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Genetic diversity of the species *Debaryomyces hansenii* and the use of chromosome polymorphism for typing of strains isolated from surface-ripened cheeses

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ABSTRACT

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Aims: To investigate the genetic diversity among strains of *Debaryomyces hansenii* and further to evaluate chromosome polymorphism determined by pulse-field gel electrophoresis (PFGE) as a tool for strain typing.

Methods and Results: In total 56 isolates of *D. hansenii* were analysed by PFGE. The isolates included type strains and other strains obtained from culture collections as well as strains collected during production of Danish surface-ripened cheeses. By use of the PFGE technique the number and size of the chromosomal bands were calculated and the total genome size estimated. The number of chromosomal bands observed was found to vary from five to 10. The most common chromosome number was found to be six and for strains with six chromosomes the total genome size was found to vary from 9·4 to 12·6 Mb. The chromosome numbers for the type strain of each variety of *D. hansenii* (*D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*) appeared to be six and seven respectively. By use of the PFGE technique it was possible to differentiate between all the investigated CBS strains and the vast majority of the dairy isolates. The dairy isolates that were found to have identical profiles (three of 56 isolates) were all isolated during production of one batch of surface-ripened cheeses and are likely to be the same strain isolated several times during cheese production. Further it was shown that the PFGE analysis did not result in a division of the two *D. hansenii* varieties, i.e. *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii* into separate groups.

Conclusion: The present study shows that the chromosomal arrangement of *D. hansenii* strains is heterogenic and does have a distinct chromosome polymorphism. Further the PFGE technique was proved to have a high discriminative power for strain typing of *D. hansenii*.

Significance and Impact of the Study: The results obtained add to the first knowledge on the genetic diversity of the species *D. hansenii*. Further the distinct chromosome polymorphism of *D. hansenii* strains as shown in this study makes the PFGE technique a useful tool for strain typing of *D. hansenii*, e.g. during cheese production.

Keywords: chromosome number, chromosome polymorphism, chromosome size, *Debaryomyces hansenii*, genetic diversity.

INTRODUCTION

The halophilic yeast *Debaryomyces hansenii* (teleomorphic form of *Candida famata*) is a highly heterogeneous species as shown by phenotypic differences such as the ability to assimilate/fermentate different carbon compounds (Seiler and Busse 1990; Nakase *et al.* 1998; van den Tempel and Jakobsen 2000), lipase and protease activity (Sørensen and Jakobsen 1997) and the ability to grow at different environmental conditions (Petersen *et al.* 2002). According to the present taxonomy *D. hansenii* is divided into two

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varieties *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*. According to Nakase and Suzuki (1985a,b) the two varieties can be discriminated by different electrophoretic mobilities of their glucose-6-phosphate dehydrogenase and by differences in their maximum temperatures for growth. While the phenotypic properties of *D. hansenii* have been investigated to some extent, the knowledge on the genetic structure including ploidy, genome size as well as chromosome numbers and sizes is rather limited. The type strain (CBS767) of *D. hansenii* var. *hansenii* has previously been reported to be haploid (van der Walt *et al.* 1977) whereas information on the ploidy of other strains belonging to the species *D. hansenii* is lacking. Recently Corredor *et al.* (2003) reported the chromosomal numbers of different *D. hansenii* strains to vary between four and 10.

Debaryomyces hansenii is involved in the fermentation of a number of food products including several types of surface-ripened cheeses. e.g. Limburger, Tilsitter, Port Salut, Trappist, Brick and the Danish Danbo. For these types of cheeses the development of an appropriate bacterial surface flora has been shown to be dependent on the metabolism of lactic acid by yeasts, in particular *D. hansenii* (Lerlercq-Perlat *et al.* 1999). Further *D. hansenii* might produce growth factors of importance for the bacteria as well as aroma components and lipolytic and proteolytic enzymes that contribute to the ripening process (Lenoir 1984; Fleet 1990; Jakobsen and Narvhus 1996). For most of the above-mentioned cheeses strains of *D. hansenii* are introduced spontaneously. The benefits moving from spontaneously fermentations to controlled fermentations are numerous (e.g. improving the quality and taste of the product) and therefore there seems to be a growing interest for the use of *D. hansenii* as a well-defined starter culture. Consequently the use of starter cultures with appropriate technological properties generates a need for fast and simple methods for typing of *D. hansenii* at strain level.

DNA techniques are valuable methods for typing of yeasts at strain level. Among the most successfully used methods are the determination of chromosome length polymorphism (CLP) by different types of pulsed-field gel electrophoresis (PFGE), e.g. orthogonal field alternation gel electrophoresis or clamped homogenous electric field gel electrophoresis. Determination of CLP has especially been used for strain typing of *Saccharomyces cerevisiae* isolated from various foods and beverages (Vezinhet *et al.* 1992; Querol *et al.* 1994; Hayford and Jespersen 1999; van der Aa Kuhle *et al.* 2001). Determination of CLP has also been proved to be useful for strain typing of other yeast species, e.g. *Yarrowia lipolytica* and *C. zeylanoides* (Deak *et al.* 2000), *C. albicans* (Monod *et al.* 1990; Doi *et al.* 1992), *C. kefyr* (Sor and Fukuhara 1989) and *C. parapsilosis* (Carruba *et al.* 1991). So far determination of CLP has never been used for strain typing of *D. hansenii* during food production actually only a limited number of techniques have been reported for strain typing of *D. hansenii*. One of the most discriminative techniques reported for typing of *D. hansenii* is mitochondrial (mt)-DNA restriction fragment length polymorphism (RFLP) (Petersen *et al.* 2001) that originally was developed for differentiation of wine and beer strains of *Saccharomyces* spp. (Aiglé *et al.* 1984; Lee and Knudsen 1985; Vezinhet *et al.* 1990). The method has been evaluated on 20 isolates of *D. hansenii* obtained from the CBS culture collection. Based on this technique 80% of the CBS isolates could be differentiated (Petersen *et al.* 2001).

