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Original article

Important genetic diversity revealed by inter-LTR PCR fingerprinting of *Kluyveromyces marxianus* and *Debaryomyces hansenii* strains from French traditional cheeses

Danièle SOHIER[1], Anne-Sophie LE DIZES[1], Dominique THUAULT[1], Cécile NEUVEGLISE[2], Emmanuel COTON[3], Serge CASAREGOLA[2]*

[1] ADRIA Développement, Z.A. de Creac'h Gwen, 29196 Quimper Cedex, France
[2] CIRM-Levures, Microbiologie et Génétique Moléculaire, INRA, CNRS, AgroParisTech, 78850 Thiverval-Grignon, France
[3] ADRIA Normandie, Boulevard du 13 juin 1944, 14310 Villers-Bocage, France

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Abstract – The genetic diversity of two major yeast species found in cheese, *Debaryomyces hansenii* and *Kluyveromyces marxianus*, was analyzed within the yeast flora in French traditional cheesemaking. Based on the amplification of sequences separating long terminal repeat (LTR) retrotransposon sequences, a molecular typing method was developed for *D. hansenii* and *K. marxianus*. This method was applied to a total of 56 *D. hansenii* strains and 61 *K. marxianus* strains, mostly isolated during fermentation and ripening of traditional cheese from Normandy and Haute-Savoie (French Alps) regions. A total of 32 and 43 robust profiles were obtained for *D. hansenii* and *K. marxianus*, respectively. Cluster analysis confirmed the large genetic diversity already shown for *D. hansenii* and revealed an even larger diversity for *K. marxianus*. After its use with *Saccharomyces cerevisiae*, the inter-LTR PCR proved to be efficient to discriminate between strains of the two species, *D. hansenii* and *K. marxianus*, isolated from the same ecological niches, confirming the high intra-specific variability of species found in cheese. This strain typing could not correlate the analyzed strains with their origin, would it be the cheese type, the cheese-making facility or the cheese batch, showing a high discrimination power. The method described here will provide a fast and reliable tool for the biodiversity study of these two major cheese yeasts.

yeast / *Debaryomyces hansenii* / *Kluyveromyces marxianus* / cheese / LTR fingerprinting

摘要 – 利用内 LTR-PCR 指纹分型方法揭示源于法国传统干酪的 *Kluyveromyces marxianus* 和 *Debaryomyces hansenii* 菌株的重要遗传多态性。在干酪加工过程的酵母菌群中，分析了源于法国传统干酪的两种重要的酵母菌 *Debaryomyces hansenii* 和 *Kluyveromyces marxianus* 的遗传多态性。基于扩增和分离长末端重复序列反转录转座子（LTRs）序列，建立了一种 *D. hansenii* 和 *K. marxianus* 的分子分型方法。该方法应用于 56 株 *D. hansenii* 和 61 株 *K. marxianus* 菌株，这些菌株大多数分离自 Normandy 和 Haute-Savoie（法国高山）地区处于发酵和成熟阶段的传统干酪。获得 *D. hansenii* 32 种区带和 *K. marxianus* 43 种区带。聚类分析证实 *D. hansenii* 具有高遗传多样性，同时揭示了 *K. marxianus* 具有更高的遗传多样性。继内 LTR-PCR 分型方法应用于 *Saccharomyces cerevisiae* 遗传多态性分析

*Corresponding author (通讯作者): serge.casaregola@grignon.inra.fr

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后，将该方法应用于分离自相同生境且高度种内变异菌株 *D. hansenii* 和 *K. marxianus* 的遗传多态性研究，该方法能有效对 *D. hansenii* 和 *K. marxianus* 进行分型。这种菌株分型方法与所研究菌株的起源、干酪类型、干酪制作设备以及干酪批次无相关性，显示了高度区分能力。该方法为干酪中的两种主要酵母菌 *D. hansenii* 和 *K. marxianus* 的遗传多态性分析提供了一个快速可靠的工具。

