequences for the type strains of *D. hansenii* var. *hansenii* (CBS767) and *D. hansenii* var. *fabryi* (CBS789).

gion size of 607 bp compared to 639 bp for all other tested strains of *D. hansenii fabryi*. No difference in ITS1-5.8S rDNA-ITS2 region size was found between *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. For each species a specific ITS1-5.8S rDNA-ITS2 region size was found, i.e. 639 bp for *D. hansenii* (except CBS8417), 620 bp for *C. zeylanoides*, 740 bp for *K. lactis*, 360 bp for *Y. lipolytica* and 850 bp for *S. cerevisiae*.

3.2. Genotyping of D. hansenii strains by ITS-PCR RFLP

The discriminative power of ITS-PCR RFLP was tested on 20 CBS strains of *D. hansenii*: 10 of *D. hansenii* var. *hansenii* and 10 of *D. hansenii* var. *fabryi* (Table 1). Of the nine restriction enzymes tested (*Bam*HI, *Dpn*I, *Fnu*4HI, *Hae*III, *Hin*dIII, *Hpa*II, *Nla*II, *Taq*I, *Sau*3AI), *Taq*I was the only enzyme that resulted in variable restriction profiles. Fig. 1 shows the *Taq*I restriction profiles for the 10 strains of *D. hansenii* var. *hansenii* (Fig. 1A) and the 10 strains of *D. hansenii* var. *fabryi* (Fig. 1B). Two restriction profiles were observed for both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. The most frequent restriction profile was observed for both *D. hansenii* var. *hansenii* (7 out of 10) and *D. hansenii* var. *fabryi* (9 out of 10); this profile consisted of four bands: 58, 109, 209 and 263 bp. The restriction profiles for the remaining three strains of *D. hansenii* var. *hansenii* consisted of three bands: 58, 263 and 318 bp, indicating a difference of