The objectives of the present study has been to obtain knowledge on the chromosomal arrangement of *D. hansenii* strains by PFGE and to determine the genetic diversity of strains isolated from Danish surface-ripened cheeses with type strains and other well-characterised strains obtained from culture collections.

MATERIALS AND METHODS

Yeast isolates

Isolates used in this study are listed in Table 1. CBS strains were obtained from Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). The isolates denoted DI were collected from eight different Danish dairies, all producers of surface-ripened cheeses of the Danbo type. The isolates denoted D were isolated during the production of one batch of surface-ripened cheeses. The isolates (DI and D) were either collected from the raw milk, the brine, the smear or from the cheese surface during ripening. The cheeses were made from pasteurized raw milk (74·6°C for 18 s). After the last pressing the cheeses were salted in saturated brine (22% w/v) at 11–13°C for 48 h, then inoculated with a slurry containing smear from the surface of 7-day-old cheeses and starter cultures of *Debaryomyces hansenii* and *Brevibacterium linens*. The cheeses were reinoculated after 4 days of ripening. After the first inoculation cheeses were stored at 22°C and 98% RH (relative humidity) for 48 h and then for approx. 20 days at 18°C and 95% RH. Finally the cheeses were stored for approx. 3 weeks at 14°C and 85% RH after which the smear was washed off and the cheeses were packed in a polyolefin (19 μm) film and further ripened for approx. 29 weeks at 7–8°C. The dairy isolates were isolated and identified as described by Petersen *et al.* (2001, 2002). The commercial starter culture for cheese ripening DH-com (*D. hansenii*) was obtained from Danisco Innovation (Copenhagen, Denmark). All isolates were purified by repeated cultivation on malt extract yeast extract glucose peptone

Table 1 Estimated size of chromosomes and total genome size for strains of *Debaryomyces hansenii*

		Size of chromosomal DNA (Mb)										
Strain	Origin	1	2	3	4	5	6	7	8	9	10	Estimated genome size (Mb)
CBS117*	CBS	3·11	2·69	2·05	1·89	1·72	1·46	1·36	1·20			15·48
CBS164*	CBS	2·90	2·53	2·16	1·84	1·58	1·53					12·54
CBS766*	CBS	2·93	2·73	2·27	1·89	1·54	1·27					12·63
CBS767*†‡	CBS	3·14	2·56	2·20	1·85	1·51	1·36					12·62
CBS772*	CBS	3·14	3·05	2·22	1·78	1·43	1·32	1·24				14·18
CBS796*	CBS	2·76	2·57	1·98	1·75	1·68	1·34					12·08
CBS1102*	CBS	3·14	2·75	2·52	2·21	2·01	1·81	1·51	1·33	1·27	0·71	19·26
CBS1792*	CBS	2·83	2·30	1·86	1·52	1·40						9·91
CBS1800*	CBS	3·04	2·58	2·14	1·86	1·56	1·39					12·57
CBS8416*	CBS	3·03	2·81	2·24	1·89	1·82	1·67	1·54	1·38			16·38
CBS789§‡	CBS	2·68	2·43	2·08	1·69	1·46	1·24	0·31				11·89
CBS1796§	CBS	2·75	2·31	2·07	1·28	1·21	0·76					10·38
CBS4373§	CBS	2·81	2·50	2·06	1·68	1·52	1·25					11·82
CBS5230§	CBS	2·71	2·36	1·96	1·70	1·49	1·22					11·44
CBS5572§	CBS	2·80	2·20	1·97	1·53	1·44	1·24	1·08				12·26
CBS6066§	CBS	2·84	2·59	2·18	1·98	1·88	1·72	1·53	1·23			15·95
CBS7254§	CBS	2·57	2·39	2·06	1·71	1·49	1·20					11·42
CBS7761§	CBS	2·88	2·37	1·94	1·52	1·33	1·13					11·17
CBS7784§	CBS	2·23	1·92	1·51	1·40	1·23	1·10					9·39
CBS8417§	CBS	3·06	2·14	1·85	1·59	1·31	1·00	0·87	0·39			12·21
DI1*	Dairy A, smear	2·72	2·38	2·03	1·89	1·65	1·41	1·02				13·10
DI2*	Dairy A, smear	3·13	2·45	2·02	1·74	1·43	1·15					11·92
DI3*	Dairy A, smear	2·88	2·43	2·04	1·76	1·51	1·39					12·01
DI4*	Dairy B, smear	2·96	2·48	2·09	1·74	1·55	0·88					11·70
DI5*	Dairy A, smear	3·11	2·69	2·18	1·98	1·87	1·65	1·33	1·18	1·03		17·02
DI6*	Dairy C, smear	3·12	2·69	2·38	2·06	1·91	1·66	1·42	1·33	0·97		17·54
DI7*	Dairy C, smear	2·69	2·03	1·88	1·63	1·52	1·35	1·04				12·14
DI8*	Dairy D, brine	2·95	2·47	2·04	1·91	1·75	1·25					12·12
DI9*	Dairy D, brine	2·95	2·53	2·09	1·99	1·86	1·48	1·34				14·24
DI10*	Dairy D, smear	3·13	2·96	2·50	2·06	1·95	1·89	1·76	1·23			17·48
DI11*	Dairy D, smear	3·10	2·73	2·42	2·02	1·89	1·70	1·43	1·32	1·04		17·65
DI12*	Dairy E, smear	3·08	2·71	2·38	2·03	1·87	1·76	1·41	1·30	1·03	0·78	18·35
DI13*	Dairy E, smear	2·89	2·66	2·19	1·99	1·88	1·59	1·50	1·37	1·05	0·85	17·97
DI14*	Dairy F, smear	2·91	2·53	2·11	1·75	1·57	0·90					11·77
DI15*	Dairy G, smear	3·13	2·67	2·05	1·98	1·76	1·44	1·10				14·13
DI16*	Dairy G, smear	3·15	2·68	2·02	1·38	1·34	1·18	1·06	0·97			13·78
DI17*	Dairy A, smear	3·16	1·99	1·91	1·78	1·71	1·54	1·45	1·31	0·96		15·81
DI22*	Dairy C, smear	2·67	2·25	2·05	1·91	1·67	1·48	1·33	0·97	0·85		15·18
DI25*	Dairy C, smear	3·09	2·73	2·17	2·02	1·89	1·68	1·44	1·35	0·99	0·82	18·18
DH-com*¶	CI	2·97	2·46	2·09	1·76	1·58	0·99					11·85

