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Characterization of *Candida krusei* strains from spontaneously fermented maize dough by profiles of assimilation, chromosome profile, polymerase chain reaction and restriction endonuclease analysis

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A.E. HAYFORD AND M. JAKOBSEN. 1999. Several isolates of *Candida krusei* from indigenous spontaneously fermented maize dough have been characterized for the purpose of selecting appropriate starter cultures and methods for their subspecifies typing. The present work describes the occurrence of *C. krusei* in Ghanaian fermented maize dough. For detailed pheno- and genotyping, 48 representative isolates were selected and comparison was made with clinical isolates of *C. krusei* and reference cultures. The techniques applied included the assimilation of carbon compounds by the API ID 32 C kit, determination of chromosome profile by pulse field gel electrophoresis, polymerase chain reaction (PCR) profiles, restriction endonuclease analysis (REA) and Southern blot hybridization. For the 48 isolates tested, 82% had the same assimilation profiles, being able to assimilate N-acetyl-glucosamine, DL-lactate, glycerol and to ferment glucose. Chromosome and PCR profiles, REA and Southern blot hybridization techniques all had a high discriminatory power and revealed DNA polymorphism, which allowed for discrimination among the strains and hence subspecific typing. On the basis of PCR and REA profiles, isolates were grouped into clusters. Southern blot hybridization appeared to be the most sensitive with respect to strain specificity. Our results demonstrated that the three methods, PCR, REA and Southern blot hybridization, were suitable tools, easy to analyse, fast (with regard to PCR) and reliable methods for the typing of *C. krusei* isolates to species and below species level. Based on the use of these techniques, we demonstrated that several strains of *C. krusei* were involved in the fermentation of maize dough from the onset and remain dominant throughout the fermentation.

INTRODUCTION

Natural fermentation of cereals is a common method of food processing in west Africa. One of the most popular of these foods, especially in Ghana, is fermented maize dough. Fermented maize dough is used to prepare a variety of staple foods in the southern coastal belt of Ghana and it contributes to a large proportion of the daily food intake. The process involves soaking of maize for 24–48 h followed by milling. The milled maize is reconstituted with water to form a stiff dough which is left to ferment in fermenting troughs for 48–72 h. Although it is one of the most important food items in Ghana, it is still produced traditionally by spontaneous fermentation, and under semihygienic conditions, which often results in a product of variable quality. Recently, the microbiology of the fermentation has been investigated; the process involves a succession of micro-organisms which leads to a selection of defined flora, comprised of *Lactobacillus fermentum*, which is the main fermenting organism, supported

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by yeasts such as *Saccharomyces cerevisiae* and *Candida krusei* (Halm *et al.* 1993; Obiri-Danso 1994; Olsen *et al.* 1995). An increase in yeast numbers during maize dough fermentation has been demonstrated (Jespersen *et al.* 1994). However, the possible role of these yeasts remains to be fully elucidated. It has been suggested that, apart from creating a favourable environment for the growth of lactic acid bacteria, these yeasts contribute to the flavour and taste of the product (Nyako and Obiri-Danso 1991; Hounhouigan *et al.* 1996). Some studies have shown that *C. krusei* has a high inhibitory action against mycotoxin-producing moulds, found on maize kernels during maize dough fermentation (Halm and Olsen 1996).

Candida krusei has been found to be dominant in most African fermented cereals such as maize and sorghum (Hamad *et al.* 1992; Halm *et al.* 1993; Hounhouigan *et al.* 1994; Jespersen *et al.* 1994), in root crops like cassava (Oyewole 1990; Amoa-Awua *et al.* 1997) and alcoholic beverages such as palm wine in Ghana, sorghum beer in west Africa and Busua beer in east Africa (Nout 1980; Kolani *et al.* 1996). In sorghum beer, *C. krusei* produces high levels of sulphur-containing amino acids (lysine, glutamic acid, aspartic acid and arginine) and has a potential as a protein additive in local human nutrition (Kolani *et al.* 1996). The acid-resistant nature of this yeast allows it to survive in acid-fermented products (Spicher and Schöder 1980). Its dominance in maize dough is, however, very significant and, in order to upgrade the traditional technology and control of the fermentation by means of starter cultures, it is important to know the exact composition of the microflora at strain level. However, from the clinical point of view, *C. krusei* is considered as an opportunistic potential pathogen in immunocompromised patients (Goldman *et al.* 1993).

Traditionally, yeasts are identified by morphological and physiological criteria which are both laborious and time-consuming. These methods are often inadequate and are influenced by environmental conditions, as a result of this misidentification frequently occurs (van der Walt 1987; van der Vossen and Hoftra 1996; Querol *et al.* 1992; Ness *et al.* 1993). This has encouraged the use of molecular techniques. Several DNA-based techniques have been employed in the typing of yeasts. Techniques such as the electrophoretic banding pattern of full length chromosomal DNA by pulse field gel electrophoresis (PFGE), orthogonal-field-alternation gel electrophoresis or clamped homogeneous electric field gel electrophoresis (CHEF) for the determination of chromosome number and size allow for species characterization (Jonge *et al.* 1986; Suzuki *et al.* 1988; Doi *et al.* 1992). These techniques have also been applied for the detection of chromosomal polymorphism within strains of *C. krusei* (Doi *et al.* 1992). Until recently, most of the characterization of *Candida* spp. has been concentrated on *C. albicans* (Iwaguchi *et al.* 1990; Mahrous *et al.* 1990; Magee *et al.* 1992). Recently, two groups of workers (Carlotti *et al.* 1994, 1996, 1997a,b; Manavathu *et al.* 1996) have used variations of molecular methods for typing of clinical strains of *C. krusei*. The techniques used by the groups included a combination of restriction endonuclease analysis (REA) and probe development from restriction fragments, which have then been used in DNA–DNA hybridization experiments. Polymerase chain reaction (PCR)-specific reactions have also been employed. The objectives of this study were to confirm the diversity among the dominant yeasts involved in the maize dough fermentation for the purpose of selecting a suitable starter, to develop methods of identification of these starters to subspecies level and to evaluate the discriminating power of the methods applied. *Candida krusei* isolates from spontaneously fermented maize dough were characterized by assimilation of carbon compound, chromosome and PCR profiles, REA and DNA hybridization.

