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Chromosomal polymorphism in the yeast species *Debaryomyces hansenii*

Mauricio Corredor, Anne-Marie Davila*, Serge Casarégola and Claude Gaillardin
*Laboratoire de Génétique Moléculaire et Cellulaire INRA UMR216, CNRS URA1925, Collection de Levures d'Intérêt Biotechnologique, Institut National Agronomique Paris-Grignon, 78850 Thiverval-Grignon, France; *Author for correspondence (e-mail: davila@grignon.inra.fr; phone: (33) 1 30 81 52 52; fax: (33) 1 30 81 54 57)*

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Abstract

Pulse field gel electrophoresis karyotypes of 41 strains of the genus *Debaryomyces*, including 35 strains confirmed as *D. hansenii* species by D1/D2 ribosomal DNA sequence analysis, were performed. Electrophoretic karyotypes of the 41 strains exhibited 4 to 10 chromosomal bands ranging between 0.7 Mb and 4.2 Mb. Among *D. hansenii* species, the patterns of strains obtained from the CBS collection and cheese isolates differed strongly from *D. hansenii* var. *hansenii* CBS767^T. Both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryii* showed chromosome length polymorphism. Electrophoretic karyotypes of the *D. hansenii* strains were analyzed by Southern hybridization with various species-specific probes isolated from *D. hansenii* var. *hansenii* CBS767^T. Repeated sequences including the F01pro, M18pro, the Ty1-*copia* retrotransposon Tdh5 and hypothetical telomeric sequence hybridized to several chromosomal bands, while a D1/D2 probe derived from the large ribosomal sub-unit hybridized only to the largest chromosome. Unique probes such as those hybridizing to actin *ACT*1, glycerol-3-phosphate dehydrogenase *GPD*1 and β-glucosidase *LAC*4 encoding genes were assigned to specific chromosomal bands of *D. hansenii* var. *hansenii* CBS767^T. These probes failed to hybridize to *D. hansenii* var. *fabryii* strongly suggesting that strains of this variety actually represent a different taxon.

Abbreviations: CBS – Centraal Bureau voor Schimmelcultures, CLIB – Collection de Levures d'Intérêt Biotechnologique, PFGE – Pulse Field Gel Electrophoresis

Introduction

The halotolerant yeast species *Debaryomyces hansenii* is found in cheese, meat, wine, beer, fruits, soil and sea water (Barnett et al. 2000). It is one of the most osmotolerant species of yeast known (Bansal and Mondal 2000), and the one most commonly found in all types of cheeses (Fleet 1990). Multiple *D. hansenii* strains have been frequently encountered but little studied until now if compared to other yeast species. The diversity of microhabitats colonized by the species suggests the existence of distinct sub-populations that have become specifically adapted to such habitats. For example, European strains are poorly fermentative, whereas Brazilian isolates have notable fermentation capabilities (Ramos et al. 1998), rendering this xylose-utilizing yeast potentially attractive for xylitol/ethanol fermentation (Parajó et al. 1995).

Developing molecular tools for identifying strains of *D. hansenii* is therefore a prerequisite to accurately monitor their biological diversity. A first indication of heterogeneity within this species was the description of the two varieties *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryii* (Nakase and Suzuki 1985). It has been shown previously that genetic polymorphism among different Spanish cheese strains of *D. hansenii* could be clearly seen by RAPD analysis of genomic

DNA and RFLP profiles of mitochondrial DNA (Romano et al. 1996). Nevertheless no clear correlation was found between geographical or ecological origin and genetic traits.

Electrophoretic karyotype analysis represents another useful approach that can be used for assessing genetic diversity. In the case of several species of the genera *Saccharomyces* (Naumova et al. 1993; Tôrök et al. 1993) and *Kluyveromyces* (Steensma et al. 1988), karyotypes have been shown to exhibit relatively low intra-specific polymorphism, whereas pronounced chromosomal polymorphism has been observed in the species *Yarrowia lipolytica* (Naumova et al. 1993; Casarégola et al. 1997), *Kluyveromyces dobzhanskii* (Belloch et al. 1998), *Saccharomyces cerevisiae* (Bidene et al. 1992) and *Candida albicans* (Doi et al. 1992).