yeasts / *Debaryomyces hansenii* / *Kluyveromyces marxianus* / 干酪 / LTR 指纹分型

Résumé – Une diversité génétique importante révélée par les empreintes de PCR inter-LTR de souches de *Debaryomyces hansenii* et *Kluyveromyces marxianus* isolées de fromages traditionnels français. La diversité génétique de deux levures majeures des fromages, *Debaryomyces hansenii* et *Kluyveromyces marxianus*, a été analysée dans la flore-levures de fromages français traditionnels. Basée sur l'amplification des séquences qui séparent les « *Long Terminal Repeats* » des rétrotransposons (LTR), une méthode de typage moléculaire a été développée pour *D. hansenii* et *K. marxianus*. La méthode a été appliquée à 56 souches de *D. hansenii* et 61 souches de *K. marxianus*, pour la plupart isolées pendant l'affinage de fromages traditionnels de Normandie et de Haute-Savoie. Un total de 32 et 43 profils robustes a été obtenu pour *D. hansenii* et *K. marxianus*, respectivement. Une analyse hiérarchique a confirmé la grande diversité génétique déjà observée pour *D. hansenii* et a révélé une grande diversité chez *K. marxianus*. Apres son utilisation chez *Saccharomyces cerevisiae*, la PCR inter-LTR s'est montrée très efficace pour discriminer entre isolats de *D. hansenii* et *K. marxianus* isolés des mêmes niches écologiques, confirmant ainsi la grande variabilité intra-spécifique des espèces présentes dans le fromage. Le typage de ces souches n'a pu être corrélé à la provenance des souches, que ce soit avec le type de fromage, la fromagerie ou le lot, indiquant un grand pouvoir de discrimination lors de ce typage. La méthode développée ici apporte un outil rapide et robuste pour étudier la biodiversité de ces deux espèces.

levures / *Debaryomyces hansenii* / *Kluyveromyces marxianus* / fromages / empreintes LTR

1. INTRODUCTION

Large-scale industrial processes relying on the use of selected starter cultures led to a low variability in the dairy microflora. Moreover, sanitation processes, such as milk pasteurization, which has a fundamental role in the control of pathogenic bacteria, also resulted in a significant reduction of the natural bacterial populations involved in naturally fermented and ripened cheese production. However, some traditional dairy products are still fermented and ripened using unselected starters and, therefore, correspond to a wide range of products with different flavors, texture and microbiological qualities [6, 29]. Moreover, the importance of raw milk as a source of strains harboring genetic diversity has been outlined in traditional cheese produced without pasteurization [9]. Finally, the existence of area typical wild strains would account for the recognized area particularity, allowing cheese labeling according to PDO (protected designation of origin). Thus, these products have been proposed as sources for new strains of interest for use in food fermentation and ripening.

Several species, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Yarrowia lipolytica*, mainly constitute the yeast flora in dairy products and cheeses [12] where they contribute to the development of texture and flavor during the ripening process [27]. The need for new strains in the dairy industry and for a deeper knowledge of the natural microflora present in typical dairy products

led to the study of the biodiversity of the most common yeast species involved in traditional cheese ripening.

To assess this biodiversity, several molecular approaches were used. For years, *Saccharomyces cerevisiae* strains were routinely characterized with RFLP (restriction fragment length polymorphism) analysis of chromosomal or mitochondrial DNA or electrophoretic karyotyping [1, 3, 32]. Techniques based on the PCR amplification of known sequences rather than repeated sequences have proved to be faster and just as efficient as RFLP analysis [14]. Yet, molecular methods for typing most non-conventional yeast species lack, mainly because of the paucity of available sequences. Repeated sequences within microsatellites [13, 28] or tRNA [23] were used as primers to generate strain-specific patterns.

