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Development of primers for detecting dominant yeasts in smear-ripened cheeses

Stéphanie Gente, Sandra Larpin, Orianne Cholet, Micheline Guéguen, Jean-Paul Vernoux and Nathalie Desmasures*

Laboratoire de Microbiologie Alimentaire, Université de Caen Basse-Normandie, Esplanade de la paix, 14032 Caen cedex, France

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PCR primers were developed for the specific detection of *Clavispora lusitaniae*, *Debaryomyces hansenii* var *hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis* and *K. marxianus* and *Yarrowia lipolytica*, yeast species commonly found on the surface of smear cheese. Forty eight representative strains frequently found in smear cheeses or taxonomically related to the target yeasts were used as templates, to validate the designed primers. The specific and selective detection of these yeasts was effective *in situ*, in Livarot smear, without yeast isolation and culture and was comparable with data obtained with a conventional method. The primers described here have thus potential for PCR studies applied to cheese. It should also be possible to use some of these primers with other substrates.

Keywords: *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, PCR primers, Smear cheese, *Yarrowia lipolytica*, Yeasts.

Smear-ripened cheeses, such as Münster, Pont l'Evêque and Taleggio, characteristically contain high levels of yeasts involved in ripening. The so-called 'smear' is the characteristic glistening rind resulting from the development of a mixed microflora of yeasts and bacteria on the surface of the cheese. The microbial ecology of the smear is complex and few systematic studies (Bärtschi et al. 1994; Eliskases-Lechner & Ginzinger, 1995a, b; Valdès-Stauber et al. 1997; Wyder & Puhan, 1999; Brennan et al. 2002) have investigated the micro-organisms found on the surface of such cheeses during ripening. Ecological investigations of the yeast flora have shown that *Debaryomyces hansenii* and *Geotrichum candidum* are the most important species in smear-ripened cheeses, followed by *Thrichosporon beigelii* and *Yarrowia lipolytica* (Brennan et al. 2004a), whereas the most common species in mould-ripened and blue cheeses are *Deb. hansenii*, *G. candidum*, *Kluyveromyces lactis*, *K. marxianus*, *Saccharomyces cerevisiae* and *Yar. lipolytica* (Cantor et al. 2004; Spinnler & Gripon, 2004). The European project QLK1-CT-2001-02228 (acronym: SCM – Smear Cheese Microflora), which involved partners from six EU countries, aimed to identify and to characterise the major yeast species and bacteria present on the surface of five smear cheeses: Limburger (Germany), Reblochon (France), Livarot (France), Tilsit (Austria) and Gubbeen (Ireland), and to study the dynamics of microbial populations during ripening. The yeast flora was found to differ considerably between these five cheeses (Brennan et al. 2004b; Goerges et al. 2004; Hohenegger et al. 2004; Larpin et al. 2004). The principal yeast species identified on the surface of the five cheeses were *Candida catenulata*, *Clavispora lusitaniae*, *Deb. hansenii*, *G. candidum*, *K. lactis*, *K. marxianus* and *Yar. lipolytica*.

Yeast identification is usually based on phenotypic, physiological and morphological criteria (Kurtzman & Fell, 1998; Barnett et al. 2000). Molecular methods are regularly used to confirm the yeast identification. Random amplified polymorphic DNA (RAPD) or restriction fragment length polymorphism (RFLP) techniques are often used to identify yeasts isolated from cheese or dairy products (Prillinger et al. 1999; Wyder & Puhan, 1999; Petersen et al. 2002; Fadda et al. 2004). However, these methods require the isolation and culture of the yeast strain. PCR-based methods associated with denaturing gradient gel electrophoresis (DGGE) have been developed to circumvent this problem, and such techniques have been validated for the analysis of wine yeasts (Cocolin et al. 2000; Prakitchaiwattana et al. 2004) and sourdough yeasts (Meroth et al. 2003). However, these methods require sequencing of the amplified products to confirm correct identification. Specific primers can be used for the simultaneous detection and identification of the target

*For correspondence; e-mail: nathalie.desmasures@unicaen.fr

micro-organism, directly in the matrix (Somer & Kashi, 2003); we chose to adopt such an approach in this study. Our objectives were (1) to construct specific PCR primers for detection and identification of the principal yeasts found on the surface of smear-ripened cheeses and (2) to assess the ability of these primers to detect these yeasts *in situ*, in Livarot cheese.