The aim of the present work was to evaluate the usefulness of karyotype analysis to approach the biodiversity of the *D. hansenii* complex and of related *Debaryomyces* species. Species specific sequences (Corredor et al. 2000) combined with sequences from a genome survey that scan about 20% of *D. hansenii* genome (Winkler et al. 2000) were also used as probes to investigate biodiversity within this species.

Materials and Methods

Strains

The strains used in this study were obtained from the CentraalBureau voor Schimmelcultures (CBS, Baarn, Netherlands) and from the Collection de Levures d'Intérêt Biotechnologique (CLIB, http://www.inra.fr/clib/). The CLIB strains have been described mostly by Baroiller and Schmidt (1990) and Romano et al. (1996). The isolation sources and the geographical origins are listed in Table 1.

Preparation of intact chromosomal DNA

All strains were cultivated in YEA medium (0.5% yeast extract, 1% glucose) at 28 °C during 48 h in order to obtain sufficient growth. The cells were harvested and washed twice in water and 50 mM EDTA. They were resuspended at a concentration of 2×10^9 cell/ml in 0.9 M sorbitol, 0.1 M Tris-HCl pH 8.0, 0.1 M EDTA, 0.28 M β-mercaptoethanol, 0.3 mg/ml zymolyase 100T and 0.5 mg/ml cytohelicase (Sigma-Aldrich, Saint Louis, USA). This suspension was mixed to an equal volume of 1% low-melting-point agarose SeaPlaque (FMC Bioproducts, Rockland, USA) prepared in sterile water, cooled to 55 °C and transferred into wells (3 mm thick, 8 mm long, 5 mm high). Once solidified, the plugs were incubated at 50 °C overnight in a lysis buffer containing 0.5 M EDTA pH 8.0, 1% SDS, and 2 mg/ml proteinase K (ICN, Irvine, USA). The lysis buffer was then eliminated and the plugs were washed with 0.5 EDTA pH 8.0 and stored at 4 °C. Prior to electrophoresis, the plugs were washed for 1 h in 10 mM Tris-HCl, 1 mM EDTA.

Pulse field gel electrophoresis

Electrophoresis was carried out with the CHEF Mapper System (Biorad, Hercules, USA). After preliminary studies, the agarose concentration (Pulse Field Certified Agarose, Biorad) was fixed at 0.8%. Gels were run in 0.5 **X** TBE buffer constantly circulated at 14 °C. Two electric field ramping programs, with a constant reorientation angle set at 120°, were applied: (1) 2.4 V/cm, 10–20 min, 48 h, (2) 3 V/cm, 2–6.6 min, 60 h. After electrophoresis, the gels were stained in ethidium bromide (0.5 μg/ml) for 30 min, then destained for 2 h in sterile water. A chromosomal preparation referred to as *Hansenula wingei* (Biorad, Hercules, USA) was used as the chromosomal standard.

Southern hybridization

Chromosomal DNA blotting on a GeneScreen (Dupont, USA) membrane was performed as previously described (Sambrook et al. 1989; Zimmermann and Fournier 1996). All the probes were produced from the type strain of *D. hansenii* var. *hansenii* CBS767^T. F01pro (AJ245420) and M18pro probes were fragments isolated by RAPD-SCAR (Corredor et al. 2000). The D1/D2 ribosomal DNA probe was obtained by PCR amplification with NL1 and NL4 primers (O'Donnell 1993). The *ACT*1-like gene probe was produced by PCR with the following primers: upper 5′-GTGTAAAGCCGGTTTTGCCG-3′ and lower 5′-AACCACCAATCCAGACGGAG-3′ (Neuvéglise, unpublished). The other probes were identified through random genomic sequencing of the CBS767 type strain (Winkler et al. 2000): BCAA005H05 hypothetical telomeric sequence, XBC0AA0D1A06 encoding β-glucosidase-like, XBC0AA001E031 glycerol-3-phosphate dehydro-

genase-like and BC0AA012G02 part of Tdh5, a Ty1-*copia* retrotransposon identified recently (Neuvéglise et al., in press). All these probes were PCR amplified, and purified from agarose gel using the Genclean II kit (Bio101, Vista, USA). Plasmids were isolated with the Plasmid mini kit (Qiagen GmbH, Hilden, Germany). Probes were labeled using the Megaprime labeling kit (Amersham Biosciences Europe GmbH, Freiburg, Germany). Membrane hybridization and washing were performed as described (Church and Gilbert 1984). ^{32}P was detected by autoradiography using a PhosphorImager Molecular Dynamics (Amersham Biosciences Europe GmbH, Freiburg, Germany).