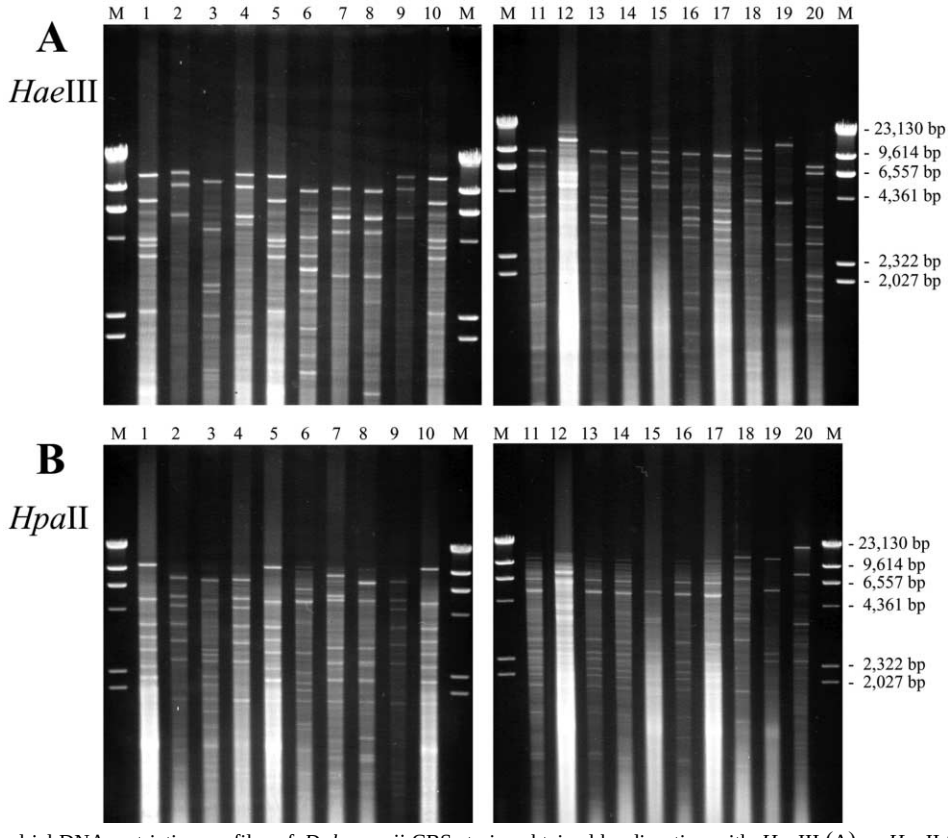

Fig. 3. Mitochondrial DNA restriction profiles of *D. hansenii* CBS strains obtained by digestion with *Hae*III (A) or *Hpa*II (B). M: marker λDNA/*Hin*dIII. Lanes 1–10 *D. hansenii* var. *hansenii*: lane 1: CBS117, lane 2: CBS164, lane 3: CBS766, lane 4: CBS767, lane 5: CBS772, lane 6: CBS796, lane 7: CBS1102, lane 8: CBS1792, lane 9: CBS1800, lane 10: CBS8416. Lanes 11–20 *D. hansenii* var. *fabryi*: lane 11: CBS789, lane 12: CBS1796, lane 13: CBS4373, lane 14: CBS5230, lane 15: CBS5572, lane 16: CBS6066, lane 17: CBS7254, lane 18: CBS7761, lane 19: CBS7784, lane 20: CBS8417.

one *Taq*I site. The restriction profile for the remaining strain of *D. hansenii* var. *fabryi* (CBS8417) consisted of four bands: 59, 153, 162 and 233 bp, i.e. the restriction profile was quite different from the restriction profiles obtained for the other strains of *D. hansenii*.

3.3. Sequence analysis of ITS1-5.8S rDNA-ITS2

The restriction profiles generated for the type strains of *D. hansenii* var. *hansenii* (CBS767) and *D. hansenii* var. *fabryi* (CBS789) were different, indicating that the two strains differ in the ITS1-5.8S rDNA-ITS2 region. In order to see whether the use of other restriction enzymes than *Taq*I could be used for subspecies typing of *D. hansenii* strains, the ITS1-5.8S rDNA-ITS2 regions for the two type strains of *D. hansenii* var. *hansenii* (CBS767) and *D. hansenii* var. *fabryi* (CBS789) were sequenced. The sequences with restriction sites are shown in Fig. 2. The size of the ITS1-5.8S rDNA-ITS2 region for the two type strains was confirmed by the sequence analysis (i.e. 639 bp). The sequences of the ITS1-5.8S rDNA-ITS2 region for the two isolates were 99.84% identical. The only difference was at position 430, where a thymine in the type strain of *D. hansenii* var. *hansenii* (CBS767) was replaced by a cytosine in the type strain of *D. hansenii* var. *fabryi* (CBS789); this base substitution resulted in the extra *Taq*I site observed for CBS789. The ITS-PCR RFLP restriction profile for CBS8417 indicated a quite different sequence of the ITS1-5.8S rDNA-ITS2 region than for the other *D. hansenii* strains. This was confirmed by sequencing (result not shown) where the sequence for *D. hansenii* var. *fabryi* (CBS8417) had a homology of 90.92% compared to the type strain of *D. hansenii* var. *fabryi* (CBS789).

The sequences were deposited at GenBank under the following accession numbers: AF210327 for *D. hansenii* var. *hansenii* (CBS767), AF210326 for *D. hansenii* var. *fabryi* (CBS789) and AF209874 for *D. hansenii* var. *fabryi* (CBS8417).

3.4. Genotyping of D. hansenii strains by mtDNA RFLP

The discriminative power of mtDNA RFLP for strain differentiation of *D. hansenii* was evaluated by examination of the 20 CBS strains of *D. hansenii* (mentioned in Section 3.2). Fig. 3 shows the mtDNA restriction profiles obtained for the 20 CBS strains by use of the restriction enzymes *Hae*III and *Hpa*II. By use of *Hae*III, seven different mtDNA restriction profiles were obtained for the 10 strains of *D. hansenii* var. *hansenii* (Fig. 3A, lanes 1–10), i.e. it was not possible to differentiate between CBS164 and CBS1800 and between CBS117, CBS772 and

Table 3
Assimilation profiles of *D. hansenii* var. *hansenii* isolates from Danish dairies