MATERIALS AND METHODS

Samples

Samples of spontaneously fermented maize dough were collected from a major commercial production site in Accra, Ghana on two separate occasions over a period of 4 years. The samples were taken before and after renovation of the production site including implementation of steeping and fermentation vessels that could be effectively cleaned. The samples comprised 500–1000 g of maize, steep water and maize dough at 24, 48 and 72 h of fermentation. Samples were taken from surfaces of steeping vessels and corn milling machines (before use) by swabbing, in order to trace the origin of the dominant yeasts. Surface layers on the maize dough were removed before sampling. Microbiological analyses were performed within 2 h of sampling.

Microbiological analysis, isolation and selection of strains for molecular typing

From all samples 10 g were homogenized in 90 ml sterile diluent (0·1% peptone, 0·8% NaCl, pH 7·2) by use of a Stomacher (Lab Blender, Model 4001; Seward Medical, London, UK) for 30 s at 'normal' speed. From appropriate 10-fold dilutions, yeasts were enumerated on Malt Agar (MA; 5398; Merck, Darmstadt, Germany) with the addition of (l^{-1}) 100 mg chloramphenicol (Chloramphenicol Selective Supplement; Oxoid, Hampshire, UK) and 50 mg tetracycline (C-4881; Sigma, St. Louis, MO, USA) and incubated at 25 °C for 7 d. From plates with the highest sample dilutions, isolates from a sector of a plate (one-third of the area) were isolated by subculturing in yeast peptone glucose broth (YPG) and streaked out until pure colonies were obtained. All isolates were examined by colony and cell morphology and further

examinations were performed with the API ID 32 C kit (Bio Merieux SA, Marcy-L'Etoile, France) according to the manufacturer's instructions. The strips were inoculated with a 72-h-old culture pregrown at 30 °C on YPG agar. The isolates were identified to species level with reference to Kreger van-Rij (1984). Strains collected over the 4-year period (Table 1) were included in the present study in order to observe and compare any diversity within the strains over this period. Forty-eight representative isolates, all phenotypically identified as *C. krusei*, were selected for further characterization, in addition to reference cultures as shown in Table 1. The cultures were maintained on YPG slants at 4 °C and recultivated every third month.

Pulse field gel electrophoresis

Yeast cultures were grown in YPG broth containing (g l^{-1} distilled water): yeast extract, 10 (0127179; Difco, Detroit, MI, USA); bactopeptone, 20 (Difco 0118170) and glucose, 40 (Merck 1·08342), pH 5·6, at 30 °C for 48 h and then successively recultivated twice for 24 h. The yeast cells were harvested by centrifugation at 3000 *g* for 5 min and washed with 8 ml buffer C (1·2 mol l^{-1} sorbitol (Sigma S-1876), 10 mmol l^{-1} Tris-HCl (Sigma T-7149), 10 mmol l^{-1} $CaCl_2$ (Sigma C-3881)). The yeasts were then treated with 200 μl 5 mg ml^{-1} zymolyase solution (120493; Seikaguru America, Igamsville, MO, USA) in 50% buffer C and 50% glycerol (Sigma G-5516) per 6 ml of yeast suspension for 1 h at 37 °C to digest the cell wall. One ml of spheroplast suspension (5×10^8 ml^{-1}) was mixed with 1 ml of 1·5% (w/v) LMP agarose (Sigma A-9414), 10·3% (w/v) saccharose (Sigma S-0389) in TES buffer, 10 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} NaCl (Sigma C-3014) and 1 mmol l^{-1} EDTA (Sigma E-5134) to form blocks. The blocks were treated with a protease solution (5 mg ml^{-1} pronase E (Sigma P-6911), 1% (w/v) N-laurylsarcosine (Sigma L-5125), 500 mmol l^{-1} EDTA) at 45 °C overnight after which the protease solution was removed and the blocks washed twice with TE buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA) at 50 °C for 1 h. The blocks were transferred to a 0·8% (w/v) NA-agarose (17-0554-02; Pharmacia LKB Biotechnology Uppsala, Sweden) gel. The PFGE was performed with an Electrophoresis Power Supply (EPS3500), GN Controller and Genenavigator (all Pharmacia LKB Biotech, Uppsala, Sweden) at 10 °C using TBE buffer (45 mmol l^{-1} Tris-base (Sigma T-8524), 44 mmol l^{-1} boric acid (Sigma B-6768), 1 μmol l^{-1} EDTA) under the following conditions: 150 V, pulse 200 s for 24 h; 100 V, pulse 700 s for 28 h according to Doi *et al.* (1992). The CHEF DNA size marker (*H. wingei* chromosome; Bio-Rad Laboratories, Hercules, CA, USA) was used for determination of chromosome size. Finally, the gel was stained with 1 mg l^{-1} ethidium bromide (Sigma E-1510) in TBE buffer for 1 h and rinsed twice with milliQ water for 5 min.

Table 1 List of yeast strains used in this study

Species	Strain and origin
Candida catenulata	CBS 565
Candida kefyr	CBS 834
Candida krusei	CBS 573
Candida krusei	(44)*
Candida krusei	(4)†
Candida krusei	(6)‡
Candida parapsilosis	CBS 7248 and 604
Candida rogosa	CBS 613
Candida tropicalis	CBS 94, 433, 644, 1920, 2321
Candida tropicalis	(185)§
Candida valida	CBS 635, 636 and 638
Cryptococcus laurentii	CBS 139
Debaromyces hansenii	CBS 164
Zygosaccharomyces rouxii	CBS 732

CBS, Centraalbureau voor Schimmelculture, Delft, The Netherlands.
* A total of 44 isolates from fermented maize dough isolated in this study (see Fig. 3).
† Previous isolates from Jespersen *et al.* (1995) designated as 18A-3, 19A-3, 19B-4 and 20B-2.
‡ A total of six isolates from clinical samples designated K24, K36, K45, K60, K62 and K65 from Carlotti *et al.* (1994). The origin of these cultures was sputum, stools, bronchial aspiration, bronchoal veolar lavage, face liquid swap and bronchial fibroscopy, respectively.
§ Isolated from maize dough in this study.