Sequencing data on some yeast species that contribute to cheesemaking such as *D. hansenii* var. *hansenii* [17] and *K. marxianus* var. *marxianus* [18], referred to further on as *D. hansenii* and *K. marxianus,* respectively, are now available. These sequence data were used to detect and describe retrotransposons [22]. Retrotransposons are mobile elements responsible for genomic polymorphism. These elements transpose via mRNA intermediates [4]. In yeasts, the large majority of retrotransposons consist of long terminal repeat (LTR) retrotransposons, the so-called Ty in *S. cerevisiae*. The most common LTR retrotransposon of *D. hansenii* is Tdh5, a member of the Ty5 family. In *K. marxianus*, only one LTR retrotransposon has been identified, Tkm1, a member of the Ty1/*copia* family [22]. Excision of the retrotransposon through a homologous recombination at the bordering LTRs leaves an isolated, or so called, solo LTR. Solo LTRs outnumber the full-length elements in the genome. These repeated sequences were successfully used for the typing of *S. cerevisiae* strains [16, 21] and of other organisms [15], through the PCR amplification of implicated sequences. Estimation of the number of LTR retrotransposons in *D. hansenii* and *K. marxianus* [11, 22] indicated that an inter-LTR PCR fingerprinting method could be developed for these species. In this work, sequences of retrotransposons present in *K. marxianus* and *D. hansenii* [22] were used to develop a method based on the PCR amplification of sequences separating LTRs in the genome, using oligonucleotide primers designed within these LTRs. The developed inter-LTR PCR method was used to carry out genomic fingerprinting of strains isolated from traditional cheeses.

2. MATERIALS AND METHODS

2.1. Yeast strains and growth conditions

Strains were obtained from the *Centre International de Ressources Microbiennes* (CIRM-Levures, http://www.inra.fr/cirmlevures) and are listed in Table I. Most of the strains were isolated during the ripening of different types of traditional French cheeses from different regions: Camembert from Normandy, Chevrotin des Aravis from the Alps (Haute-Savoie) and Saint-Nectaire from Massif-Central [2, 10, 20]. Few strains were from Spanish Roncal cheese [28]. Strains were cultured at 28 °C overnight with agitation in liquid YPD medium (glucose 1% – Sigma Aldrich, St. Quentin, France; Bacto yeast extract 1% and Bacto peptone 1% – BD, Le Pont de Claix, France).

2.2. Oligonucleotidic primers

LTR sequences were aligned using the ClustalX program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and primers were designed in the conserved regions, i.e. oligonucleotides DH8 and DH9 from the *D. hansenii* LTR retrotransposon Tdh5 (Accession No. AJ439552) and

Table IA. List of the 56 *D. hansenii* strains used in this study.

Biotope of origin	Geographical area of sampling	Strains
Camembert (raw milk)	Normandy, France	CLIB 607, 608, 609, 656, 684, 685, 686, 702
Chevrotin des Aravis (raw milk goat's cheese)	Haute-Savoie, France	CLIB 594, 616, 617, 618, 657, 659, 661, 662, 663, 664, 665, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 689, 690, 691, 692, 693, 695, 696, 697, 698, 701
Saint-Nectaire cheese	Massif Central, France	CLIB 622, 623, 626, 627, 628
Roncal cheese	Navarra, Spain	CLIB 236, 238, 239
Cheese	France	CLIB 249
Cheese	Russia	CLIB 541
Beer	Denmark	CLIB 197^T
N/A	Japan	CLIB 195
Foodstuff	France	CLIB 542
Human	Hungary	CLIB 545
Environment	USA	CLIB 539

N/A, not available.

Table IB. List of the 61 *K. marxianus* strains used in this study.

Biotope of origin	Geographical area of sampling	Strains
Camembert (raw milk)	Alençon, Normandy, France	CLIB 720, 735, 736, 755, 756, 757, 758, 759, 760, 761, 765, 766, 767, 768, 769, 770, 771, 772, 773, 775, 776 and TL 91, 27, 38, 46a, 48, 73, 89, 90, 99, 100, 121, 134, 136, 138, 166, 201, 202
Camembert (raw milk)	Elsewhere, Normandy, France	CLIB 777, 780, 783, 784, 785, 787, 788 and TL 220, 221, 225, 240, 265, 267, 269, 270, 277, 285, 287, 291, 294, 295, 297, 298

oligonucleotides KM1 and KM2 from the *K. marxianus* LTR retrotransposon Tkm1 (Accession No. AJ439546). Primers used in this study are described in Table II.