Carbon compound	Assimilation group[a]						Total (%)
	A	B	C	D	E	F	
Galactose	+	+	+	+	+	+	100
Actidione	−	−	−	−	−	−	0
Saccharose	+	+	+	+	+	+	100
N-acetyl-glucosamine	+	+	+	+	+	+	100
DL-lactate	+	+	+	+	+	+	100
L-arabinose	+	+	+	+	+	+	100
Cellobiose	+	+	+	+	+	+	100
Raffinose	+	+	+	+	+	+	100
Maltose	+	+	+	+	+	+	100
Trehalose	+	+	+	+	+	+	100
2-keto-gluconate	+	+	+	+	+	+	100
α-methyl-D-glucoside	+	+	+	+	+	+	100
Mannitol	+	+	+	+	+	+	100
Lactose	+	+	+	+	+	+	100
Inositol	−	−	−	−	−	−	0
Sorbitol	+	+	+	+	+	+	100
D-xylose	+	+	+	+	+	+	100
Ribose	+	−	+	−	+	−	65
Glycerol	+	+	+	+	+	+	100
Rhamnose	+	+	+	+	−	+	90
Palatinose	+	+	+	+	+	+	100
Erythritol	−	−	−	+	+	−	70
Melibiose	−	−	−	−	−	−	0
Glucuronate	−	+	+	+	+	−	60
Melezitose	+	+	+	+	+	+	100
Gluconate	+	+	+	+	+	+	100
Levulinate	+	+	+	+	+	+	100
Glucose	+	+	+	+	+	+	100
Sorbose	+	+	+	+	+	+	100
Glucosamine	+	+	+	+	+	+	100
Esculin	−	−	−	−	−	−	0
Percentage of isolates	30	25	20	10	10	5	

[a]A: MD5, MD11, MD15, MD16, MD22, MD25. B: MD4, MD6, MD14, MD17, DH-com. C: MD8, MD10, MD12, MD13. D: MD2, MD3. E: MD1, MD7. F: MD9.

CBS8416. By use of *Hpa*II (Fig. 3B, lanes 1–10) restriction profiles different from those obtained with *Hae*III were seen. The frequency of recognition sites for the enzyme *Hpa*II is higher than for *Hae*III resulting in more bands by use of *Hpa*II. However, the clustering of the strains were the same as for *Hae*III except that CBS767 could not be separated from CBS164 and CBS1800. By use of *Hae*III, eight different mtDNA restriction profiles were obtained for the 10 strains of *D. hansenii* var. *fabryi* (Fig. 3A, lanes 11–20), i.e. it was not possible to differentiate between CBS789 and CBS5230 and between CBS6066 and CBS7254. Also for *D. hansenii* var. *fabryi* the mtDNA restriction profiles obtained by use of *Hpa*II (Fig. 3B, lanes 11–20) consisted of more bands compared to those obtained by use of *Hae*III. For *Hpa*II seven different mtDNA restriction profiles were obtained, i.e. it was not possible to differentiate between CBS789, CBS4373, CBS5230 and CBS6066.

By cluster analysis of the combined results of restriction analysis with *Hae*III and *Hpa*II (result not shown), the 20 CBS strains could be divided into 16 different clusters. None of the clusters contained strains of both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. A similarity of 36% was seen between the type strain of *D. hansenii* var. *hansenii* (CBS767) and the type strain of *D. hansenii* var. *fabryi* (CBS789).

3.5. Phenotyping dairy isolates

The 20 dairy isolates (DI1-17, DI22, DI25 and DH-com) obtained from seven different Danish dairies (Table 1) had micro- and macromorphological characteristics typical for *D. hansenii*. All isolates were identified as *D. hansenii* var. *hansenii* by their inability to grow at 36 °C. The isolates were further characterised by use of the API ID 32 C assimilation kit. The assimilation profiles of the 20