Data analysis

The gel photographs were digitized in the TIFF (Tagged Images File Format) format and analyzed with the Taxotron® Package (Institut Pasteur, France), according to Machado et al. (1998). Band migration distances were compared with the RestrictoScan® program and converted into molecular weights with the RestrictoTyper® program.

Results

Electrophoretic karyotypes of various Debaryomyces *species*

Pulsed field gel electrophoresis (PFGE) of chromosomal DNA was performed on 33 isolates of *D. hansenii*, the type strains of *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryii* and six other *Debaryomyces* species (see Table 1). Results for a set of strains are presented in Figure 1 (a, b, c). The chromosomes were resolved into 4 to 10 bands (see Figure 1,2) ranging in size from 0.7 Mb to 4.2 Mb with most bands migrating between 1.3 Mb to 3.0 Mb. Some *D. hansenii* strains exhibited smaller chromosome bands than most, like CLIB609 and CLIB624, 1 Mb, or CBS6574, 0.9 Mb, while CLIB237 was the only strain with a chromosome band of about 4.2 Mb (Figure 2). These results differ from those for most type strains of the non-*D. hansenii* species which exhibit at least three large chromosomal bands (2.8 Mb, 3.1 Mb and 3.8 Mb) like *D. tamarii* CBS4333^{T}, *D. nepalensis* CBS5921^{T}, *D. castellii* CBS2923^{T}, *D. pseudopolymorphus* CBS2008^{T} and *D. maramus* CBS1958^{T} (see Figure 1c). *D. castelli* CBS2923^{T}, on the other