*Strains of the variety *D. hansenii* var. *hansenii*.

†Haploid strain (van der Walt *et al.* 1977). Ploidy of other strains unknown.

‡Type strain.

§Strains of the variety *D. hansenii* var. *fabryi*.

¶Commercial isolate, Danisco Innovation, Copenhagen, Denmark.

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), 10·0 g glucose (Merck) per litre distilled water] added 20% (v/v) glycerol.

Pulsed-field gel electrophoresis

The yeast cultures were pregrown in Yeast Peptone Glucose broth containing per litre of distilled water 10·0 g yeast extract (Difco), 20·0 g bactopeptone (Difco) and 40·0 g glucose (Merck), pH = 5·6, at 25°C for 48 h and then successively recultivated twice for 24 h. *D. hansenii* chromosomal DNA was prepared in agarose plugs as described by Jespersen *et al.* (2000). For each isolate 1/4 agarose plug was transferred to a 0·8% (w/v) NA-agarose gel (Amersham Biosciences, Uppsala, Sweden).

The PFGE was performed with a PFGE DR-III unit (Bio-Rad, Hercules, CA, USA). During electrophoresis TBE buffer [45 mM Tris-base (Sigma, St. Louis, MO, USA), 44 mM boric acid (Sigma), 1 μM EDTA) was used as running buffer. The buffer was changed every 24 h. Running conditions for the resolution of chromosomal DNA molecules were a 200 s switch interval at 150 V for 24 h followed by a 700 s interval at 100 V for 48 h. Yeast DNA-PFGE markers (*Saccharomyces cerevisiae* YNN295, Amersham Biosciences and *Hansenula wingei*, Bio-Rad) were used for determination of chromosomal sizes. Finally the gel was stained with 1 mg ethidium bromide (Sigma) per litre TBE buffer for 1 h and rinsed twice with milliQ water for 15 min. The gels were visualized with u.v. transillumination and photographed. Estimation of chromosomal DNA sizes were made by use of the Kodak 1D Image Analysis Software, version 3·5 (Eastman Kodak Company, Rochester, NY, USA).

Cluster analysis

The cluster analyses were carried out by use of the computer program BioNumerics version 1·0 (Applied Maths, Kortrijk, Belgium). The similarities between chromosomal 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).

RESULTS

Chromosome number and genome size for CBS strains of *Debaryomyces hansenii*

Among the 20 CBS isolates (Table 1) chromosome polymorphism was evident and included both the size and the number of chromosomal bands. Figure 1a shows the chromosome profiles for the 20 CBS strains and Table 1 gives the number and estimated sizes of chromosomal bands. Of the investigated CBS strains the only strain with a known ploidy is the type strain of *D. hansenii* var. *hansenii* (CBS767) that previously has been reported to be haploid (van der Walt *et al.* 1977). For this strain six chromosomal bands were