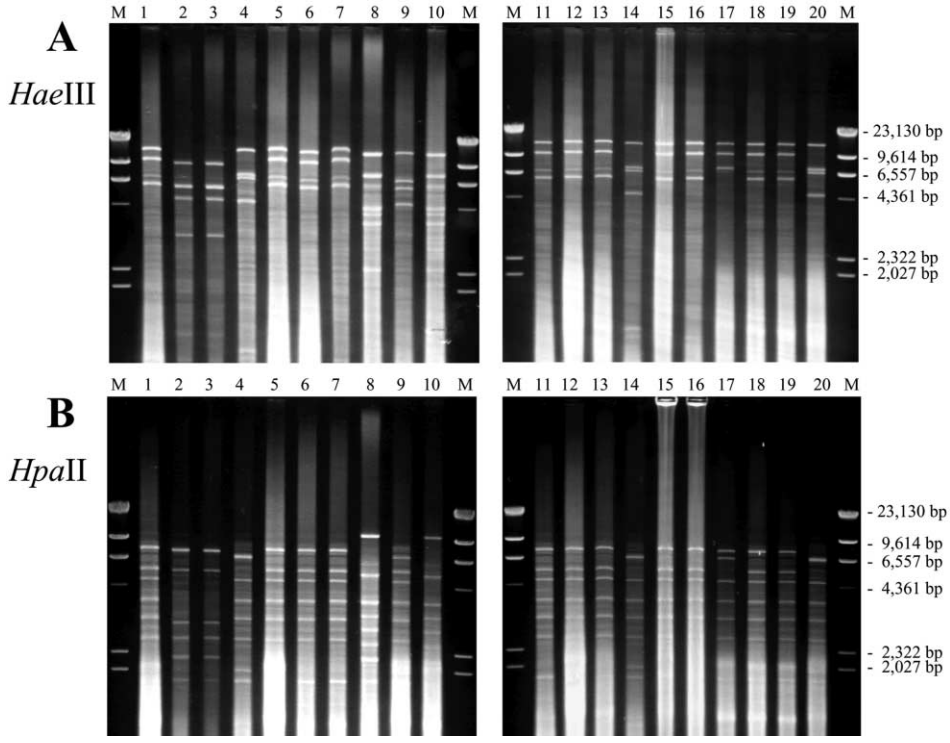

Fig. 4. Mitochondrial DNA restriction profiles of *D. hansenii* var. *hansenii* dairy isolates obtained by digestion with *Hae*III (A) or *Hpa*II (B). M: marker λDNA/*Hin*dIII, lane 1: DI1, lane 2: DI2, lane 3: DI3, lane 4: DI4, lane 5: DI5, lane 6: DI6, lane 7: DI7, lane 8: DI8, lane 9: DI9, lane 10: DI10, lane 11: DI11, lane 12: DI12, lane 13: DI13, lane 14: DI14, lane 15: DI15, lane 16: DI16, lane 17: DI17, lane 18: DI22, lane 19: DI25, lane 20: DH-com.

isolates are shown in Table 3. All isolates had assimilation profiles typical for *D. hansenii*, six different assimilation profiles were observed. The diversity was caused by differences in the ability to assimilate the following four carbon compounds: ribose, rhamnose, erythritol and glucoronate.

3.6. Genotyping of dairy isolates by mtDNA RFLP

The mtDNA restriction profiles obtained for the 20 dairy isolates by digestion with the restriction enzymes *Hae*III and *Hpa*II are shown in Fig. 4. By use of *Hae*III (Fig. 4A, lanes 1–20), six different