2.3. Fingerprinting conditions

Genomic DNA was extracted using the Dneasy Plant Kit (Qiagen, Les Ulis, France) and quantified by fluorimetry with PicoGreen (Invitrogen, Cergy-Pontoise, France) following the manufacturer's instructions. Primers were synthesized and purified by HPLC (Proligo, Évry, France). Amplification reactions were performed in a 50-µL volume containing the total genomic DNA quantity required: 1 $\mu mol \cdot L^{-1}$ of each primer, 500 $\mu mol \cdot L^{-1}$ of dNTP, 1.25 U of *Taq* DNA polymerase and 5 µL of 10 X PCR buffer (Q-Biogen, Illkirch, France). Total genomic DNA quantities corresponded to 20 ± 5 ng for *D. hansenii* strains and to 45 ± 5 ng for *K. marxianus* strains. PCR conditions using the primer

Table II. Oligonucleotidic primers used in this study and the associated Simpson's diversity index.

Species	Name	Sequence	Simpson's diversity index (%)
K. marxianus	KM1	5′-GTTGGTATAATATCTGG-3′	98.5
K. marxianus	KM2	5′-TTCTAAGGTCCCTACTAC-3′	
D. hansenii	DH8	5′-CTCAATTTATTCTGACTTCGC-3′	96.5
D. hansenii	DH9	5′-GATTGTTGTTGAAGCTATCATTGG-3′	

pair DH8/DH9 were as follows: 94 °C for 4 min, 4 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 44 °C for 30 s and 72 °C for 2 min with a final extension completed at 72 °C for 4 min. PCR conditions with the primer pair KM1/KM2 were as follows: 94 °C for 4 min, four cycles of 94 °C for 30 s, 38 °C for 30 s and 72 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 41 °C for 30 s and 72 °C for 2 min with a final extension at 72 °C for 4 min. PCR amplification was performed with an I-Cycler thermocycler (BIORAD, Les Ulis, France). A total of 35 μL of each reaction mixture was loaded on a 2% agarose gel (wt/vol) (Q-Biogen, France) with 1 X TBE electrophoresis buffer (Q-Biogen, Illkirch, France) containing 0.2 $mg \cdot mL^{-1}$ ethidium bromide and run at 120 V in a SUB-CELL GT electrophoresis system (BIORAD, Les Ulis, France) for 3 h.

2.4. Data analysis

All PCR amplification profiles were analyzed with the Bionumerics program (Applied Maths, Ghent, Belgium) [31]. The performed analysis included (i) normalization of electrophoresis patterns to compensate for minor differences in migration, (ii) subtraction of a non-linear background from the patterns and comparison based on the rolling disk principle, (iii) calculation of Pearson's coefficient for similarity between patterns and (iv) clustering of the patterns using the unweighted pair group method with arithmetic averages [30]. The inter-LTR PCR discriminatory level was evaluated using Simpson's diversity index D ($D = 1 - 1/N\ (N-1)\ \Sigma x_j\ (x_j - 1)$), where N is the number of strains and x_j is the number of strains per group [31].

3. RESULTS

3.1. Inter-LTR PCR amplification discriminating performances

Oligonucleotidic primers were designed to match conserved regions of the LTRs aligned with ClustalX (data not shown) and used to PCR amplify genomic DNA from various strains of *D. hansenii* and *K. marxianus* species. Different primer pairs were tested; those leading to the most discriminating results were selected and used throughout this study (Tab. II). To ensure repeatability of the PCR inter-LTR fingerprinting method, different conditions of amplification were tested with the genomic DNA of three strains in independent experiments as described by Gente et al. [13] (data not shown).