Polymerase chain reaction amplification

Yeast strains were grown at 30 °C for 72 h on MYGP agar containing (g l^{-1} distilled water): malt extract, 3·0 (Difco 85177); yeast extract, 3·0 (Difco 0127179); glucose, 10·0 (Merck 1·08342); bacto peptone, 5·0 (Difco 0118170) and agar, 15 (Difco 014001), pH 5·6. From the plate a yeast suspension containing approximately 10^8 cells ml^{-1} sterile milliQ water was made. Denaturation was performed on 100 μl yeast suspension at 95 °C for 10 min and thereafter cooled to 4 °C. The supernatant fluid was used as the source of template for the PCR reaction, which was performed according to the method described by Carlotti *et al.* (1997a). The primers used were oligonucleotide which flanked the species-specific variable region of CKRS-1 that is located in the non-transcribed spacer 2 of the intergenic region of rRNA genes of *C. krusei*. Sequences were as follows: Ano1, 5′-GGC CAA CAC ATA CAT ACC TT-3′ and Ano2, 5′-GGT AGG ATA CTA ACC ACA GC-3′. The reaction was performed on a 50 μl reaction volume containing 2·5 μl supernatant fluid,

2·5 units Taq polymerase (M186E; Promega, Madison, WI, USA), 5 μl buffer (Promega M190G), 25 mmol l^{-1} $MgCl_2$ (Promega A351H), 200 μmol l^{-1} of each deoxynucleotide (Promega U124O) and 1 μmol l^{-1} of each primer. Amplification was carried out in a Thermal Cycler (Gene amp PCR system 2400; Perkin Elmer, Norwalk, CT, USA) according to the following protocol: 92 °C for 4 min, followed by 32 cycles at 55 °C for 30 s, extension at 72 °C for 2 min and termination at 72 °C for 10 min, thereafter the reaction product was cooled to 4 °C. The PCR products were then separated by electrophoresis. The amplification product (20 μl) was applied to a submerged horizontal 1% type II medium EEO agarose gel (Sigma Chemical Co.) and run at ambient temperature for 2 h at 80 V using 1 × Tris-borate-EDTA; 1 kb DNA Ladder (GibcoBRL, Life Technologies, Taastrup, Denmark) was used as marker. The gel was stained with 1 mg ethidium bromide (Sigma E-1510) l^{-1} milliQ water for 20 min and rinsed twice in milliQ water for 20 min.

Cluster analysis

The gels were visualized with a u.v. transilluminator (Pharmacia LKB Macrovue) and photographed with a land camera (Polaroid MPE, Cambridge, MA, USA). Band patterns were scanned and data collected by use of the GelScan XL 2400 program (Pharmacia LKB Biotechnology AB), normalized and further processed by use of Gelcompar 4·0 Software (Applied Maths, Kortrijk, Belgium) which was also used for generation of the cluster analyses.

DNA preparation. Yeast chromosomal DNA for hybridization experiments was extracted according to the method of Scherer and Stevens (1987). After centrifugation (3000 *g* for 20 min) of 5 ml overnight cultures the pellet was treated with 4 μl of 2-mercaptoethonal, 0·5 mg zymolase (Seikaguru Corporation 120493) and 40 μl of 10% SDS. DNA was precipitated with 2-isopropanol. DNA preparations were stored in sterile water at −20 °C until use.

Restriction digest (restriction endonucleaase analysis)

DNA (0·75 μg) samples were separately digested at 37 °C with 10 U of *Eco*RI, *Hind*III or *Hinf*I (Boehringer Mannheim, Mannheim, Germany) for 5 h. DNA fragments were separated electrophorically on 0·8% type II medium EEO agarose (Sigma Chemical C) in Tris-borate-EDTA buffer. Electrophoresis was performed at 40 V for 18 h according to Carlotti *et al.* (1994). Gels were stained and photographed as described above. For Southern blot hybridization, chromosomal DNA digested with *Hinf*I enzyme was used.

Southern blot hybridization

The REA gels (*Hinf*I digest) were transferred to a positively charged nylon membrane (Boehringer Mannheim 1417 240) by vacuum blotting using a VacuGene XL blotting unit (Pharmacia LKB Biotech, Uppsala, Sweden) and Vacugene XL vacuum blotting pump (Pharmacia LKB Biotechnology AB). The blotting was performed according to the manufacturer's instructions at 50 mbar, depurination (0·25 mol l^{-1} HCl (Merck 1·00317)) for 30 min, denaturation (1·5 mol l^{-1} NaCl (Sigma C-3014), 0·5 mol l^{-1} NaOH (Merck 1·06498)) for 30 min, neutralization (1·0 mol l^{-1} Tris-base (Sigma T-8542), 1·5 mol l^{-1} NaCl, pH 7·5) for 30 min and transfer (20 × SSC, 3·0 mol l^{-1} NaCl (Sigma C-3014), 300 mmol l^{-1} trisodium citrate (Merck 1·06448)) for 60 min. The nylon membrane was cross-linked with u.v. at $1{\cdot}2 \times 10^5$ μJ cm^{-2} for 20 s using a cross-linker (Hoefer UVC500; Pharmacia LKB Biotechnology AB). A DNA fingerprinting probe CkF1,2 obtained from Arnaud Carlotti (Université Claude Bernard-Lyon, France) was used at a concentration of 10 ng μl^{-1} for the hybridization. Probe labelling and hybridization were conducted as described using the ECL direct nucleic acid labelling and detection system kit (Pharmacia LKB, Biotech, Uppsala, Sweden), and as recommended by the manufacturer.

RESULTS

Microbiological analysis

Yeast isolates selected from malt agar plates were phenotypically identified to species level. *Candida krusei* isolates were characterized by being flat, off-white to greyish colonies with irregular margins fringed with pseudomycelium on MYPG agar, with formation of a creeping pellicle when grown in broth, and growth at 37 °C. The composition of yeast flora during processing of maize dough is shown in Table 2. Based on the API test performed on selected isolates (27) from 24 h fermentation plates, 49% of these isolates were *Sacch. cerevisiae*, 15% *C. krusei* and 36% *Candida* spp. and others. Of 27 isolates selected from 48 h fermentation plates, 55% were *Sacch. cerevisiae*, 35% *C. krusei* and 10% other yeasts. Isolates (27) from 72 h fermentation comprised 26% *Sacch. cerevisiae*, 63% *C. krusei* and 11% other yeasts. In order to trace the origin of the yeasts during fermentation, processing equipment such as the milling machine was microbiologically monitored before use. The yeast composition on the corn milling machine comprised 51% *C. krusei* and 48% other *Candida* species with no *Sacch. cerevisiae* being isolated (Table 2).