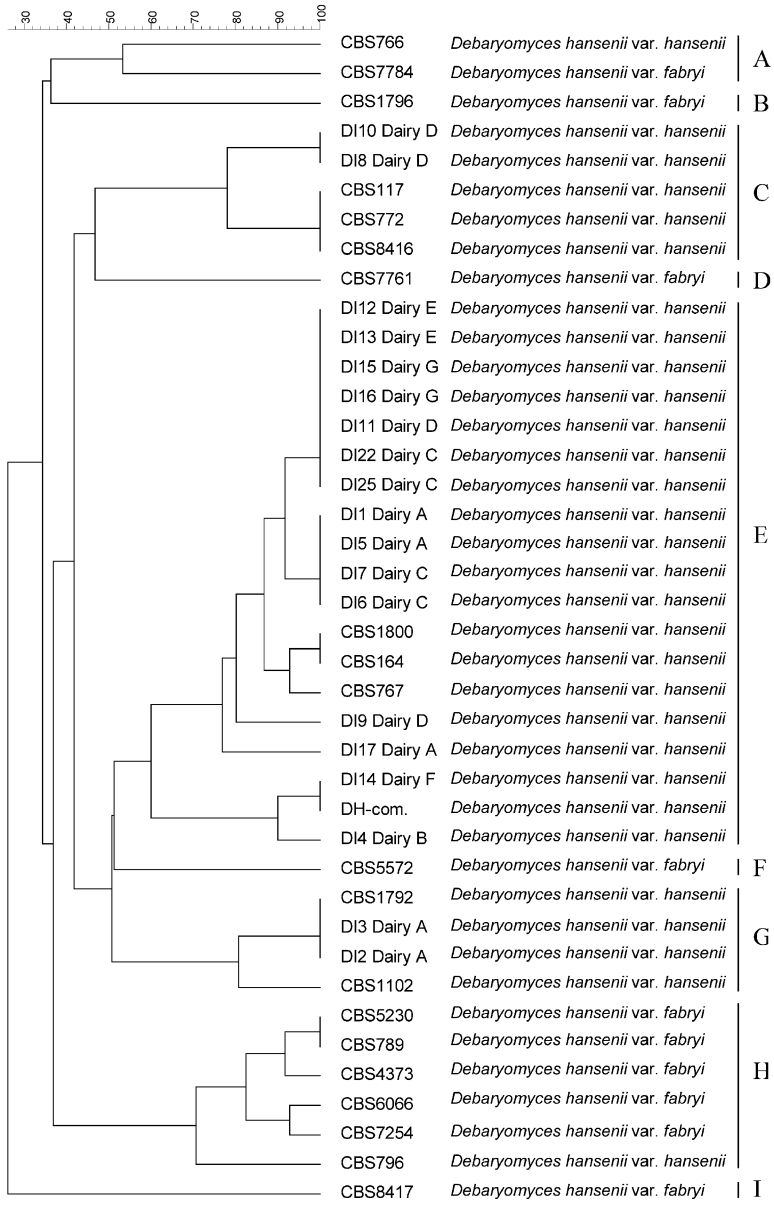

Fig. 5. Dendogram showing the clustering of *D. hansenii* strains (20 CBS strains and 20 dairy isolates) based on their mtDNA restriction profiles obtained by digestion with *Hae*III and *Hpa*II. The dendogram is achieved by use of the Dice coefficient and the unweighted pair group algorithm with arithmetic averages (UPMGA).

mtDNA restriction profiles were obtained for the 20 dairy isolates, i.e. the technique could not separate isolates within the following four groups: [DI1, DI5, DI6, DI7, DI11, DI12, DI13, DI15, DI16, DI22, DI25], [DI4, DI14, DH-com], [DI2, DI3] and [DI8, DI10]. By digestion with *Hpa*II (Fig. 4B, lanes 1–20) eight different mtDNA restriction profiles were obtained, the difference from the groups obtained by use of *Hae*III were that the isolates DI1, DI5, DI6 and DI7 separated from DI11, DI12, DI13, DI15, DI16, DI22, DI25 and DI4 were separated from DI14 and DH-com. As a consequence, cluster analysis of the combined results of restriction analysis with *Hae*III and *Hpa*II (result not shown) divided the 20 dairy isolates into eight clusters, each representing one of the restriction profiles obtained by use of *Hpa*II.

No obvious correlation was found between the eight mtDNA restriction profiles and the six assimilation profiles obtained for the dairy isolates. By combination of the mtDNA restriction profiles and the assimilation profiles 12 groups were obtained.