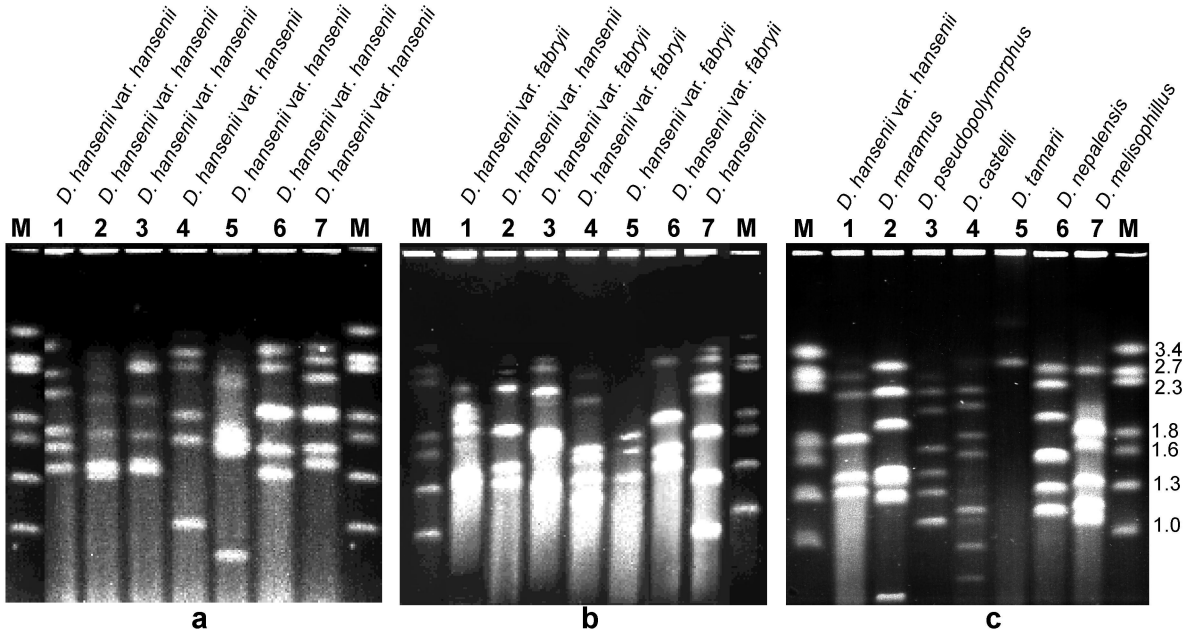

Figure 1. CHEF karyotypes in 0.8% agarose gel. (a), *D. hansenii* var. *hansenii* strains: 1-CBS1962, 2- CLIB607, 3- CLIB608, 4- CLIB609, 5-CLIB611, 6- CLIB613, and 7- CLIB616. (b) lanes 1,3,4,5,6: *D. hansenii* var. *fabryii* strains, lanes 2 and 7: *D. hansenii* var. *hansenii* strains: 1-CLIB606, 2- CBS767^{T}, 3- CBS789^{T} 4- CBS5572, 5- CBS4373, 6- CBS5139, and 7- CBS6574. (c), 1- CBS767^{T} *D. hansenii* var. *hansenii*, 2-CBS1958^{T} *D. maramus*, 3- CBS2008^{T} *D. pseudopolymorphus*, 4-CBS2923^{T} *D. castellii*, 5- CBS4333^{T} *D. tamarii*, 6- CBS5921^{T} *D. nepalensis*, and 7-CBS6344^{T} *D. melissophilus*. M- *H. wingei* standard. Sizes are indicated on the right in megabases

hand, exhibited small chromosomal bands ranging from 0.7 Mb to 2.2 Mb. The genome size of the above type strains ranged from 9.8 to 12.4 Mb.

Polymorphism of chromosomal bands within D. hansenii

In PFGE different strains of both *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryii* exhibited a strong chromosome-length polymorphism (Figure 2). For the sake of clarity we divided the chromosomal patterns in three groups: namely group 1, characterized by large chromosomal bands (over 2.4 Mb); group 2, exhibiting only medium sized chromosomal bands (from 2.3 Mb to 1.7 Mb); group 3, displaying small chromosomal bands (under 1.5 Mb). All strains