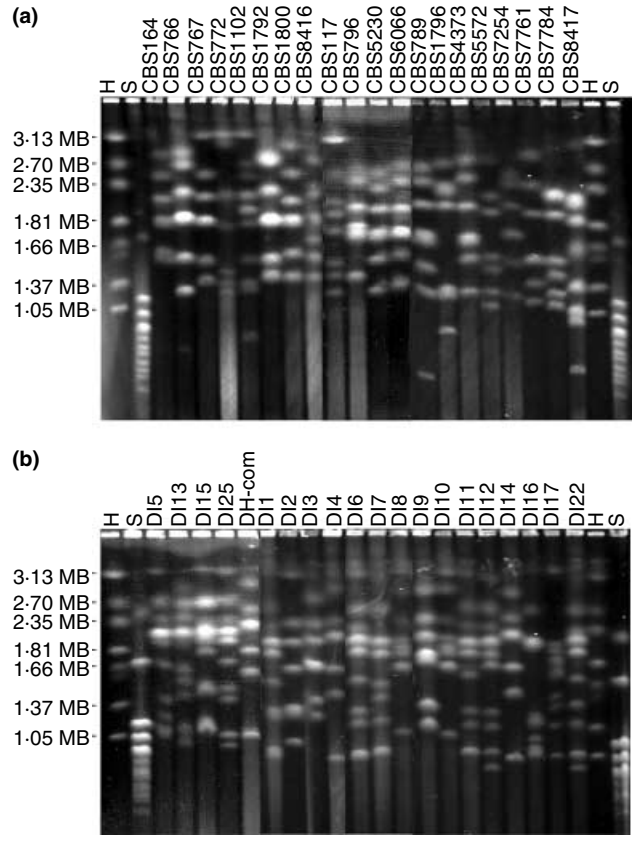

Fig. 1 Pulse-field gel electrophoresis of *Debaryomyces hansenii* strains under a 200 switch interval at 150 V for 24 h followed by a 700 s switch interval at 100 V for 48 h. Markers: H, *Hansenula wingei*; S, *Saccharomyces cerevisiae*

observed by the PFGE technique with sizes from 1·36 to 3·14 Mb. The genome size of the entire genome could be estimated to approx. 12·6 Mb. For the type strain of *D. hansenii* var. *fabryi* (CBS789) seven chromosomal bands were observed with sizes from 0·31 to 2·68 Mb and the genome size was estimated to approx. 11·9 Mb. Of the remaining 18 stains, six chromosomal bands were observed for 10 strains. On average the genome size for the 11 strains with six chromosomal bands were 11·6 ± 1·0 Mb. For one strain (CBS1792) only five chromosomal bands were observed and the total genome size for this particular strain was found to be approx. 9·91 Mb. As two chromosomal bands corresponding to a size of 1·86 and 2·83 Mb show a significantly increased fluorescence intensity compared with the other bands it is reasonable to believe that this strain (CBS1792) actually has a higher number of chromosomes than five and that the observation of five bands is a result of co-migration of chromosomes. A higher number of chromosomal bands than six could be observed for the remaining eight CBS strains. For these strains the number of chromosomal bands varied

between seven and 10 with sizes from 0·31 to 3·14 Mb. Due to the additional number of chromosomal bands a huge variation in the estimated genome size was found (9·4–19·3 Mb) among these eight strains. However, some of the chromosomal bands were very close in size, e.g. for strain CBS1102 chromosomal bands with sizes of 1·27 and 1·33 Mb were seen. This could indicate that these chromosomal bands were two copies of the same chromosome and thereby that the strains have another ploidy than haploid. Most likely they are diploid or aneuploid strains.

Chromosome number and genome size for dairy isolates of *Debaryomyces hansenii*

Among the 20 dairy isolates (DI1–DI17, DI22, DI25 and DH-com) chromosome polymorphism were evident and included both the size and the number of chromosomal bands observed. The chromosome profiles for the dairy isolates are shown in Fig. 1b. Table 1 gives the number and estimated sizes of chromosomal bands. Six chromosomal bands were observed for six of the dairy isolates with sizes from 0·90 to 3·13 Mb. On average the genome size for these isolates were 11·9 ± 0·2 Mb. For the remaining isolates a higher number of chromosomal bands were observed. For these isolates the number of chromosomal bands varied between seven and 10 with sizes from 0·78 to 3·16 Mb. Due to the high numbers of chromosomal bands observed the estimated genome size among these isolates varied between 12·1 and 18·4 Mb. As for the CBS strains some of the chromosomal bands were very close in size, e.g. for isolate DI16 chromosomal bands with sizes of 1·34 and 1·38 Mb were seen, which could indicate that these chromosomal bands are two copies of the same chromosome.

The genetic diversity of CBS and dairy strains of *D. hansenii*

Figure 2 shows a cluster analysis based on the chromosomal profiles of the 20 CBS strains and the 20 dairy isolates obtained from seven different dairies (Table 1). All the isolates investigated had unique profiles. The 40 isolates could be divided in 10 clusters with a similarity level of