3.7. Clustering CBS strains of D. hansenii with dairy isolates

Fig. 5 shows a cluster analysis of the 20 CBS strains and the 20 dairy isolates based on their mtDNA restriction profiles obtained by digestion with *Hae*III and *Hpa*II, respectively. In total 23 different combined restriction profiles were obtained. The 40 isolates could be divided into nine clusters with a similarity level of maximum 52%, none of the clusters included both dairy isolates and *D. hansenii* var. *fabryi* CBS strains. Five of the nine clusters represented more than one isolate (cluster A, C, E, G and H). Cluster E (47.5% of all isolates) included 80% of the dairy isolates and 30% of the 10 *D. hansenii* var. *hansenii* CBS strains, whereas no CBS strains of *D. hansenii* var. *fabryi*. The remaining 20% of the dairy isolates were included in cluster C and G; cluster C (12.5% of all isolates) included 10% of the dairy isolates and 30% of the *D. hansenii* var. *hansenii* CBS strains and cluster G (10% of all isolates) included 10% of the dairy isolates and 20% of the *D. hansenii* var. *hansenii* CBS strains. The remaining 20% of the *D. hansenii* var. *hansenii* CBS strains were included in cluster A and cluster H together with CBS strains of *D. hansenii* var. *fabryi*. The remaining strains of *D. hansenii* var. *fabryi* clustered in cluster B, D, F and I. The *D. hansenii* var. *fabryi* strain CBS8417 were distinct from all other investigated isolates of *D. hansenii* and could be differentiated at a similarity level of 28%.

4. Discussion

In the present study ITS-PCR could be used for differentiation of yeast species of importance for surface ripened cheeses. For each of the investigated species a specific ITS1-5.8S rDNA-ITS2 region size was found. The sizes of the ITS1-5.8S rDNA-ITS2 regions obtained for the yeast species investigated in this study confirmed an earlier study by Esteve-Zarzoso et al. (1999) who analysed 132 yeast species obtained from culture collection. One strain of *D. hansenii* var. *fabryi* (CBS8417) analysed in this study was atypical by having a ITS1-5.8S rDNA-ITS2 region size 32 bp lower than all other investigated isolates of *D. hansenii*. However, this strain was also found to differ in other aspects and even though the assimilation profile of this strain is typical for *D. hansenii*, the genetic diversity obtained by sequence analysis of the ITS1-5.8S rDNA-ITS2 region and mtDNA RFLP (Figs. 3, 5) indicates that this strain might not be associated to the species *D. hansenii*.

From the restriction analysis of the ITS1-5.8S rDNA-ITS2 region of 20 *D. hansenii* strains, it could be concluded that the ITS-PCR RFLP method is not suitable for typing of *D. hansenii* at strain level. Only one out of nine investigated restriction enzymes (*Taq*I) demonstrated genetic diversity within the strains. This enzyme divided the 20 strains of *D. hansenii* into three groups. Sequence analysis of the ITS1-5.8S rDNA-ITS2 region for the type strain of *D. hansenii* var. *hansenii* (CBS767) and *D. hansenii* var. *fabryi* (CBS789) confirmed this observation as an identity of 99.84% (corresponding to a difference in one basepair) found between the ITS1-5.8S rDNA-ITS2 regions of the two type strains. This observation together with the restriction analysis of several strains (ten of each variety) indicates that the two varieties within *D. hansenii* are very closely related.

ITS-PCR RFLP is earlier reported useful for typing of yeasts at subspecies level. In a study by

Baleiras Couto et al. (1996), they investigated 15 isolates of *S. cerevisiae* from wine and beer; the isolates were divided into three and four groups by use of *Taq*I and *Mse*I, respectively. Accordingly, they concluded the method valuable for subspecies typing of *S. cerevisiae*. Guillamón et al. (1998) found ITS-PCR RFLP useful for differentiation of yeasts at species level in an investigation comprising 33 yeast species from beer and wine belonging to 11 different genera. For each species, a specific restriction pattern were found by use of one of the restriction enzymes *Cfo*I, *Hae*III and *Hin*fI. Recently, Esteve-Zarzoso et al. (1999) confirmed this result in an extended study comprising 132 yeast species belonging to 25 genera, including both telemorphic and anamorphic ascomycetous and basidiomycetous yeasts. Based on the results obtained in the present study for *D. hansenii* and the observations done by Guillamón et al. (1998) and Esteve-Zarzoso et al. (1999), ITS-PCR RFLP seems not to be useful for typing of yeasts at strain level. However, as different ITS-PCR RFLP restriction profiles have been observed between strains within the same species by use of specific restriction enzymes as reported in the present study and by Baleiras Couto et al. (1996), the evaluation of the ITS-PCR RFLP method for species recognition requires investigation on a large number of isolates in order to ensure species specific restriction profiles.