Materials and Methods

Yeast strains and culture conditions

A selection of 48 reference and commercial strains of yeasts of 27 species or varieties, was chosen to test cross-reaction (Table 1). Commercial cultures of *Deb. hansenii*, *G. candidum* and *K. lactis* were also selected because these species are used in smear cheese processing. The strains were maintained at −80 °C in 25% glycerol.

Geotrichum candidum strains were initially cultured in malt extract broth (MEB, VWR) for 48 h at 25 °C, with shaking at 120 rpm (Novotron, VWR, Fontenay-sous-Bois, France). Potato dextrose agar plates (AES Laboratoire, Combourg, France) covered with a cellophane film were then inoculated with 100 µl of this preculture. Plates were incubated for 48 h at 25 °C in the dark, and the mycelium was then recovered from the cellophane film with a sterile spatula.

The other yeast strains were cultured (both precultures and study cultures) for 48 h at 25 °C, with shaking at 200 rpm (Novotron, VWR), in 300-ml Erlenmeyer flasks containing 50 ml MEB.

Lactose assimilation in strains of *K. marxianus*, CLIB 396 and CLIB 527, was done as described by Yarrow (1998). The tests were carried out in liquid yeast nitrogen base (Difco, Biovalley, Marne la Vallée, France) supplemented with 0·5% lactose (Sigma-Aldrich, Saint Quentin Fallavier, France), at 25 °C, without shaking. Growth was observed after 1, 2 and 3 weeks.

DNA isolation from collection strains

For *Geotrichum* and *Galactomyces* strains, the mycelium was frozen in liquid nitrogen and ground for 90 s, at 30 oscillations/s, in a Retsch GmbH MM200 mixer (Bioblock, Illkirch, France). DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions.

For the other yeasts, MEB cultures were centrifuged at 7000 ***g*** (4 °C, 15 min, Eppendorf centrifuge 5810R, Bioblock). The pellet was suspended in 1·5 ml sterile TE buffer (100 µM-Tris, 2 µM-EDTA, pH 8·0) and the pH was adjusted to 7·0±0·1 by adding 1 M-NaOH. The mixture (1 ml) was transferred to a fresh 2 ml tube and centrifuged at 10 000 ***g*** (4 °C, 5 min). The supernatant was discarded and the pellet dissolved in 1 ml sterile distilled water and centrifuged (4 °C, 10 000 ***g***, 5 min). The cell pellet was disrupted with 0·6 g glass beads (0·1 mm diameter) in 0·4 ml lysis buffer (10 mM-Tris pH 8, 1 mM-EDTA, 100 mM-NaCl, 20 g Triton/l, 10 g SDS/l.). Phenol : chloroform : isoamyl alcohol (25 : 24 : 1; Fluka, Saint Quentin Fallavier, France) was added (0·4 ml) and the tube vortexed for 3 to 6 min, depending on the yeast strain concerned, using an MM200 grinder. After centrifugation (4 °C, 15000 ***g***, 10 min), the supernatant was shaken (v/v) with chloroform : isoamyl alcohol (24 : 1) and the DNA was precipitated from the aqueous phase by freezing at −20 °C in absolute ethanol, washed twice with 1 ml 70% ethanol and dried under vacuum. The DNA pellet was resuspended in 50 µl sterile distilled water and incubated for 1 h at 37 °C with 2 µl 10 mg.ml^{-1} RNase A solution (Sigma Aldrich). The DNA was stored overnight at 4 °C and then frozen at −20 °C for longer term storage.

Isolation of DNA from Livarot cheese

Batches of Livarot cheese manufactured by three dairies, designated A, B, and C, were studied. Cheese samples were taken on the day before salting, the day before packaging and at the end of ripening. The smear was scrapped from the surface with a sterile scalpel and frozen at −80 °C until analysis. DNA was extracted from 1 g of the smear, as described by Duthoit et al. (2003), including a final purification step, using the Wizard DNA clean-up system, as recommended by the supplier (Promega, Charbonnières, France).

Design of specific PCR primers

Database interrogations (DDBJ, EMBL, GenBank), using BLAST program (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990) and target sequences made it possible to design primers for the specific detection of *Clav. lusitaniae*, *Deb. hansenii*, *G. candidum*, *K. lactis*, *K. marxianus* and *Yar. lipolytica*. CLUSTALW (Thompson et al. 1994) alignments were generated to ensure that no hybridization could occur with other known DNA sequences from yeast species (listed in Table 1). We also tested previously described primers for *Yar. lipolytica* and *K. marxianus* (Kanbe et al. 2002). The PCR primers (Table 2) used in this study were prepared by Invitrogen (Cergy Pontoise, France).