As shown in Table 3 various assimilation profiles were obtained for 48 *C. krusei* isolates from the various steps of fermented maize dough processing. Most of the isolates were

Table 2 Composition of yeast flora on maize kernels, steeping tank, steep water, corn mill and during maize dough fermentation

Sample	No. of isolates picked	Composition
Maize kernels	26	Mixed population dominated by *Candida* spp.
Steeping tank	12	*Candida* spp.
Steep water (24 h)	27	Mixed population of *Candida* spp., *Saccharomyces* spp., *Debaryomyces* spp. and *Candida krusei* (3%)
Corn mill	26	*Candida krusei* (51%), other *Candida* spp. (48%)
Fermentation (24 h)	27	*Saccharomyces cerevisiae* (49%), *Candida krusei* (15%), others (36%)
Fermentation (48 h)	27	*Saccharomyces cerevisiae* (55%), *Candida krusei* (35%), others (10%)
Fermentation (72 h)	27	*Saccharomyces cerevisiae* (26%), *Candida krusei* (63%), others (11%)

Table 3 Assimilation profiles (API-32C) of *Candida krusei* strains isolated from spontaneously fermented maize dough

Carbon compound	*C. krusei**
Galactose	6/48
Actidione	—
Saccharose	—
N-acetyl-glucosamine	48/48
DL-lactate	48/48
L-arabinose	—
Cellobiose	—
Raffinose	—
Maltose	—
Trehalose	—
2-Keto-gluconate	—
α-Methyl-D-glucoside	—
Mannitol	—
Lactose	—
Inositol	1/48
Sorbitol	—
D-xylose	—
Ribose	—
Glycerol	43/48
Rhamnose	—
Palatinose	—
Erythritol	—
Melibiose	—
Glucoronate	—
Melezitose	—
Glucunate	—
Levulinate	—
Glucose	48/48
Sorbose	1/48
Glucosamine	—
Aesculin	—

* No. of assimilation-positive strains for 48 *C. krusei*.

able to utilize N-acetyl-glucosamine, DL-lactate, glycerol and glucose; this profile makes up 82% of the strains, including the type strain of *C. krusei* (CBS 573) and all clinical strains (Table 1). Twelve per cent of the strains could assimilate galactose, 2% sorbose and 2% inositol. However, 10% of the strains (18 A-3, 19 A-3, 171, 190 and 199) could not utilize glycerol, which identified them, with a 67% probability, as *C. valida* or, with a 25·4% probability, as *C. krusei* according to their API score. There was no correlation between the assimilation profiles and the time of sampling nor the stage of fermentation. In general, all the strains were phenotypically similar, including clincal isolates. We selected 44 isolates of *C. krusei* from the present work, four isolates from previous (Jespersen *et al.* 1994) work and six clinical isolates from Carlotti *et al.* (1994) for further characterization using DNA-based methods. The later isolates (clinical) were included for comparison.

Pulse field gel electrophoresis

Chromosome profiles were performed for all 48 isolates of *C. krusei* and other *Candida* spp. (results not shown). Some representative isolates of *C. krusei* and other *Candida* spp. are shown in Fig. 1a. Chromosome profiles for *C. krusei* isolates were typical for *C. krusei,* with chromosomes ranging from approximately 3·13 to 1·2 mbp. Isolates with phenotypic characteristics of *C. valida* (18 A-3, 19 A-3171, 190 and 199) had a typical *C. krusei* chromosome profile (Fig. 1b) and were different from the chromosome profiles of *C. valida* reference (CBS 635, 636, 638) strains included in the study. Results are shown for strain 18 A-3 (Fig. 1a, lane 9) and *C. valida* strains CBC 636 and CBS 638 (Fig. 1b, lanes 5 and 6). For all *C. krusei* strains two bands typical of this species were approximately 1·33 and 1·26 mbp (determined from the standard marker used) (Fig. 1b). These bands were absent from other *Candida* spp. tested (Fig. 1a). Figure 1b shows an example of the chromosome profiles of *C. krusei* strains from maize and from clinical samples (Fig. 1b lanes 2–6 and 8 and 9–12, respectively). The approximate sizes of the five bands observed were about 3·2, 2·8, 2·6, 1·33 and 1·26 mbp. The

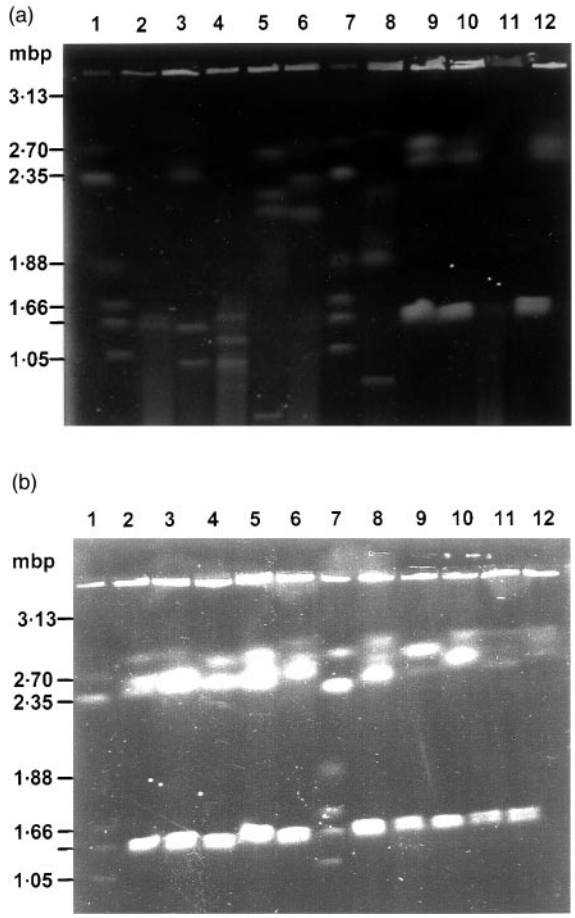


Fig. 1 (a) Chromosome profiles determined by pulse field gel electrophoresis of *Candida* species and *Candida krusei* isolates from fermented maize dough. Lanes 1 and 7, marker; lane 2, *C. krusei* CBS 573^{T}; lane 3, *C. tropicalis* CBS 94; lane 4, *C. kefyr* CBS 834; lanes 5 and 6, *C. valida;* lane 8, *C. rogosa* CBS 613; lanes 9–12, *C. krusei* (18 A-3, 19B-4, CBS 573^{T} and 20B-2). (b) Chromosome profiles of *C. krusei* isolates from maize dough and clinical source. Lanes 1 and 7, marker; lanes 2–6 and 8, maize isolates (174, 168, 122, 132, 82 and 65); lanes 9–12, clinical isolates (K24, K36, K45 and K60)