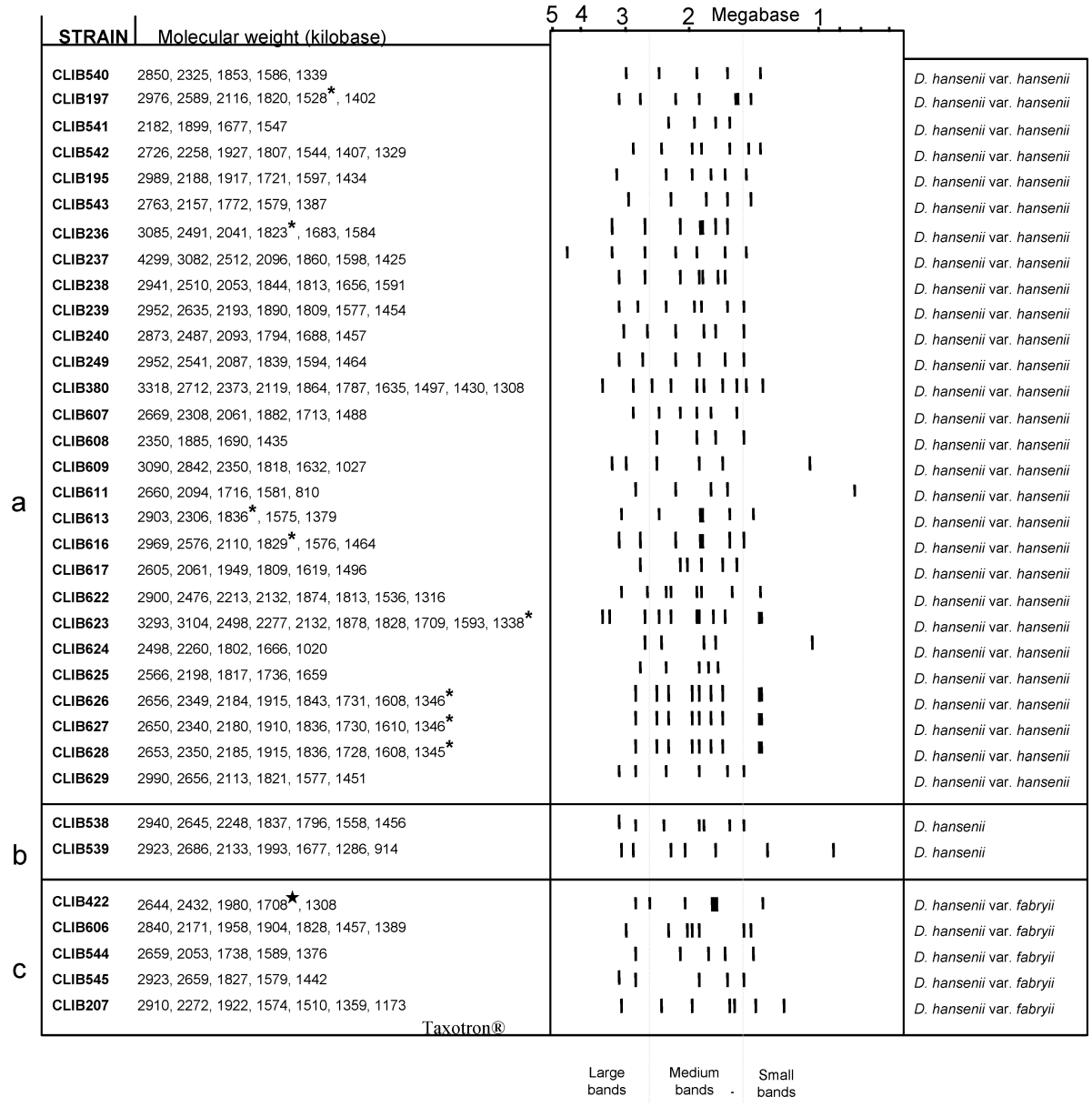

Figure 2. Analysis of chromosomal patterns of *D. hansenii* using the Taxotron® Package. Group a, *D. hansenii v*ar. *hansenii* strains; group b, *D. hansenii* without variety and group c, *D. hansenii* var. *fabryii*. Comigrating bands are indicated by * in the case of double bands and by ★ for triple bands

shared medium sized chromosomal bands of 2.9, 2.3, 2.1, and 1.8 Mb. Most patterns consisted of 6 chromosomal bands, although some strains exhibit 5 to 10 chromosomal bands, e.g. see CLIB611 and CLIB380 in Figure 2. The genome sizes were measured by using the RestrictoTyper® program. Taking into account comigrating bands, the genome size of *D. hansenii* var. *hansenii* CBS767^T was estimated to be 12.7 Mb, in the same range as *D. hansenii* var. *fabryii* CBS789^T (11.7 Mb to 13.4 Mb) (Figure 1, lane 2).

Southern hybridization analysis

We hybridized successively *D. hansenii* CBS767^T karyotype with the repeated probes F01pro, M18pro and D1/D2, the Ty1-*copia* retrotransposon Tdh5, a repeated probe, a hypothetical telomeric sequence and 4 single copy gene probes (*ACT*1-like, *LAC*4-like and *GPD*1-like). Two to six chromosomes hybridized to the F01pro sequence (Figure 3, lane 4), to the Ty1-*copia* retrotransposon sequence (Figure 3, lane 6) and to the hypothetical telomeric sequence (Figure 3, lane 7), indicating that these sequences were present on several chromosomes. On the contrary, the *ACT*1-like, M18pro, *GPD*1-like and *LAC*4-like probes identified single chromosomal bands, suggesting that these sequences were unique or repeated in the same chromosome. Alternatively, several chromosomes could have comigrated during the electrophoretical separation. Indeed, it has been previously shown that M18pro was a mildly repeated sequence (Corredor et al. 2000).