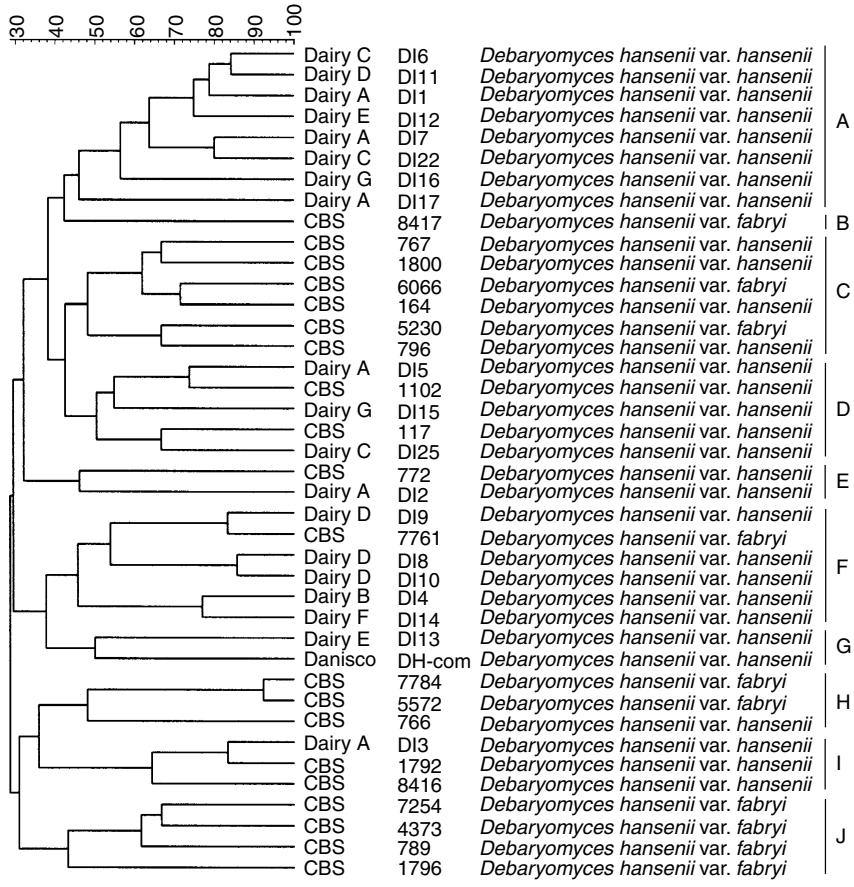

Fig. 2 Dice/UPMGA dendogram showing the clustering of *Debaryomyces hansenii* strains (20 CBS strains, 19 dairy isolates and one commercial starter culture) based on determination of CLP by PFGE

maximum 43%. The dairy isolates were divided into six of the clusters (A, D, E, F, G, I) and could not be differentiated from the CBS strains as a number of the clusters (D, E, F, I) included both CBS and dairy strains. Further, it was not possible to differentiate the dairy isolates into dairy-specific clusters as, e.g. cluster A included isolates from five of seven dairies. Among the CBS strains cluster analysis did not result in a division of the two *D. hansenii* varieties, i.e. *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii* into separate groups. Although all the dairy isolates were identified as *D. hansenii* var. *hansenii* (Petersen *et al.* 2001) they were found to cluster together with both CBS strains of *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, which shows that it is not possible to differentiate between the two varieties by their chromosome polymorphism.

Use of PFGE for typing of *D. hansenii* isolates during the production of Danish surface-ripened cheeses

Figure 3 shows a cluster analysis based on chromosomal profiles of 16 isolates obtained from a Danish dairy during the production of one batch of surface-ripened cheeses. For the 16 isolates 13 different chromosomal profiles were obtained indicating that several different strains are involved in the fermentation of surface-ripened cheeses. In cluster A, a 100% similarity between three isolates (D83311, D10349 and D18335) was observed. These three isolates were respectively isolated from the inoculation slurry, the cheese surface after inoculation and the cheese surface after 7 days of ripening. As in cluster A, a 100% similarity was observed for the strains in cluster K (D9311, D9312). Both isolates were obtained from the cheese surface after brining. Compared with the high discriminative power of PFGE resulting in a 100% separation of all *D. hansenii* strains as showed in Fig. 2 it is reasonable to believe that these isolates actually are the same strain isolated several times during cheese production. Some of the other isolates were very closely related, e.g. the strains in cluster A had a similarity level of 78% with the strain in cluster B (isolated from the cheese surface after reinoculation). Further the strains in cluster K had a similarity level of more than 94% with the strain in cluster L (D10341, isolated from the cheese surface after reinoculation). None of these dairy isolates denoted D (Fig. 3) had identical chromosomal profiles to either the CBS strains (Figs 1a and 2) or the dairy isolates denoted DI (Figs 1b and 2). The high number of different profiles shows that several different strains are involved during the production of Danish surface-ripened cheeses.

DISCUSSION

The type strain of *D. hansenii* (CBS767) has previously been reported to be haploid (van der Walt *et al.* 1977). The number of chromosomes for this specific strain of *D. hansenii* (CBS767) was in the present study found to be six suggesting that the chromosome number of *D. hansenii* is six. On the PFGE gel, bands with multiple chromosomes

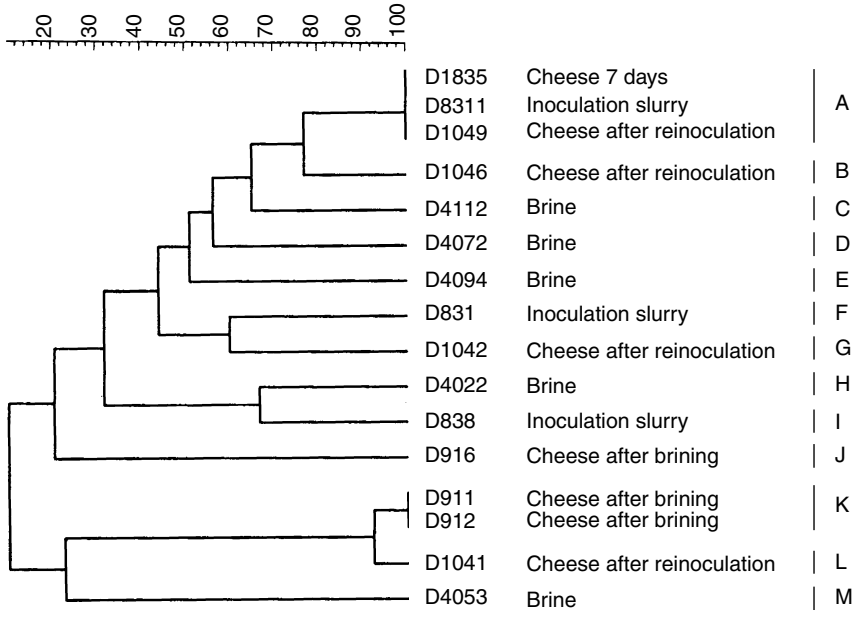

Fig. 3 Dice/UPMGA dendogram based on determination of CLP by PFGE showing the clustering of *Debaryomyces hansenii* strains isolated during the production of one batch of Danish surface-ripened cheeses