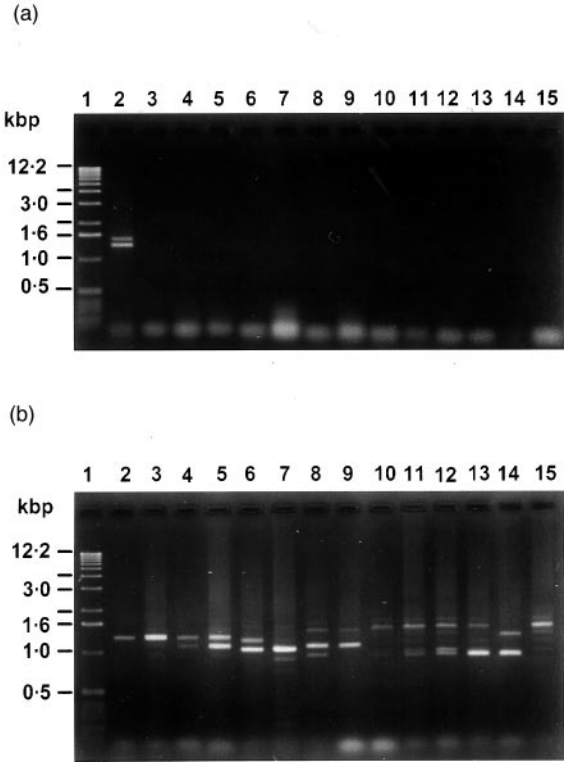


Fig. 2 (a) Specificity of the polymerase chain reaction (PCR) with primer pair Arno1 and Arno2. Lane1, 1 kb DNA ladder (GibcoBRL); lane 2, *Candida krusei* (CBS 573^{T}); lanes 3–5, *C. valida* (CBS 635, 636 and 638); lane 6, *C. tropicalis* (CBS 64); lane 7, *C. kefyr* (CBS 834); lane 8, *C. rogosa* (CBS 613); lane 9, *Cryptococcus laurentii* (CBS 139); lane 10, *Zygosaccharomyces rouxii* (CBS 732); lane 11, *C. cantenulata* (CBS 656); lanes 12 and 13, *C. parasilosis* (CBS 7248 and 604); lane 14, *Debaramyces hansenii* (CBS 164); lane 15, blank. (b) Examples of PCR fingerprinting of *C. krusei* isolates from fermented maize dough showing representation of clusters. Lanes 2 and 3, 168 and 204 (cluster 1); lane 4, 79 (cluster 1); lanes 5 and 6, 194 and 19 A-3 (cluster 1); lanes 7–9, 18 A-3, 19B-4 and 41 (cluster 2); lanes10–13, 10, 76, 132 and 193 (cluster 4); lane 14, 131 (cluster 5); lane 15, 202 (cluster 6)

profiles were, in general, quite homogeneous. Chromosome polymorphism was detectable at high molecular size range (2·6–3·2 mbp); three bands can be seen for some strains while two bands occurred in other strains (Fig. 1b).

Polymerase chain reaction and cluster analysis

Forty-eight maize isolates, six clinical isolates and the type strain (CBS 573^{T}) of *C. krusei*, as well as other *Candida* spp., were tested using primers Arno1 and Arno2 (Carlotti *et al.* 1997a). Some representative isolates are shown in Fig. 2. The primers used were specific for *C. krusei*; all other species included in this study had no PCR product with these primers (Fig. 2a) including the closely related *C. valida* (Fig. 2a, lanes 3–5). *Candida tropicalis, C. kefyr* and *C. rogosa*, often found in maize dough, had no PCR product (Fig. 2a, lanes 6–8). Two major amplification products of about 1·6 and 1·4 kb were observed for the type strain (CBS 573^{T}) (Fig. 2a, lane 2). Several PCR products were obtained for *C. krusei* strains, the sizes ranged from about 2·0 to 1·0 kb, and varied in number and sizes depending on the strain (Fig. 2b). The diversity among the isolates was evident although some isolates had similar profiles. To compare the differences among the strains isolated from different stages of fermentation and also with

clinical strains, cluster analysis was performed based on their PCR product. This was designed to facilitate the identification of the underlying genetic relatedness between the strains of different origin and between strains from food and clinical sources.

The results of cluster analysis based on the PCR profiles of 51 *C. krusei* isolates from fermented maize dough, clinical sources and one reference strain are presented in a dendrogram shown in Fig. 3. Six clusters were defined at a similarity level of about 67%. Cluster 1 comprised 23 *C. krusei* strains from the various steps of maize dough fermentation (steep water, 24, 48 and 72 h fermentation) including isolates from the corn milling machine. Cluster 1 did not contain any of the clinical isolates. Strain 183 appeared to be alone, but links with cluster 1 at 58% similarity. Cluster 2 comprised a mixture of *C. krusei* clinical strains and strains from 72 h fermentation, whereas Cluster 3 contained only clinical strains. Cluster 4 comprised 17 *C. krusei* strains from the different steps of fermentation except for 24 h. Strain no. 191 appears to be a straggler within cluster 4 at 67% similarity. Cluster 5 contains two strains, one each from 48 and 72 h, respectively. Finally, Cluster 6 is made up of two strains from the corn mill, one of the clinical strains and the type strain (CBS 573) of *C. krusei*. There was no correlation between PCR profiles and the time of sampling, neither the period nor the stage of fermentation. Clinical isolates seemed to be confined to two related clusters (2 and 3).

Restriction endonuclease analysis and cluster analysis

We tested three enzymes, *Eco*RI, *Hind*III and *Hinf*I, on all isolates from fermented maize dough. Representative isolates are shown in Fig. 4. All three enzymes generated distinguishable band patterns. *Eco*RI and *Hind*III enzymes generated variable band patterns within the different strains of *C. krusei*, but gave a lot of background. Depending on the strain, *Eco*RI and *Hind*III digest yielded one to six visible fragments in the range of 9–3 kb. Two visible fragments of about 3·6 kb and 2·9 $\pm$ 0·1 kb from *Eco*RI digest were observed in all *C. krusei* strains. Clearer band patterns were obtained for *Hinf*I digests (Fig. 4, lanes 4, 7, 11 and 14); this was easier to use with the Gel Compar program. The *Hinf*I digest generated 9–12 bands spanning the size range of about 6·1–1·0 $\pm$ 0·1 kb. These experiments were repeated to ascertain their reproducibility. Calculated band sizes from two separate gels revealed an error range of $\pm$0·1 kb. Cluster analysis based on the *Hinf*I digest of *C. krusei* strains revealed a total of 11 clusters at about 45% similarity as illustrated in Fig. 5. Cluster I included isolates from 24, 48 and 72 h fermentation, one isolate from a previous isolation (Jespersen *et al.* 1994) and all clinical isolates (K24, K36, K45, K62) except one (K60) which remained in a single cluster II. Cluster III comprised isolates from the corn mill, steep water, 48 and 72 h fermentation; no 24 h isolates were observed in this cluster. Strain numbers 187, 124 and 125 remained in separate clusters IV, VI and VII, respectively. Cluster V comprised two isolates from 48 h fermentation. Cluster VIII comprised corn mill and 72 h fermentation isolates as did cluster IX, with the exception of one isolate from 24 h fermentation. Cluster X contained isolates from previous studies (19 A-3), the corn mill, 24, 48 and 72 h fermentation and the type strain of *C. krusei* (CBS 573^T). Strain no. 75 had a separate cluster XI.