PCR conditions

Amplifications were carried out in a PTC 200 thermal cycler (MJC Research, Waltham, USA). The reaction mixture (50 µl) contained 100 pmol of each primer, 0·2 mM of each deoxyribonucleoside triphosphate (Invitrogen), 1 × PCR buffer without $MgCl_2$, 1·5 mM-$MgCl_2$ (Qbiogene, Illkirch, France), 80–100 ng yeast DNA or 5 µl Livarot smear DNA, and 3 U *Taq* DNA polymerase (Qbiogene). DNA fragments were amplified as described in Table 2. Aliquots (10 µl) of the amplified products were subjected

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Table 1. Characteristics of the strains used for primer validation

Species	Strain designation[α]	Origin	Country
Candida catenulata	CBS 565^T	Faeces of man with dysentery	Puerto Rico
	CBS 6174	Milk from cow with mastitis	The Netherlands
Clavispora lusitaniae	CBS 4413^T	Caecum of pig	Portugal
	CBS 5299	Milk from cow with mastitis	Greece
Cryptococcus albidus var. *albidus*	CBS 142	Atmosphere	Japan
Debaryomyces hansenii var. *hansenii*	CBS 766	Cheese	Russia
	CBS 767^T	Cherry	Denmark
	Scom3	Commercial strain (cheese ripening)	France
Debaryomyces hansenii var. *fabryii**	CLIB 606	Dairy atmosphere	France
*Debaryomyces marama**	CBS 1958^T	Atmosphere	New Zealand
*Debaryomyces nepalensis**	CBS 5921^T	Soil	Nepal
*Galactomyces citri-aurantii**	CBS 175.89^T	Soil from orange orchard	Zimbabwe
*Galactomyces geotrichum**	CBS 772.71^T	Soil	Puerto Rico
*Galactomyces reesii**	CBS 179.60^T	Cold water retting of *Hibiscus cannabinus*	Indonesia
Geotrichum candidum	ATCC 204307	Pont l'Evêque cheese	France
	UCMA 96	Pont l'Evêque cheese	France
	CBS 110.12	Milk	France
	Scom1	Commercial strain (cheese ripening)	France
	Scom2	Commercial strain (cheese ripening)	France
*Geotrichum europaeum**	CBS 866.68^T	Wheat field soil	Germany
*Geotrichum pseudocandidum**	CBS 626.83^T	Stomach of elk	France
Issatchenkia occidentalis	WSYC 312^T	Unknown	
	WSYC 328	Unknown	
*Kluyveromyces dobzhanskii**	CBS 2104^T	*Drosophila pseudoobscura*	USA
Kluyveromyces lactis var. *lactis*	CBS 683^T	Gassy cheese	United Kingdom
	CBS 739	Cheese	Italy
	CBS 1067	Buttermilk	The Netherlands
	Scom4	Commercial strain (cheese ripening)	France
	Scom5	Commercial strain (cheese ripening)	France
Kluyveromyces marxianus	CBS 712^T	unknown	
	CBS 1557	Stracchino cheese	Italy
	CBS 8415	Brine bath in cheese factory	The Netherlands
	CLIB 396	Unknown	
	CLIB 527	Unknown	
*Kluyveromyces wickerhamii**	CBS 2745^T	Gut of *Drosophila* sp.	USA
Pichia anomala	CBS 5759	Unknown	USA
Pichia fermentans	CBS 187	Buttermilk	The Netherlands
Pichia membranifaciens	CBS 107^T	Unknown	
Rhodotorula mucilaginosa var. *mucilaginosa*	CBS 316	Unknown	
Saccharomyces cerevisiae	CBS 1171^T	Brewer's top yeast	The Netherlands
Torulaspora delbrueckii	CBS 1146	Unknown	
Yarrowia lipolytica	CBS 6124.1	Maize-processing	USA
	CBS 6124.2	Maize-processing	USA
	CBS 6317	Probably dairy product	USA
	CLIB 791	Goat's cheese	France
	CLIB 880	Idiazabal cheese	Spain
Zygosaccharomyces cidri	CBS 4575^T	Cider	France
Zygosaccharomyces rouxii	CBS 732	Concentrated black-grape must	Italy