will display a significantly increased fluorescence intensity compared with single chromosomal bands of a similar size (Doi *et al.* 1992). However, for the type strain of *D. hansenii* var. *hansenii* (CBS767) no variation in the fluorescence intensity was observed between the bands indicating that no multiple chromosomal bands were present thereby supporting our theory of six chromosomes in *D. hansenii* var. *hansenii*. PFGE has previously been successfully used for karyotyping of *Geotrichum candidum* strains (Gente *et al.* 2002). In that study it was shown that the determination of chromosome numbers by microscopic karyotyping could be confirmed by PFGE.

In the present study, 55% of the analysed strains were found to have a higher number of chromosomal bands than six. As the ploidy of these strains is unknown it is not possible to determine whether these additional chromosomal bands are true chromosomes or multiple alleles of the same chromosomes seen for nonhaploid strains. However, as some of the chromosomal bands are very close in size, the additional number of chromosomal bands might for nonhaploid strains be due to multiple alleles of the same chromosome generated by duplication or deletion of sequences (Zolan 1995). Further translocations might generate multiple alleles that vary in size (Zolan 1995). As the predominant chromosome number seen for all the investigated strains including the haploid type strain of *D. hansenii* var. *hansenii* is six it is therefore most likely that the original chromosome number of *D. hansenii* is six and further that some of the strains might be diploid or even aneuploid. Variability in chromosome numbers has also been reported for a number of other yeast species. Doi *et al.* (1992) found variability in chromosome numbers for a number of species, e.g. strains of *C. krusei* were found to have seven to nine chromosomes, *C. kefyr* (imperfect form of *Kluyveromyces marxianus*, Lachance 1998) strains were found to have 17–19 chromosomes and *C. glabrata* strains were found to have 13–14 chromosomes. Naumov *et al.* (2001) observed variability in chromosome numbers for *Hansenula anomala* strains (now recognized as *Pichia anomala*, Kurtzman 1998) that were found to have nine to 12 chromosomes. The lower variation within chromosomes observed for other yeast species confirm our hypothesis that the six to 10 chromosomal bands observed for the *D. hansenii* strains is not in all cases the exact chromosome number but due to the fact that some of the bands close in size might be copies of the same chromosome.

In the present study, the genome size for strains with six chromosomes was found to vary between 9·4 and 12·6 Mb. Previously the size of other genomes has been found to vary such as for *C. albicans* (29·3–32·6 Mb), *C. kefyr* (27·4–31·1 Mb), *C. stellatoidea* (now recognized as *C. albicans*, Meyer *et al.* 1998) (32·3–33·9 Mb), *C. tropicalis* (29·5–31·4 Mb), *C. parapsilosis* (25·8–26·8 Mb), *C. krusei* (19·3–20·2 Mb), *C. guilliermondii* (imperfect form of *Pichia guilliermondii*, Kurtzman 1998) (11·5–11·6 Mb), *C. glabrata* (13·7–14·4 Mb) (Doi *et al.* 1992) and *Saccharomyces cerevisiae* (13·5–14·5 Mb) (Olson 1991). As further seen the genome size varies to a great extent between different yeast species. The size of the *D. hansenii* genome as found in the present study is comparable with the genome size for yeast species as, e.g. *C. guilliermondii*, *C. glabrata* and *S. cerevisiae*.

As mentioned earlier determination of CLP has successfully been used for typing of *S. cerevisiae* isolates from a wide range of food products although the chromosome profiles reported for *S. cerevisiae* are much more homogeneous compared with those observed among *D. hansenii* isolates. For *S. cerevisiae* a common chromosomal pattern with only minor variations in the numbers and sizes of chromosomal bands are generally seen (Briones *et al.* 1996; Naumov *et al.* 1998; Hayford and Jespersen 1999; Jespersen *et al.* 2000; Pataro *et al.* 2000). Contrary, it seems impossible to recognize a general chromosomal pattern for *D. hansenii* strains investigated and huge variations are seen both in size and number of chromosomal bands. These variations make the PFGE technique a useful tool for strain typing of *D. hansenii*.