Southern hybridization with the CkF1,2 probe

For further characterization of *C. krusei* isolates from maize dough, *Hinf*I DNA digests of some *C. krusei* isolates from maize dough and all clinical isolates were probed with the CkF1,2 probe obtained from Carlotti *et al.* (1994). The probe hybridized to all *C. krusei* isolates and not to other *Candida* spp. included in the study (Fig. 6a). The banding patterns obtained for some *C. krusei* isolates are shown in Fig. 6a–d. About two to seven bands of variable sizes (depending on strain) ranging from 6·1 to 2·0 kb were obtained. Except for strain numbers 121 and 131 (Fig. 6c, lanes 6 and 7) and strain numbers 45 and 80 (Fig. 6d, lanes 2 and 3), which were identical pairs, all the other strains had individual band patterns. Of 38 strains (isolates from maize dough) tested, 34 possible different fragment sizes hybridized to the probe. The probe hybridized to four fragment sizes of about 4·0, 3·8, 3·5 and 3·2 kb of *Hinf*I digest of the type strain of *C. krusei* (Fig. 6a, lane 1).

DISCUSSION

Fermented maize dough processing is a spontaneous fermentation and requires no added inoculum, therefore organisms found in the dough are either from the environment or from the raw materials used. We confirmed, in this study, previous observations made by other workers (Halm *et al.* 1993; Obiri-Danso 1994; Hounhouigan *et al.* 1994; Jespersen *et al.* 1994), on this and similar products in west Africa, that *C. krusei* and *Sacch. cerevisiae* are the predominant yeasts in maize dough (characterization of the latter is addressed elsewhere; Hayford and Jespersen 1999). The occurrence of *C. krusei* on the corn milling machine indicated one of several routes through which natural inoculation could occur, as well as sources of the organism. The importance of *C. krusei* in many African fermented products is continually being emphasized and the need for proper identification procedures is important when technological properties as well as development of starter cultures are being considered. We evaluated the use of molecular methods for the characterization of *C. krusei* isolates, from various stages of maize dough processing,

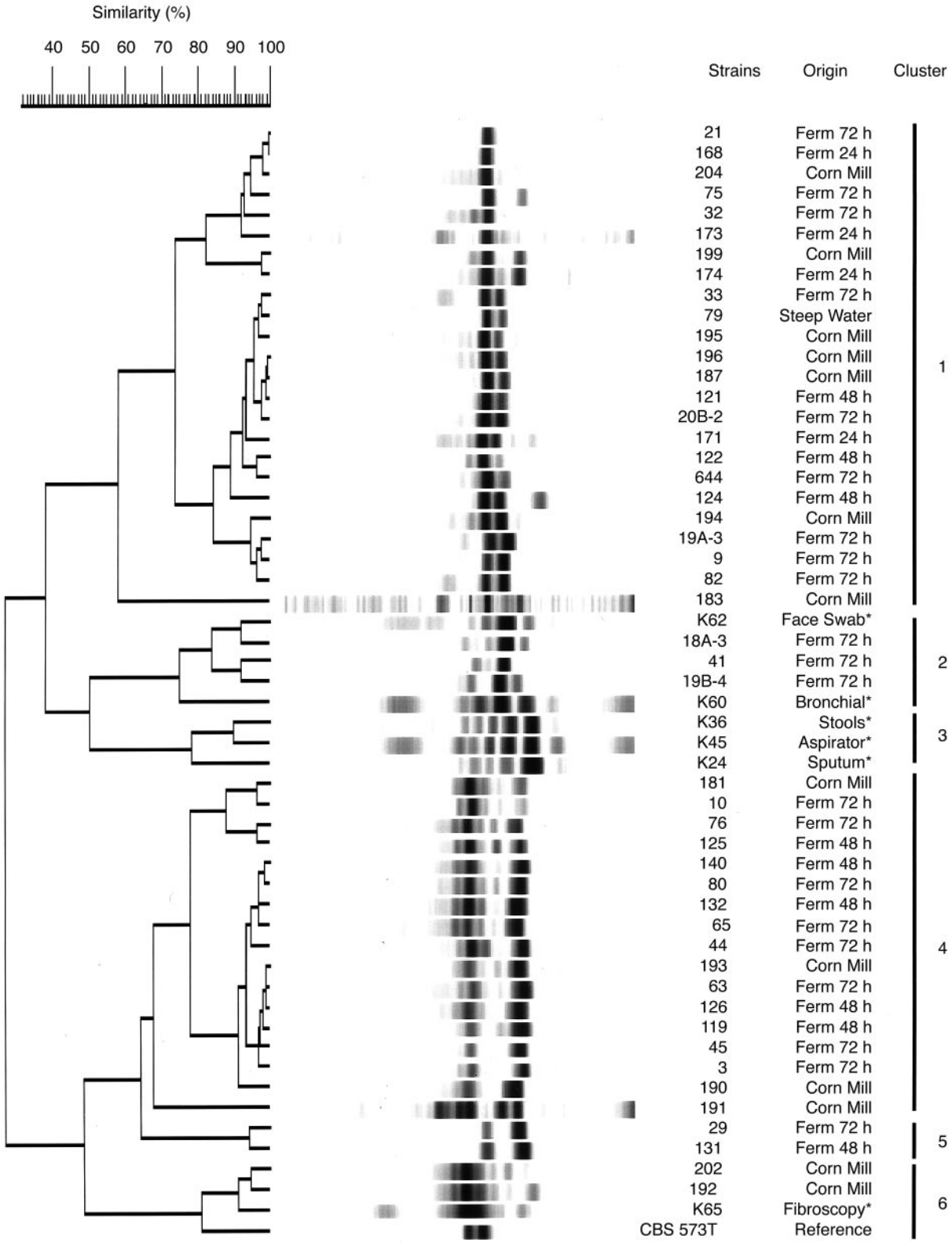


Fig. 3 Dendrogram of similarity among 48 food isolates, six clinical and one reference strain (CBS 573^T) of *Candida krusei* based on their polymerase chain reaction product with Arno1 and Arno2 primers. Similarity was based on Pearson product moment correlation coefficients and UPMGA clustering