All the *D. hansenii* var. *hansenii* strains were tested with the repeated probes F01pro (Corredor et al. 2000) and D1/D2. A set of hybridized electrophoretic karyotypes is shown in Figure 4. Hybridization with F01pro probe detected between 2 and 4 bands, ranging in size from 1.8 Mb to 2.1 Mb (Figure 4a, see also Figure 3, lane 4). On the same karyotypes, the ribosomal D1/D2 probe hybridized only to the largest

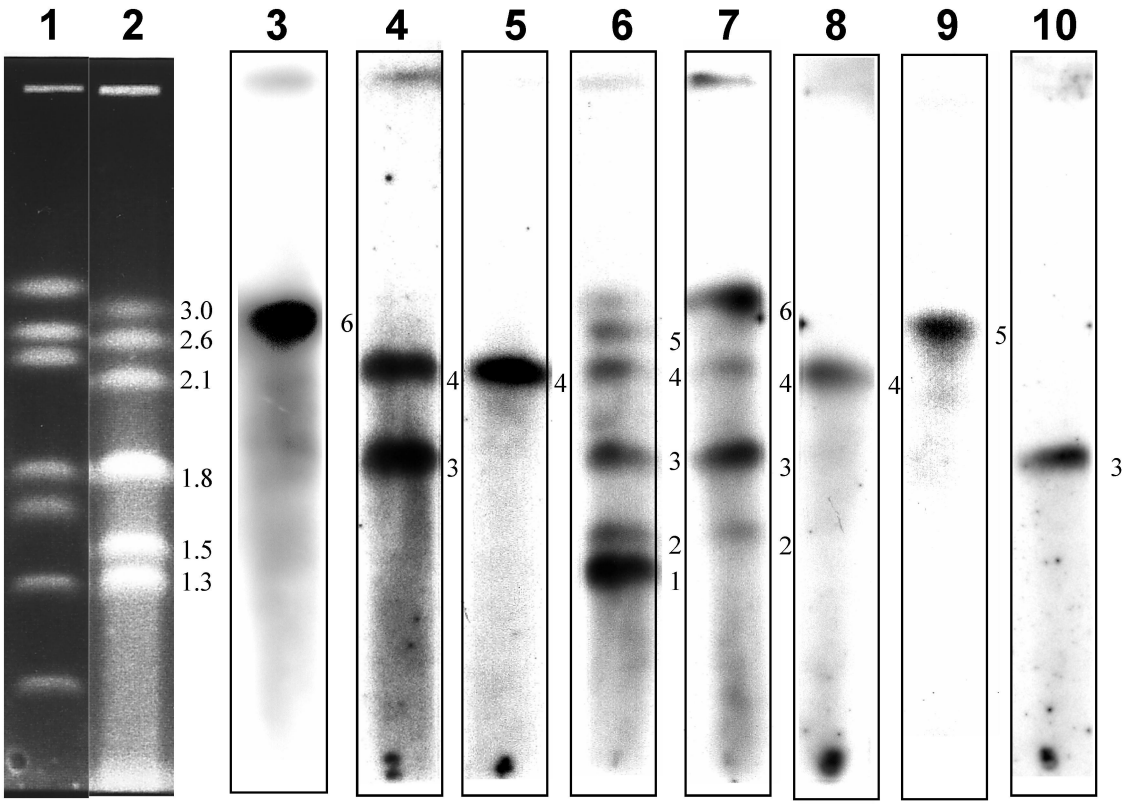

Figure 3. Southern hybridization of the PFGE karyotypes of *D. hansenii* CBS767^T with different DNA probes. Ethidium bromide stained karyotype of *H. wingei* (lane 1) and CBS767^T (lane 2). Hybridization of CBS767^T karyotype with: D1/D2 sequence (lane 3), F01pro (lane 4), M18pro (lane 5), the Ty1-*copia* retrotransposon Tdh5 (lane 6), hypothetical telomeric sequence (lane 7), *GPD*1-like (lane 8), *LAC*4-like (lane 9), *ACT*1-like (lane 10). Numbers corresponding to chromosome bands are shown.