*,tested as taxonomically related species; [α], **ATCC**: American Type Culture Collection; **CBS**: Centraal Bureau voor Schimmelculture; **CLIB**: Collection de Levures d'Intérêt Biotechnologique; **UCMA**: Université de Caen Microbiologie Alimentaire; **WSYC**: Weihenstephan Yeast Collection; **T**, type strain

to electrophoresis in 1% agarose gels, in 1 × TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA, pH 8·2). The gels were stained with ethidium bromide (5 $\mu g.ml^{-1}$) and visualised under UV light. A 123 bp polymer was used as a molecular weight marker (Invitrogen).

DNA sequencing

The identification of strain CBS 766 as *Deb. hansenii* was confirmed by sequencing, as previously described by Martorell et al. (2005). The ITS1-ITS4, NL1-NL4 and

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Table 2. Sequences of primers and PCR conditions for selected yeasts

Species	Primer names	Sequence 5′–3′	Reference	PCR conditions
Cavispora lusitaniae	OCL1 OCL2	CTCTCCAGGACGCCAAAACC CCGTACTCGTAAACACCTGAC	This study	4 min at 94 °C; 25 cycles of 1 min at 94 °C, 30 s at 64 °C and 30 s at 72 °C; 5 min at 72 °C.
Debaryomyces hansenii	GDH1 GDH2	GAACTACTTCCTGCTATCCG CCACCCGACTACAGATCTC	This study	4 min at 94 °C; 25 cycles of 1 min at 94 °C, 30 s at 60 °C and 30 s at 72 °C; 5 min at 72 °C.
Geotrichum candidum	CGLFOC CGLROC	TCCATTGAGACCGAGCTC GTAGATGGAGTTCTCGTT	This study	4 min at 94 °C; 20 cycles of 1 min at 94 °C, 25 s at 54 °C and 20 s at 72 °C; 5 min at 72 °C.
Kluveromyces lactis & K. marxianus	KLOC1 KLOC2	ATCGGGTACCTTCAATGG TTGCGCACGGATCTGTAAC	This study	4 min at 94 °C; 20 cycles of 1 min at 94 °C, 25 s at 54 °C and 20 s at 72 °C; 5 min at 72 °C.
Kluyveromyces marxianus	CKFF35 CKFR104	CTTCCAAAGGTCAGAAGTATGTCC CACGAAATCGTTAGGAACTTCAC	Kanbe et al. 2002	4 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 66 °C and 2 min at 72 °C; 5 min at 72 °C.
Yarrowia lipolytica	YLLF35 YLLR59	AAGACGGCAAGATTTACACCCAGA TTCAAAGCCAGTCTAGTGCCATTG	Kanbe et al. 2002	4 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 68 °C and 2 min at 72 °C; 5 min at 72 °C.
Yarrowia lipolytica	YLEXG1 YLEXG2	GAGAAGGCTCTTTCCTGGG AAAAGGCCCAGTAGCCAATGGG	This study	4 min at 94 °C; 25 cycles of 1 min at 94 °C, 30 s at 60 °C and 30 s at 72 °C; 5 min at 72 °C.

CA21-CA22R primer sets were used for the PCR amplification of 5·8S-ITS, the D1/D2 domain and the actin gene (*ACT1*), respectively. Sequences were aligned using CLUSTALW (Thompson et al. 1994).

Results and Discussion

Construction and validation of DNA primers in pure cultures

Primers were designed for *Clav. lusitaniae, Deb. hansenii, G. candidum, K. lactis* and *Yar. lipolytica*. We were unable to design specific primers for *C. catenulata* based on the DNA sequences available in international databases and from previous studies.

Specificity, for all the PCR primers designed, was assessed by amplification from purified genomic DNA extracted from the five target yeasts, from their closest relatives (marked * in Tables 1 & 3) (Kurtzman & Robnett, 1998; Kurtzman, 2003; de Hoog & Smith, 2004) and from yeast species commonly found in smear cheeses (Table 1). Detection limits for the various yeast species have been reported elsewhere (Larpin et al. 2006).