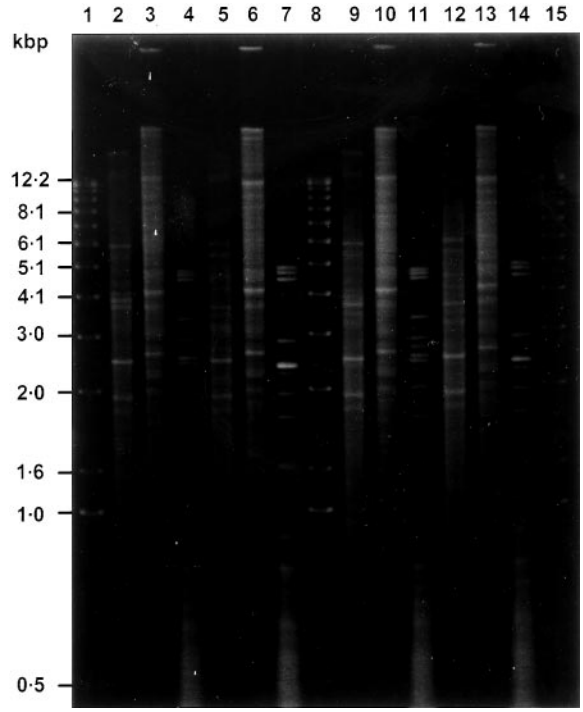


Fig. 4 Examples of restriction digest with *Eco*RI, *Hind*III and *Hinf*I of *Candida krusei* isolates from maize dough. Lanes 1, 8 and 15, 1 kb ladder (marker); lanes 2–4, 132 (*Eco*RI, *Hind*III and *Hinf*I); lanes 5–7, 82 (*Eco*RI, *Hind*III and *Hinf*I); lanes 9–11, 44 (*Eco*RI, *Hind*III and *Hinf*I); lanes 12–14, 32 (*Eco*RI, *Hind*III and *Hinf*I)

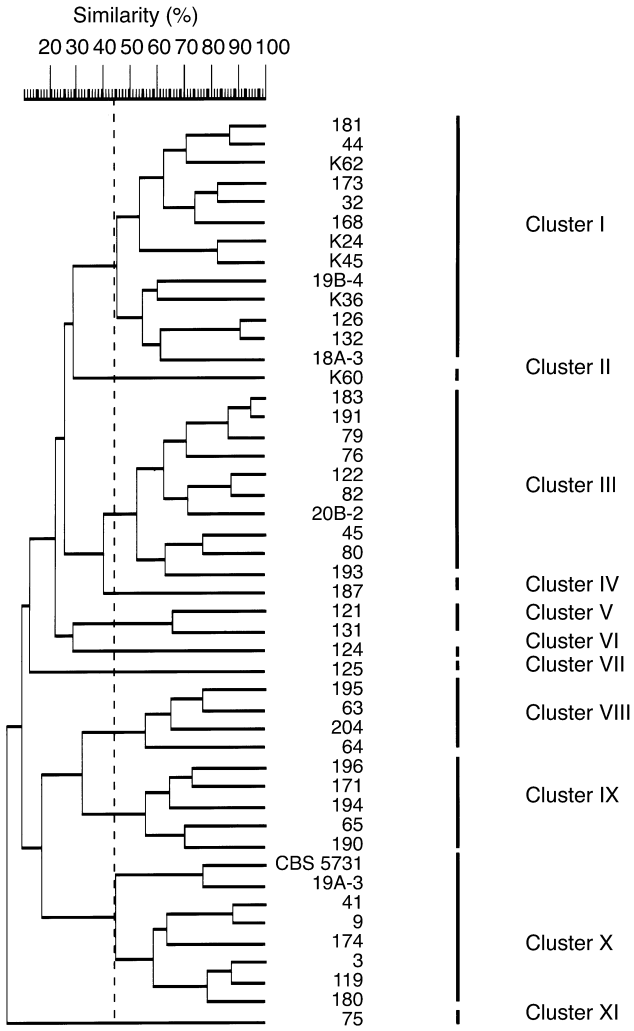
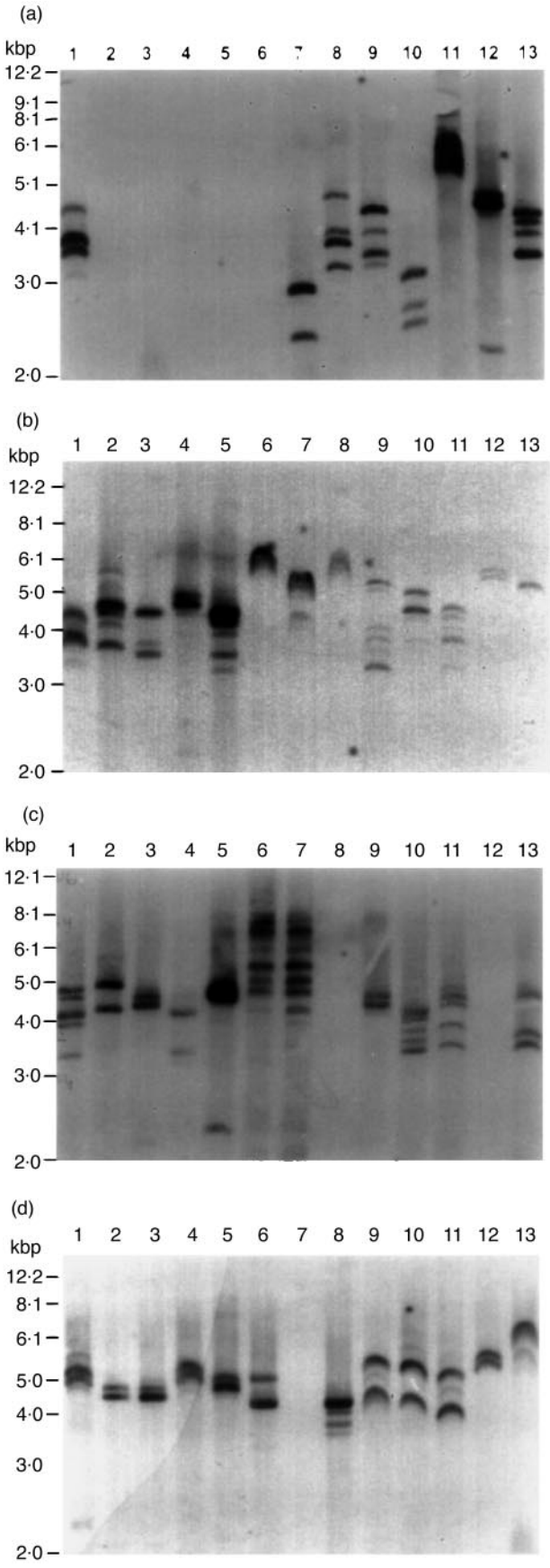