Extensive restriction fragment length polymorphism was observed between *D. hansenii* strains by use of mtDNA RFLP. In general, the CBS strains showed a higher genetic variability than the dairy isolates; however, they also varied more phenotypically (results not shown) than the dairy isolates. This observation indicates that the dairy isolates are more closely related than the CBS strains. The discriminative power of the mtDNA RFLP was increased by use of a combination of two restriction enzymes. For the CBS strains, the use of *Hae*III resulted in a greater polymorphism than *Hpa*II; the opposite was observed for the dairy isolates. Romano et al. (1996) used mtDNA RFLP for subspecies differentiation of *D. hansenii* and *C. zeylanoides* isolates from Spanish cheeses. They observed a high level of genetic variability by use of *Hae*III and *Ava*II for *D. hansenii*. By use of one of these enzymes, the 28 isolates of *D. hansenii* were divided into five groups. The isolates of *C. zeylanoides* were differentiated by use of *Bst*NI and *Hpa*II; 27 isolates were divided into five groups. It is confirmed by the present study that the use of *Hae*III shows a high level of genetic variability for *D. hansenii*. The level of discrimination depends on the selected restriction enzymes and we suggest a combination of *Hae*III and *Hpa*II for differentiation of *D. hansenii* isolates.

Cluster analysis of the 20 CBS strains and the 20 dairy isolates based on their mtDNA restriction profiles obtained by digestion with *Hae*III and *Hpa*II (Fig. 5), showed a similarity level of maximum 52% that the dairy isolates only clustered with the CBS strains of *D. hansenii* var. *hansenii*. This is in accordance with the identification of all the dairy isolates as *D. hansenii* var. *hansenii* due to their inability to grow at 36 °C. Some of the clusters contained isolates of both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, which show that mtDNA RFLP cannot be used to differentiate taxonomically between the two varieties and confirm their close relatedness.

The cluster analysis (Fig. 5) showed that within some of the seven dairies (dairy A, C, D) more than one predominant strain of *D. hansenii* were observed. This observation indicates that several strains might be involved in the ripening process of surface ripened cheeses.

According to the differences in pheno- and genotypic characteristics observed for the dairy isolates, their technological properties are also presumed to vary. Further a succession of strains during the ripening process might occur as investigated previously for other species in other fermented products (Hayford and Jespersen, 1999; Hayford and Jakobsen, 1999). Sabate et al. (1998) used mtDNA RFLP to investigate the succession between *S. cerevisiae* strains during wine fermentation. In connection to surface ripened cheeses, mtDNA RFLP appears to be useful for investigation of the microbial succession between strains of *D. hansenii* during the ripening process. Further investigations of the correlation between the microbial succession and the technological properties of *D. hansenii* strains during the production of surface ripened cheeses should be carried out.

In conclusion, the results of the present study suggest that ITS-PCR is useful for differentiation of yeasts species of importance for surface ripened

cheeses, whereas ITS-PCR RFLP was found not to be useful for typing of *D. hansenii* at strain level. Further mtDNA RFLP was found to have a high discriminative power for typing of *D. hansenii* strains and were proved to obey the requirement of a simple method for subspecies typing of *D. hansenii*.

Acknowledgements

This work is part of the FØTEK programme supported by the Danish Dairy Research Foundation (Danish Dairy Board) and the Danish government. The authors are grateful to Tina Schmidt Christensen for excellent technical assistance.

References

Aiglé, M., Erbs, D., Moll, M., 1984. Some molecular structures in the genome of lager brewing yeasts. Am. Soc. Brew. Chem. 42, 1–7.

Baleiras Couto, M.M., Eijsma, B., Hoftra, H., Huis in't Veld, J.H.J., van der Vossen, J.M.B.M., 1996. Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. Appl. Environ. Microbiol. 62, 41–46.