Debaryomyces hansenii. Primers were designed for *Deb. hansenii* var. *hansenii* (GDH1 and GDH2) based on the F01pro sequence (accession number AJ245420) identified by Davila et al. (2001). When used under the PCR conditions as described in Table 2, these primers gave no cross reactions with the closest related species (*Deb. marama* and *Deb. nepalensis*) or the closely related variety *Deb. hansenii* var. *fabryii*. Furthermore, no amplification occurred with various yeast species commonly found in smear cheeses, in accordance with the results of Corredor et al. (2000) for the F01pro probe. A single band of approximately 200 bp was observed for both the *Deb. hansenii* reference strain CBS 767^T and the commercial strain, Scom3.

No reproducible DNA amplification was observed with CBS 766 (Table 3). Sequencing confirmed that CBS 766 belonged to the species *Deb. hansenii* (26S rDNA sequence 100% identical to that of strain CBS 767^T) but it was not possible to determine whether this strain belonged to variety *hansenii* or *fabryii* (11 and 12 mismatches in 602 bp for the partial *ACT1* gene sequences for actin of *Deb. hansenii* var *hansenii* CBS 767^T (accession number AJ 508505) and *Deb. hansenii* var *fabryii* CBS 789^T (accession number AJ 508504), respectively). Many phenotypic studies have shown that *Deb. hansenii* is a highly heterogeneous species (Seiler & Busse, 1990; Nakase et al. 1998; Petersen et al. 2002). Petersen & Jespersen (2004) and Petersen et al. (2001) showed that this species is highly diverse genetically. Petersen et al. (2001) showed, in their investigation of the mtDNA restriction profiles of various *Deb. hansenii* strains, that CBS 766 was readily differentiated from the other strains of this species. The relationship of CBS 766 to *Deb. hansenii* var. *hansenii* is thus called to question. This may also account for the lack of amplification with primers GDH1 and GDH2, providing further evidence for the specificity of our primers.

Geotrichum candidum. A few *G. candidum* sequences were available in databases. The *CGL* gene, encoding cystathionine-γ-lyase (accession number AJ511875), was

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Table 3. PCR products obtained with each pair of primers

Primers tested: Amplicon expected size (bp): Yeast species		OCL1/OCL2	GDH1/GDH2	CGLFOC/CGLROC	KLOC1/KLOC2	CKFF35/CKFR104	YLEXG1/YLEXG2	YLLF35/YLLR59
		246	200	200	245	694	256	245
Candida catenulata	CBS 565^T	–	–	–	–	–	–	–
	CBS 6174	–	–	–	–	–	–	–
Clavispora lusitaniae	CBS 4413^T	+	–	–	–	–	–	–
	CBS 5299	+	–	–	–	–	–	–
Cryptococcus albidus var. *albidus*	CBS 142	–	–	–	–	–	–	–
Debaryomyces hansenii var. *hansenii*	CBS 766	–	–	–	–	–	–	–
	CBS 767^T	–	+	–	–	–	–	–
	Scom3	–	+	–	–	–	–	–
Debaryomyces hansenii var. *fabryii**	CLIB 606	nd	–	nd	nd	nd	nd	nd
*Debaryomyces marama**	CBS 1958^T	nd	–	nd	nd	nd	nd	nd
*Debaryomyces nepalensis**	CBS 5921^T	nd	–	nd	nd	nd	nd	nd
*Galactomyces citri-aurantii**	CBS 175·89^T	nd	nd	+	nd	nd	nd	nd
*Galactomyces geotrichum**	CBS 772·71^T	nd	nd	–	nd	nd	nd	nd
*Galactomyces reesii**	CBS 179·60^T	nd	nd	–	nd	nd	nd	nd
Geotrichum candidum	ATCC 204307	–	–	+	–	–	–	–
	UCMA 96	–	–	+	–	–	–	–
	CBS 110·12	–	–	+	–	–	–	–
	Scom1	–	–	+	–	–	–	–
	Scom2	–	–	+	–	–	–	–
*Geotrichum europaeum**	CBS 866·68^T	nd	nd	–	nd	nd	nd	nd
*Geotrichum pseudocandidum**	CBS 626·83^T	nd	nd	–	nd	nd	nd	nd
Issatchenkia occidentalis	WSYC 312^T	–	–	–	–	–	–	–
	WSYC 328	–	–	–	–	–	–	–
*Kluyveromyces dobzhanskii**	CBS 2104^T	nd	nd	nd	–	nd	nd	nd
Kluyveromyces lactis	CBS 683^T	–	–	–	+	–	–	–
var. *lactis*	CBS 739	–	–	–	+	–	–	–
	CBS 1067	–	–	–	+	–	–	–
	Scom4	–	–	–	+	–	–	–
	Scom5	–	–	–	+	–	–	–
Kluyveromyces marxianus	CBS 712^T	–	–	–	+	+	–	–
	CBS 1557	–	–	–	+	+	–	–
	CBS 8415	–	–	–	+	+	–	–
	CLIB 396	–	–	–	+	+	–	–
	CLIB 527	–	–	–	+	+	–	–
*Kluyveromyces wickerhamii**	CBS 2745^T	nd	nd	nd	–	nd	nd	nd
Pichia anomala	CBS 5759	–	–	–	–	–	–	–
Pichia fermentans	CBS 187	–	–	–	–	–	–	–
Pichia membranifaciens	CBS 107^T	–	–	–	–	–	–	–
Rhodotorula mucilaginosa var. *mucilaginosa*	CBS 316	–	–	–	–	–	–	–
Saccharomyces cerevisiae	CBS 1171^T	–	–	–	–	–	–	–
Torulaspora delbrueckii	CBS 1146	–	–	–	–	–	–	–
Yarrowia lipolytica	CBS 6124·1	–	–	–	–	–	+	+
	CBS 6124·2	–	–	–	–	–	+	+
	CBS 6317	–	–	–	–	–	+	+
	CLIB 791	–	–	–	–	–	+	+
	CLIB 880	–	–	–	–	–	+	+
Zygosaccharomyces rouxii	CBS 732	–	–	–	–	–	–	–
Zygosaccharomyces cidri	CBS 4575^T	–	–	–	–	–	–	–