Several DNA-based methods have been developed for identification of the species *D. hansenii* (Nishikawa *et al.* 1998; Esteve-Zarzoso *et al.* 1999; Andrighetto *et al.* 2000; Corredor *et al.* 2000; Petersen *et al.* 2001). However, none of these methods are usable for strain typing. So far the only methods reported for strain typing at intraspecies level are mtDNA RFLP (Romano *et al.* 1996; Petersen *et al.* 2001), RAPD (Romano *et al.* 1996) and Fourier-transform infrared microscopy (FT-IR) (Wenning *et al.* 2002). The 20 CBS strains of *D. hansenii* as well as the 20 dairy isolates denoted DI have previously been investigated by mtDNA RFLP using two different restriction enzymes (*Hae*III and *Hpa*II) (Petersen *et al.* 2001). In that study 23 different mtDNA profiles were obtained for the 40 isolates. In the present study all 40 isolates could be separated by PFGE indicating that the PFGE technique has a much higher discriminative power than mtDNA RFLP. Most of the isolates that were found to have identical mtDNA profiles were, although separated by the PFGE technique, found to cluster together, i.e. [DI8 and DI10] were clustered together as well as [CBS164 and CBS1800] and [DI3 and CBS1792] and [DI11, DI12, DI16 and DI22] and [DI1, DI6 and DI7]. As for the PFGE technique mtDNA RFLP was not able to distinguish between *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*. Romano *et al.* (1996) also used mtDNA RFLP for strain typing of *D. hansenii*. They analysed 28 strains of *D. hansenii* isolated during the production of Roncal and Idiazabal cheeses and by this method the strains could be separated into five groups. The same 28 strains were additionally analysed by RAPD by use of $(CTG)_5$ primers, the strains were separated in two groups. FT-IR was analysed for strain

typing of 21 strains *D. hansenii* obtained from culture collections (Wenning *et al.* 2002). By this method it was found that 91% of the strains had individual FT-IR spectra. Of the reported methods for strain typing of *D. hansenii* so far, PFGE do have the highest discriminative power due to the fact that all analysed strains in the present study have an individual PFGE profile.

In conclusion, the results of the present study show that the chromosomal arrangement of *D. hansenii* is very heterogenic with a distinct chromosome polymorphism. The average number of chromosomes in *D. hansenii* is most likely to be six for which the total genome size varies between 9·4 and 12·6 Mb. However, additional variations might be seen in both chromosome numbers and genome sizes. Furthermore, PFGE has been proved to have a very high discriminative power for strain typing of *D. hansenii* and was found valuable for strain differentiation of *D. hansenii* during the production of Danish surface-ripened cheeses.

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REFERENCES

van der Aa Kuhle, A., Jespersen, L., Glover, R.L.K., Diawara, B. and Jakobsen, M. (2001) Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. *Yeast* **18**, 1069–1079.

Aiglé, M., Erbs, D. and Moll, M. (1984) Some molecular structures in the genome of lager brewing yeasts. *Journal of the American Society of Brewing Chemistry* **42**, 1–7.

Andrighetto, C., Psomas, E., Tzanetakis, N., Suzzi, G. and Lombardi, A. (2000) Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeast isolated from dairy products. *Letters in Applied Microbiology* **30**, 5–9.

Briones, A.I., Ubeda, J. and Grando, M.S. (1996) Differentiation of *Saccharomyces cerevisiae* strains isolated from fermenting must according to their karyotype patterns. *International Journal of Food Microbiology* **28**, 369–377.

Carruba, G., Pontieri, E., Bernardis, F.D., Martino, P. and Cassone, A. (1991) DNA fingerprinting and electrophoretic karyotype of environmental and clinical isolates of *Candida parapsilosis*. *Journal of Clinical Microbiology* **29**, 916–922.

Corredor, M., Davila, A. Gaillardin, C. and Casaregola, S. (2000) DNA probes specific for the yeast species *Debaryomyces hansenii*: useful tools for rapid identification. *FEMS Microbiology Letters* **193**, 171–177.

Corredor, M., Davila, A., Casarègola, S. and Gaillardin, C. (2003) Chromomal polymorphism in the species *Debaryomyces hansenii*. *Antonie van Leeuwenhoek* **83**, 215–222.

Deak, T., Chen, J. and Beuchat, L.R. (2000) Molecular characterization of *Yarrowia lipolytica* and *Candida zeylanoides* isolated from poultry. *Applied and Environmental Microbiology* **66**, 4340–4344.

Doi, M., Homma, M., Chindamporn, A. and Tanaka, K. (1992) Estimation of chromosome number and size by pulsed-field gel electrophoresis (PFGE) in medically important *Candida* species. *Journal of General Microbiology* **138**, 2243–2251.

Esteve-Zarzoso, B., Bellodh, C., Uruburu, F. and Querol, A. (1999) Identification of yeasts by RFLP analysis of the 5·8S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology* **49**, 329–337.

Fleet, G.H. (1990) Yeast in dairy products – a review. *Journal of Applied Bacteriology* **68**, 199–211.

Gente, S., Desmasures, N., Jacopin, C., Plessis, G., Beliard, M., Panoff, J.M. and Guéguen, M. (2002) Intra-species chromosome-length polymorphism in *Geotrichum candidum* revealed by pulsed field gel electrophoresis. *International Journal of Food Microbiology* **76**, 127–134.

Hayford, A.E. and Jespersen, L. (1999) Characterization of *Saccharomyces cerevisiae* strains from spontaneously fermented maize dough by profiles of assimilation, chromosome polymorphism, PCR and *MAL* genotyping. *Journal of Applied Microbiology* **86**, 284–294.

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

Jespersen, L., van der AaKuhle, A. and Petersen, K.M. (2000) Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeast. *International Journal of Food Microbiology* **60**, 43–53.

Kurtzman, C.P. (1998) *Pichia* E.C. Hansen emend. Kurtzman. In *The Yeasts; a Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 273–352. Amsterdam: Elsevier.