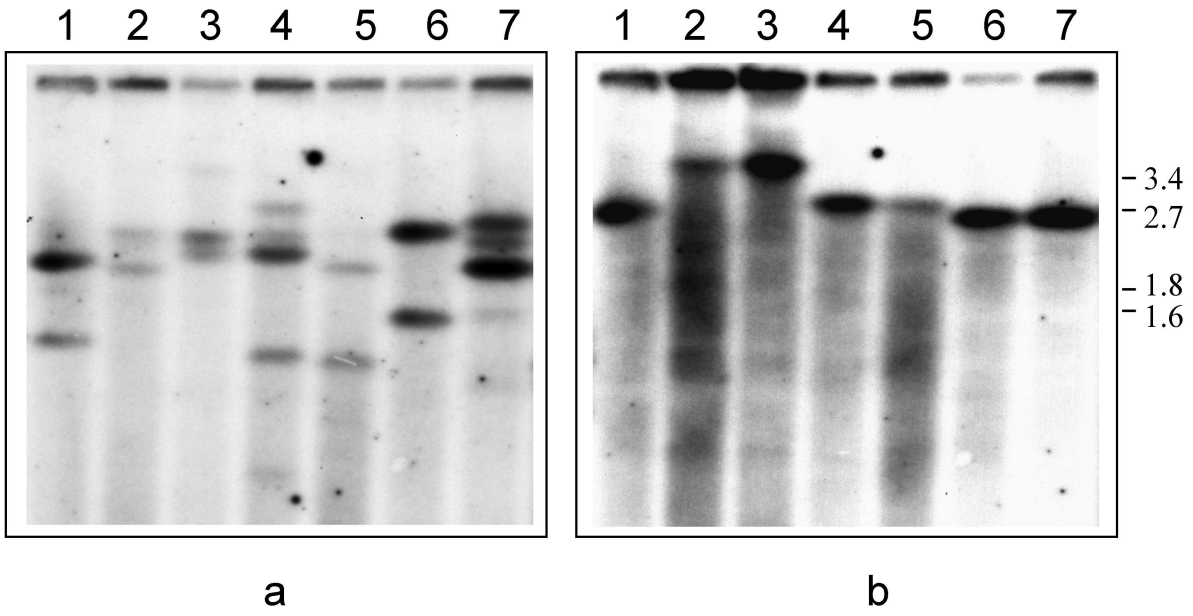

Figure 4. Assignment of chromosomes carrying rDNA or the repeated F01pro sequence. PFGE karyotypes of *D. hansenii* strains were hybridised with the following probes: (a), F01pro and (b), D1/D2. Strains tested: 1- CBS1962, 2- CLIB607, 3- CLIB608, 4- CLIB609, 5- CLIB611, 6- CLIB613, 7- CLIB616

chromosomal band (Figure 4b, see also Figure 3, lane 3), as was also observed for all *D. hansenii* strains tested (data not shown).

Discussion

As in a number of other yeast species, (Bidene et al. 1992; Casarégola et al. 1997), a significant level of chromosome length polymorphism was seen in *D. hansenii* affecting both the number and size of chromosomal bands (see Figure 2). Thus PFGE appears to permit accurate discrimination at the strain level. In a previous study, the CLIB607 and CLIB608 strains appeared to share very similar RFLP profiles using the F01pro probe and physiological characteristics (Corredor et al. 2000); however, these strains could be clearly differentiated through PFGE. Using PFGE, we observed no correlation between band size distribution and habitat or geographical origin as previously shown by RFLP analysis of mitochondrial DNA of Spanish cheese isolates (Romano et al. 1996).

Karyotype analysis thus confirms that strains of *D. hansenii* differ markedly at the level of genomic organization. Chromosomal length polymorphism in some yeast species has been shown to result from variation in the number of repeated sequences whereas variation in the number of chromosomal bands may reflect aneuploidy. In addition, chromosomal rearrangements are likely to play a key role in the observed variability. In particular, reciprocal translocation has been shown to be a frequent event in *Saccharomyces cerevisiae* laboratory strains or wild isolates (Casarégola et al. 1998; Rachidi et al. 1999).

All the *D. hansenii var. hansenii* species strains tested hybridized to the moderately repeated probes F01pro, M18pro, the Ty1-*copia* retrotransposon Tdh5 and hypothetical telomeric sequence probes but failed to do so to chromosomes of *D. hansenii var fabryii*. This strongly supports the view that these two varieties actually represent two different species as has been suggested by several authors (Nakase and Suzuki 1985; Prillinger et al. 1999). Hybridization results indicate furthermore that all *D. hansenii var. hansenii* strains constitute a single species within which different strains share a common genetic repertoire distributed differently among chromosomes. A similar situation has been reported for strains of the *Y. lipolytica* species (Casarégola et al. 1997).