*,tested as taxonomically related species; +, visible band of the expected size; –, no visible band; nd, not determined

used for the design of a pair of specific primers, CGLFOC/CGLROC. Cystathionine-γ-lyase is involved in the α-γ-elimination of cystathionine and methionine, leading to the production of methanethiol, a sulphur compound that contributes to the aroma of soft cheeses. These primers gave no cross reactions with the most closely related species described by de Hoog & Smith (2004): *Galactomyces geotrichum*, *Gal. reesii*, *G. europaeum* and *G. pseudocandidum*. However, a positive reaction was observed with *Galactomyces citri-aurantii*. No amplification occurred with various yeast species commonly found in smear cheeses. Although cystathionine-γ-lyase has also been isolated from *Sac. cerevisiae* (Ono et al. 1992), no DNA amplification was observed with these primers and the type strain of this species (Table 3). A ~200 bp PCR product was obtained with CGLFOC and CGLROC for *G. candidum* ATCC 204307, UCMA 96, CBS 110·12, Scom1 and Scom2 and for *Gal. citri-aurantii* CBS 175·89 (Table 3).

The taxonomy of the genus of filamentous yeast-like fungi, *Geotrichum* Link is complex. In a review of the taxonomy of this fungus (de Hoog et al., 1998), 11 species were classified within this genus. The species *Gal. citri-aurantii/G. citri-aurantii* were separated in 1988 (Butler et al. 1988) from *Gal. geotrichum/G. candidum*, based on the ability to cause sour rot on citrus. The amplification of both *Gal. citri-aurantii* and *G. candidum* is consistent with the very close link between these two taxa. As the two species have very different substrates, the primers CGLFOC/CGLROC can be validated for *in situ* analysis. Until recently, *G. candidum* was considered to be the anamorph of *Gal. geotrichum*. In 2004, de Hoog & Smith (2004) proposed that *Gal. candidus* should be considered the new teleomorph of *G. candidum*. The lack of amplification with the reference strain of *Gal. geotrichum* (CBS 772·71) (Table 3) confirms that the primers used are selective.

Kluyveromyces lactis *and* K. marxianus. A pair of primers (KLOC1/KLOC2) targeting the *lac*4 gene (accession number M84410), encoding β-galactosidase, was designed for *K. lactis*, because this intracellular enzyme has not been found in other yeast species commonly isolated during cheese ripening. With our primers, no cross reactions occurred with the most closely related species, *K. dobzhanskii* and *K. wickerhamii*. However, a positive reaction was observed with *K. marxianus*. No reaction was observed for the other yeast species commonly found in smear cheeses.