Lachance, M.A. (1998) *Kluyveromyces* van der Walt emend. van der Walt. In *The Yeasts; a Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 227–247. Amsterdam: Elsevier.

Lee, S.Y. and Knudsen, F.B. (1985) Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. *Journal of the Institute of Brewing* **91**, 169–173.

Lenoir, J. (1984) The surface flora and its role in the ripening of cheese. *International Dairy Federation Bulletin* **171**, 3–20.

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

Meyer, S.A., Payne, R.W. and Yarrow, D. (1998) *Candida* Berkhout. In *The Yeasts; a Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 454–573. Amsterdam: Elsevier.

Monod, M., Porchet, S., Baudraz-Rosselet, F. and Frenk, E. (1990) The identification of pathogenic yeast strains by electrophoretic analysis of their chromosomes. *Journal of Medical Microbiology* **32**, 123–129.

Nakase, T. and Suzuki, M. (1985a) Taxonomic studies on *Debaryomyces hansenii* (Zopf) Lodder Kreger-van Rij and related species. I. Chemotaxonomic investigations. *Journal of General Applied Microbiology* **31**, 49–69.

Nakase, T. and Suzuki, M. (1985b) Taxonomic studies on *Debaryomyces hansenii* (Zopf) Lodder Kreger-van Rij and related species. II. Practical discrimination and nomenclature. *Journal of General Applied Microbiology* **31**, 71–86.

Nakase, T., Suzuki, M., Phaff, H.J. and Kurtzman, C.P. (1998) *Debaryomyces* Lodder & Kreger-van Rij Nom. Cons. In *The Yeast; a Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 157–163. Amsterdam: Elsevier.

Naumov, G.I., Naumova, E.S. and Sniegowski, P.D. (1998) *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* are associated with exudases of North American oaks. *Canadian Journal of Microbiology* **44**, 1045–1050.

Naumov, G.I., Naumova, E.S. and Schnürer, J. (2001) Genetic chracterization of the nonconventional yeast *Hansenula anomala*. *Research in Microbiology* **152**, 551–562.

Nishikawa, A., Sugita, T. and Shinoda, T. (1998) Rapid identification of *D. hansenii/Candida famata* by polymerase chain reaction. *Medical Mycology* **37**, 101–104.

Olson, M.V. (1991) Genome structure and organization in *Saccharomyces cerevisiae*. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energitics* ed. Broach, J.R., Pringle, J. and Jones, E. pp. 1–39. Cold Spring Habor, NY: Cold Spring Habor Laboratory Press.

Pataro, C., Guerra, J.B., Petrillo-Peixoto, M.L., Mendonça-Hagler, L.C., Linardi, V.R. and Rosa, C.A. (2000) Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentation in Brazil. *Journal of Applied Microbiology* **89**, 24–31.

Petersen, K.M., Møller, P.L. and Jespersen, L. (2001) DNA typing methods for differentiation of *Debaryomyces hansenii* strains and other yeast related to surface ripened cheeses. *International Journal of Food Microbiology* **69**, 11–24.

Petersen, K.M., Westall, S. and Jespersen, L. (2002) Microbial succession of *Debaryomyces hansenii* strains during the production of surface ripened cheeses. *Journal of Dairy Science* **85**, 478–486.

Querol, A., Barrio, E. and Ramon, D. (1994) Population dynamics of natural *Saccharomyces* strains during wine fermentations. *International Journal of Food Microbiology* **21**, 315–323.

Romano, A., Casaregola, S., Torre, P. and Gaillardin, C. (1996) Use of RAPD and mitochondrial DNA RFLP for typing of *Candida zeylanoides* and *Debaryomyces hansenii* yeast strains isolated from cheese. *Systematic and Applied Microbiology* **19**, 255–264.

Sørensen, B.B. and Jakobsen, M. (1997) The combined effect of temperature, pH and NaCl on growth of *Debaryomyces hansenii* analysed by flow cytometry and predictive microbiology. *International Journal of Food Microbiology* **34**, 209–220.

Seiler, H. and Busse, M. (1990) The yeasts of cheese brines. *International Journal of Food Microbiology* **11**, 289–304.

Sor, F. and Fukuhara, H. (1989) Analysis of chromosomal DNA patterns of the genus *Kluyveromyces*. *Yeast* **5**, 1–10.

van den Tempel, T. and Jakobsen, M. (2000) The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures for production of Danablu. *International Dairy Journal* **10**, 263–270.

Vezinhet, F., Blondin, B. and Hallet, J.N. (1990) Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of ecological strains of *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **32**, 568–571.

Vezinhet, F., Hallet, J-N. Valade, M. and Poulard, A. (1992) Ecological survey of wine yeast strains by molecular methods of identification. *American Journal of Enology and Viticulture* **43**, 83–86.

van der Walt, J.P., Taylor, M.B. and Liebenberg, N.V.D.W. (1977) Ploidy, ascus formation and recombination in *Torulaspora* (*Debaryomyces*) *hansenii*. *Antonie van Leeuwenhoek* **43**, 205–218.

Wenning, M., Seiler, H. and Scherer, S. (2002) Fourier-transform infrared microspectroscopy, a novel and rapid tool for identification of yeasts. *Applied and Environmental Microbiology* **68**, 4717–4721.

Zolan, M.E. (1995) Chromosome-length polymorphism in fungi. *Microbiology Review* **59**, 686–698.