Fig. 5 Dendrogram of similarity among *Candida krusei* strains from maize dough based on their *Hinf*I digest fragments. Similarity is based on Pearson product moment correlation coefficients and UPGMA clustering

and included six clinical isolates for comparison. The API system could be used for identification only to species level and for phenotyping. In support of this, only one or two isolates had different assimilation profiles. The majority of the strains (82%) had the same assimilation profile, making strain differentiation impossible. These profiles, however, were typical for *C. krusei* (Kurtzman and Fell 1998). Although assimilation profiles were not suitable for strain differentiation, some information on the technological properties of these isolates was evident by this (conventional) method, in that all the strains could assimilate DL-lactate which indicated that they were capable of utilizing lactic acid produced by *Lact. fermentum* in this product. Being acid-resistant, these organisms were able to survive and grow in the acid environment of about pH 3·7 (normal pH of maize dough). Nout (1991) hypothesized that the extent of acidification in cereal lactic fermentation is regulated by yeast growth. In the present investigation the conventional method was not completely reliable in differentiating between *C. krusei* and *C. valida*, due to the similarity in their assimilation and fermentation profile. It is becoming evident that phenotypic identification based on morphological and biochemical characterization often leads to misidentification (van der Vossen and Hoftra 1996). This, apparently, is due to the fact that yeasts are often subject to mutation and deletions in their chromosomes, thus any physiological property of a yeast belonging to a genus may be unstable under certain conditions, leading occasionally to total misidentification (van der Walt 1987).

We further used several molecular techniques described by various authors to confirm the diversity among our strains. With PFGE, *C. krusei* and *C. valida* were differentiated based on the number and size of their chromosomes. The two prominent bands occurring at approximately 1·33–1·26 mbp in all *C. krusei* strains distinguished them from *C. valida* and other *Candida* spp. The chromosome profiles obtained for *C. krusei* isolates from both maize dough and clinical isolates



matched one of three profiles observed by Doi *et al.* (1992). Chromosome length polymorphism was not easily detectable but evident in the present study due to difficulty in separation of the larger size chromosomes. Doi *et al.* (1992) concluded that different methods (separately) may be necessary for the separation of larger chromosomes and another method for the separation of smaller chromosomes. The PFGE technique was more precise for species identification and, to some extent, subspecies identification. The method had a higher discriminatory power than the API system.

The PCR, REA and Southern blot hybridization technique together confirmed the variations among the strains. The variability in the band patterns obtained with the REA method was sufficient to differentiate all strains tested, as with the PCR method, although fragment sizes in the latter method appeared more similar. In some cases with the PCR technique differences between strains were differences in intensity of band patterns. However, REA produced distinct patterns, and variations within the strains were based on the number and sizes of the *Hinf*I fragments. The disadvantage of the REA compared with the PCR is the occurrence of numerous fragments which made visual analysis difficult. On the other hand, PCR was fast, easy to handle and specific for *C. krusei* which makes it easier for quick identification. With Southern hybridization the band patterns were more individualistic, characteristic for each strain and this provided an estimate of the degree of genomic relationship between strains. This method had the highest discriminating power. For example, in cases where two strains may have the same PCR band pattern or closely related REA pattern, the Southern blot pattern had a strain-specific DNA band pattern.

←

Fig. 6 (a) Examples of hybridization of CkF1,2 to *Hinf*I digest of whole cell DNA of *Candida krusei* strains from fermented maize dough and reference cultures. Lane 1, *C. krusei* (CBS 573^T); lane 2, *C. tropicalis* (CBS 94); lane 3, *C. kefyr* (CBS 834); lanes 4 and 5, *C. valida* (CBS 636 and 638); lane 6, *C. rogosa* (CBS 613); lanes 7–13, *C. krusei* (18 A-3, 19B-4, 20B-2, 19 A-3, 194, 193 and 187). (b) Examples of hybridization of CkF1,2 to *Hinf*I digest of whole cell DNA of *C. krusei* strains from fermented maize dough. Lane 1, 174; lane 2, 168; lane 3, 122; lane 4, 132; lane 5, 82; lane 6, 65; lane 7, 171; lane 8, 190; lanes 9–13, K24, K36, K45, K60 and K62 (clinical isolates). (c) Examples of hybridization of CkF1,2 to *Hinf*I digest of whole cell DNA of *C. krusei* strains from fermented maize dough. Lane 1, 79; lane 2, 173; lane 3, 126; lane 4, 124; lane 5, 119; lane 6, 121; lane 7, 131; lane 8, blank; lane 9, 44; lane 10, 32; lane 11, 41; lane 12, blank; lane 13, 9. (d) Examples of hybridization of CkF1,2 to *Hinf*I digest of whole cell DNA of *C. krusei* strains from fermented maize dough. Lane 1, 3; lane 2, 45; lane 3, 80; lane 4, 180; lane 5, 181; lane 6, 183; lane 7, *C. tropicalis* (185); lane 8, 191; lane 9, 196; lane 10, 204; lane 11, 195; lane 12, 63; lane 13, 75

The only drawback with this method was the time factor involved in the hybridization experiments.

Interestingly, all the clusters formed based on both PCR and REA methods had strains from the various steps of the fermentation represented in each cluster. Previously isolated strains were also found in different clusters, clustering with strains isolated from the present study. This suggests that various strains are involved in the fermentation from the onset and remain dominant throughout the fermentation. The fact that previous isolates did not group separately may suggest stability in the genetic variation over the 4-year period. However, it seemed that clinical isolates tended to group in either the same cluster or closely related clusters. This may suggest some genetic differences between clinical and food strains. This, however, remains to be elucidated. The specificity of the primers and probe was confirmed in accordance with the group who first developed and reported on it (Carlotti *et al.* 1994, 1996, 1997a,b). This study is the first, to our knowledge, to report and analyse molecular variations among natural strains of *C. krusei* from the various steps of traditional maize dough fermentation. It has revealed the high level of genetic diversity that exists among the strains involved in the fermentation and possible genetic variation between food and clinical strains of *C. krusei*.

We conclude that the methods evaluated in this study are suitable for typing of our *C. krusei* strains. The most suitable method for quick identification would be the PCR method. However, the Southern hybridization method could be advantageous for confirmation of starter cultures.

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