The taxonomy of the genus *Kluyveromyces* has been revised by Kurtzman (2003). This genus comprises *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. marxianus*, *K. nonfermentans* and *K. wickerhamii*, which have a number of properties in common. The phenetic heterogeneity of *K. marxianus* has been reviewed (Kurtzman & Fell, 1998) and accounts for difficulties in the differentiation of this species from other related species, especially *K. lactis*, which was previously considered to be a variety of *K. marxianus* (Kurtzman & Fell, 1998) and is found on the same substrates. The similarity between these two species may account for the amplification observed with primers KLOC1/KLOC2 for both *K. lactis* and *K. marxianus*. A PCR product of ~245 bp in size was obtained from the ten *K. lactis* and *K. marxianus* strains tested. *K. lactis* species is known to use lactose as a carbon source, whereas lactose utilisation is variable among *K. marxianus* strains (Barnett et al. 2000). For the two *K. marxianus* strains tested, one assimilated lactose and the other did not. Thus, at least part of the *lac*4 gene is present within the genome of *K. marxianus*, as DNA amplification occurred independently of lactose use. However, it is important to distinguish between *K. lactis* and *K. marxianus* if we are to understand cheese ecology. A specific amplification was obtained (Table 3) for the *K. marxianus* strains, using the primers designed by Kanbe et al. (2002), targeting the topoisomerase II gene (accession number AB049138). We were able to distinguish between *K. lactis* and *K. marxianus* (Table 3), using a combination of our primers and those described by Kanbe et al. (2002).

Yarrowia lipolytica. A pair of PCR primers (YLEXG1/YLEXG2, Table 2) was designed from the sequence of the *exg*1 gene (accession number Z46972), which encodes an exo-1, 3-β-glucanase from *Yar. Lipolytica*, according to Esteban et al. (1999). Specific DNA amplification (256 bp) was observed with these primers, for the five *Yar. lipolytica* reference strains (Table 3). With primers targeting the topoisomerase II of *Yar. lipolytica*, a specific amplicon of 245 bp (Table 3) was observed, as described by Kanbe et al. (2002). Whatever the primer set used, no amplification was observed with the other yeasts (Table 3). The genus *Yarrowia* includes only one species, so no other representatives were tested.

Clavispora lusitaniae. The gene *URA3* (accession number AF450297) (François et al. 2004), encoding orotidine-5′-phosphate decarboxylase, was used as a target sequence for *Clav. lusitaniae*. With the OCL1 and OCL2 primers, a 246 bp PCR product was obtained for the two strains of *Clav. lusitaniae*, CBS 4413^T and CBS 5299. No cross reactions occurred with the other species tested (Table 3). The genus *Clavispora* includes only one species, so no other representatives were tested.

Validation of PCR primers in situ *in Livarot cheese*

The Livarot samples tested in this study were previously investigated with a conventional method. Four hundreds and fifty two yeast isolates were identified from the surface of the cheese to species level; Table 4 shows the distribution of these species during ripening of the cheeses in the three batches (Larpin et al. 2004).

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Table 4. Overview of previous identification and distribution of 452 yeast isolates (Larpin *et al.*, 2004) obtained from Livarot smears in this study

	Dairy A			Dairy B			Dairy C		
	J+2†	J+24	J+60	J+3	J+17	J+60	J+3	J+21	J+58
Debaryomyces hansenii	12	14	3	nd	nd	nd	21	3	3
Geotrichum candidum	23	36	40	30	42	34	6	42	28
Kluyveromyces lactis	10	nd	nd	5	nd	nd	23	nd	nd
Yarrowia lipolytica	2	nd	5	nd	1	18	nd	1	10
Clavispora lusitaniae	nd	nd	nd	nd	nd	nd	nd	nd	nd
Other yeast species	3	nd	2	13	7	3	nd	3	9

nd: not detected

†J+2 and J+3 correspond to the day before salting, J+17, J+21 and J+24 correspond to the day before packaging, J+58 and J+60 correspond to the end of ripening