The identification of single copy probes encoding glycerol-3-phosphate dehydrogenase, β-glucosidase and actin, which are localized on distinct chromo-

Table 1. List of strains

Strain	Species	Origin	Country	Reference
CBS 766	*Debaryomyces hansenii* var. *hansenii*	cheese	Russia	
CBS 767[T]	*Debaryomyces hansenii* var. *hansenii*	cherry	Denmark	
CBS 789[T]	*Debaryomyces hansenii* var. *fabryii*	interdigital mycotic lesion	Germany	
CBS 1098	*Debaryomyces hansenii* var. *hansenii*	cheese	Russia	
CBS 1102	*Debaryomyces hansenii* var. *hansenii*	beef	France	
CBS 1958[T]	*Debaryomyces maramus*	atmosphere	New Zealand	
CBS 1962	*Debaryomyces hansenii* var. *hansenii*	unknown	Japan	
CBS 2008[T]	*Debaryomyces pseudopolymorphus*	tanning fluid	France	
CBS 2659	*Debaryomyces hansenii*	apple	Italy	
CBS 2923[T]	*Debaryomyces castellii*	soil	Sweden	
CBS 4333[T]	*Debaryomyces tamarii*	Tamari-soya	unknown	
CBS 4373	*Debaryomyces hansenii* var. *fabryii*	wine	South Africa	
CBS 5139	*Debaryomyces hansenii* var. *fabryii*	human skin	Hungary	
CBS 5572	*Debaryomyces hansenii* var. *fabryii*	soil	New Zealand	
CBS 5921[T]	*Debaryomyces nepalensis*	soil	Nepal	
CBS 6089	*Debaryomyces hansenii* var. *hansenii*	grape juice	France	
CBS 6344[T]	*Debaryomyces melissophilus*	gut of honey bee	South Africa	
CBS 6574	*Debaryomyces hansenii*	sea water	unknown	
CLIB 236	*Debaryomyces hansenii* var. *hansenii*	Roncal cheese	Spain	Romano et al. (1996)
CLIB 237	*Debaryomyces hansenii* var. *hansenii*	Roncal cheese	Spain	Romano et al. (1996)
CLIB 238	*Debaryomyces hansenii* var. *hansenii*	Roncal cheese	Spain	Romano et al. (1996)
CLIB 239	*Debaryomyces hansenii* var. *hansenii*	Roncal cheese	Spain	Romano et al. (1996)
CLIB 240	*Debaryomyces hansenii* var. *hansenii*	Roncal cheese	Spain	Romano et al. (1996)
CLIB 249	*Debaryomyces hansenii* var. *hansenii*	cheese	France	Romano et al. (1996)
CLIB 380	*Debaryomyces hansenii* var. *hansenii*	goat cheese	France	Baroiller and Schmidt (1990)
CLIB 606	*Debaryomyces hansenii* var. *fabryii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 607	*Debaryomyces hansenii* var. *hansenii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 608	*Debaryomyces hansenii* var. *hansenii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 609	*Debaryomyces hansenii* var. *hansenii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 611	*Debaryomyces hansenii* var. *hansenii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 613	*Debaryomyces hansenii* var. *hansenii*	Camembert cheese	France	Baroiller and Schmidt (1990)
CLIB 616	*Debaryomyces hansenii* var. *hansenii*	goat cheese	France	Baroiller and Schmidt (1990)
CLIB 617	*Debaryomyces hansenii* var. *hansenii*	goat cheese	France	Baroiller and Schmidt (1990)
CLIB 622	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 623	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 624	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 625	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 626	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 627	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 628	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)
CLIB 629	*Debaryomyces hansenii* var. *hansenii*	Saint Nectaire cheese	France	Corredor et al. (2000)

[T] Type strain of genus *Debaryomyces*

somal bands, will permit more precise assessment of the types of genetic rearrangements responsible for this high degree of polymorphism.

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