Belloch, C., Barrio, E., Uruburu, F., Garcia, M.D., Querol, A., 1997. Characterization of four species of the genus *Kluyveromyces* by mitochondrial DNA restriction analysis. Syst. Appl. Microbiol. 20, 397–408.

Eliskases-Lechner, F., Ginizinger, W., 1995a. The yeast flora of surface ripened cheeses. Milchwissenschaft 50, 458–462.

Eliskases-Lechner, F., Ginizinger, W., 1995b. The bacterial flora of surface ripened cheeses with special regard to coryneforms. Lait 75, 571–584.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.

Fleet, G.H., 1990. Yeast in dairy products—a review. J. Appl. Bacteriol. 68, 199–211.

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., Sabaté, J., Barrio, E., Cano, J., Querol, A., 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch. Microbiol. 169, 387–392.

Hayford, A.E., Jakobsen, M., 1999. Characterization of *Candida krusei* strains from spontaneously fermented maize dough by profiles of assimilation, chromosome profile, polymerase chain reaction and restriction endonuclease analysis. J. Appl. Microbiol. 1, 29–40.

Hayford, A.E., Jespersen, L., 1999. Characterization of *Saccharomyces cerevisiae* strains from spontaneously fermented maize dough by profiles of assimilation, chromosome polymorphism, PCR and *Mal* genotyping. J. Appl. Microbiol. 86, 284–294.

Jakobsen, M., Narvhus, J., 1996. Yeast and their possible beneficial and negative effects on the quality of dairy products. Int. Dairy J. 6, 755–768.

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.

Lee, S.Y., Knudsen, F.B., 1985. Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. J. Inst. Brew. 91, 169–173.

Lenoir, J., 1984. The surface flora and ITS1-5.8S rDNA-ITS2 role in the ripening of cheese. Int. Dairy Fed. Annu. Bull. 171, 3–20.

Lerlercq-Perlat, M.N., Oumer, A., Bergere, J.L., Spinnler, H.E., Corrieu, G., 1999. Growth of *Debaryomyces hansenii* on a bacterial surface-ripened soft cheese. J. Dairy Res. 66, 271–281.

Nakase, T., Suzuki, M., Phaff, H.J., Kurtzman, C.P., 1998. *Debaryomyces* Lodder and Kreger-van Rij Nom. Cons. In: Kurtzman, C.P., Fell, J.W. (Eds.), 4th edn. The Yeast: A Taxonomic Study, vol. 26. Elsevier, Amsterdam, pp. 157–163.

Oda, Y., Yabuki, M., Tonomura, K., Fununaga, M., 1997. A phylogenic analysis of *Saccharomyces* species by sequence of 18S–28S rRNA spacer regions. Yeast 13, 1243–1250.

Prillinger, H., Molnár, O., Eliskases-Lechner, F., Lopandic, K., 1999. Phenotypic and genetypic identification of yeasts from cheese. Antonie van Leeuwenhoek 75, 267–283.

Querol, A., Barrio, E., Ramon, D., 1992. A comparative study of different methods of strain characterisation. Syst. Appl. Microbiol. 15, 439–446.

Reps, A., 1993. Bacterial surface-ripened cheeses. In: Fox, P.F. (Ed.), 2nd edn. Cheese, Chemistry, Physics and Microbiology, vol. 2. Chapman and Hall, London, UK, pp. 137–172.

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.

Sabate, J., Cano, J., Querol, A., Guillamón, J.M., 1998. Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. Lett. Appl. Microbiol. 26, 452–455.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chainterminating-inhibitors. Proc. Natl. Acad. Sci. 74, 5463–5467.

Seiler, H., 1986. Identification of cheese-smear coryneform bacteria. J. Dairy Res. 53, 439–449.

Valdés-Stauber, N., Scherer, S., Seiler, H., 1997. Identification of yeasts and coryneform bacteria from the surface microflora of brick cheeses. Int. J. Food Microbiol. 34, 115–119.

Vezinhet, F., Blondin, B., Hallet, J.N., 1990. Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of ecological strains of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 32, 568–571.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols, vol. 38. Academic Press, San Diego, p. 317.