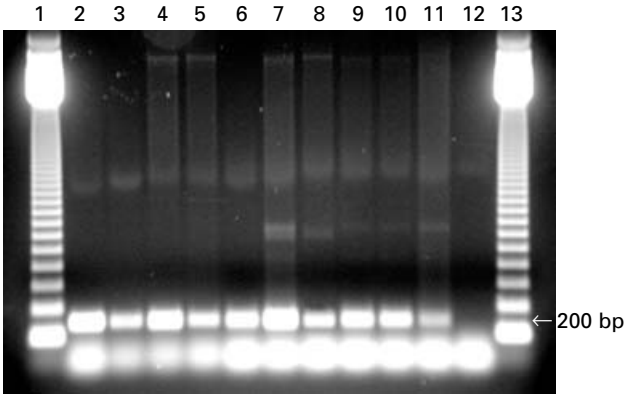

Fig. 1. *In situ* detection of *Geotrichum candidum* in Livarot smears, using the CGLFOC/CGLROC primers. Lanes 1 and 13: molecular weight marker; lane 2: *G. candidum* CBS 110·12; lane 3–5: Livarot smear from dairy A, lane 3: 2 d into ripening; lane 4: 24 d into ripening; lane 5: 60 d into ripening; lane 6–8: Livarot smear from dairy B, lane 6: 3 d into ripening; lane 7: 17 d into ripening; lane 8: B 60 d into ripening; lane 9–11 Livarot smear from dairy C, lane 9: 3 d into ripening; lane 10: C 21 days into ripening; lane 11: C 58 d into ripening; lane 12: negative control.

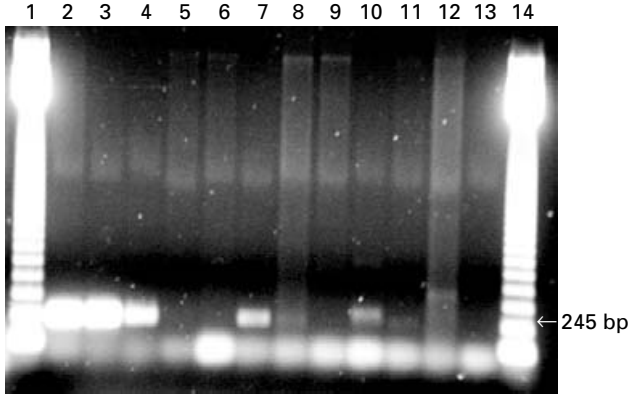

Fig. 2. *In situ* detection of *Kluyveromyces lactis* in Livarot smears, using the KLOC1/KLOC2 primers. Lanes 1 and 14: molecular weight marker; lane 2: *K. lactis* CBS 683; lane 3: *K. marxianus* CBS 1557; lane 4–6: Livarot smear from dairy A, lane 4: 2 d into ripening; lane 5: 24 d into ripening; lane 6: 60 d into ripening; lane 7–9: Livarot smear from dairy B, lane 7: 3 d into ripening; lane 8: 17 d into ripening; lane 9: B 60 d into ripening; lane 10–12 Livarot smear from dairy C, lane 10: 3 d into ripening; lane 11: C 21 days into ripening; lane 12: C 58 d into ripening; lane 13: negative control.

G. candidum was detected in the crude DNA extracts of all smear samples (Fig. 1), confirming the results obtained with a conventional approach (Table 4). The PCR approach confirmed the presence of *K. lactis* in all three batches, at the early stage of ripening. This species was not detected by PCR in the middle and late stages of ripening (Fig. 2). For *Deb. hansenii* and *Clav. lusitaniae,* the results obtained with our primer sets (data not shown) also confirmed those obtained with a culture-based approach (Table 4). *Yar. lipolytica* was detected *in situ* with the YLLF35 and YLLR59 primers (Kanbe et al. 2002), in all three batches at the end of ripening (data not shown). With the conventional approach (Table 4), this species was also detected primarily at the end of ripening in Livarot cheese. Conversely, we were unable to detect this species (data not shown) with the YLEXG1 and YLEXG2 primers. Thus, for the same DNA extracts, primers targeting the topoisomerase II gene seem to give more sensitive detection of *Yar. lipolytica* in the complex matrix than primers targeting the exo-1, 3-β-glucanase.

In conclusion, primer set specificity was validated to the species or subspecies level for *Clav. lusitaniae, Deb. hansenii* var *hansenii* and *Yar. lipolytica*. The primers designed for *G. candidum*/*Gal. citri-aurantii* may be considered species-specific, as these two yeasts have different substrates. *K. lactis* and *K. marxianus* can be differentiated, by coupling our primer set to that developed by Kanbe et al. (2002). *In situ*, single amplicons of the same size as obtained from the relevant pure culture were detected, validating these primers for the detection of target yeasts in mixed populations in Livarot smear. The primers described here have thus potential for PCR studies applied to cheese.

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It should also be possible to use some of these primers with other substrates.

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