Fingerprinting profiles were then generated for 56 *D. hansenii* strains and 61 *K. marxianus* strains mainly isolated from various traditional French cheeses [2, 20]. The genetic diversity was then assessed by examining the clustering of the typing profiles obtained (Figs. 1 and 2). The selected primer pairs ensure a Simpson's diversity index higher than 95% (Tab. II). The amplified

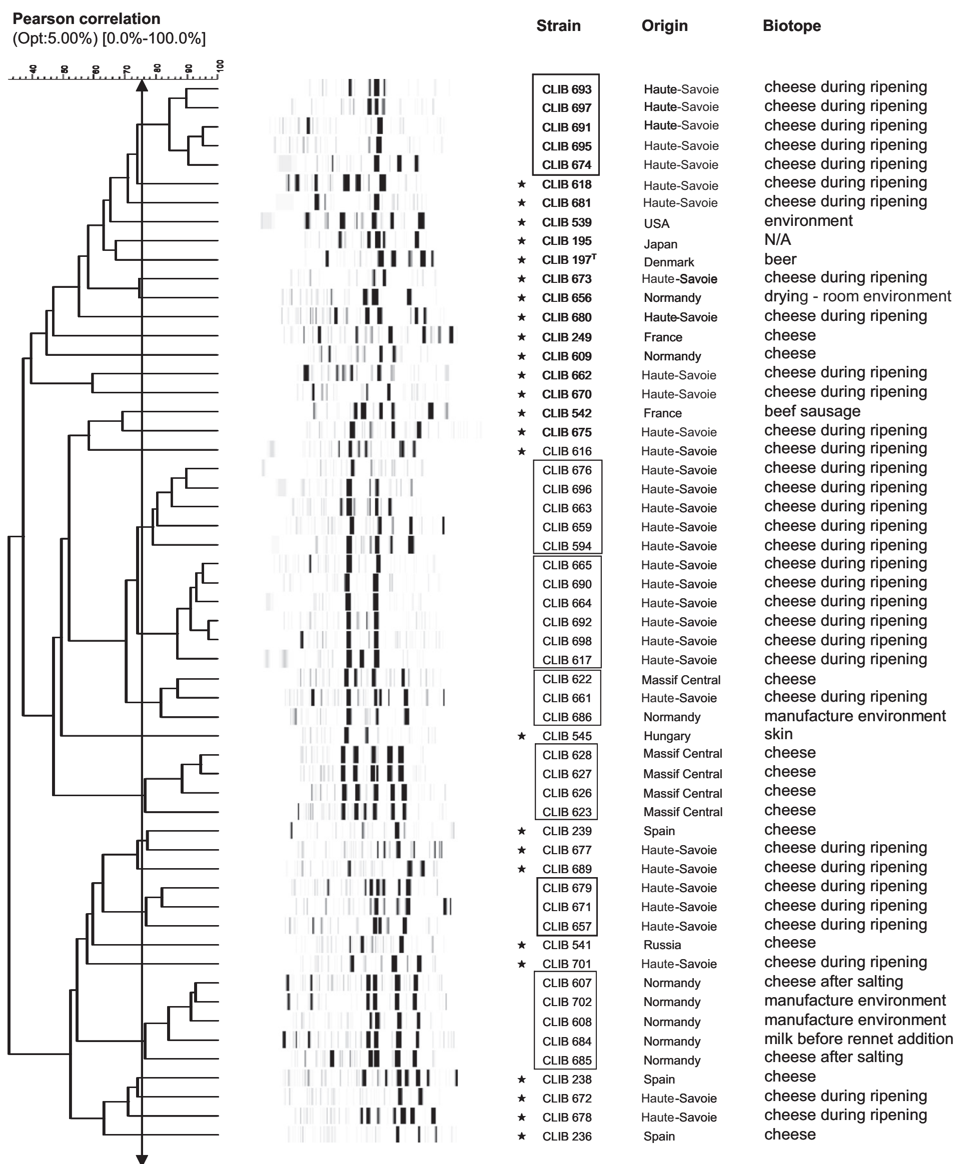

Figure 1. *D. hansenii* strains inter-LTR fingerprinting. Origin and biotope of isolation of the 56 studied *D. hansenii* strains is indicated. Stars indicate strains displaying a unique profile. Strains with similar profiles are boxed. The vertical bar indicates the 75% similarity cut-off. Note that, although the strains CLIB 239 and CLIB 677 display similarity below the 75% threshold, they were not grouped in a cluster as their respective profiles are clearly different. N/A: not available.

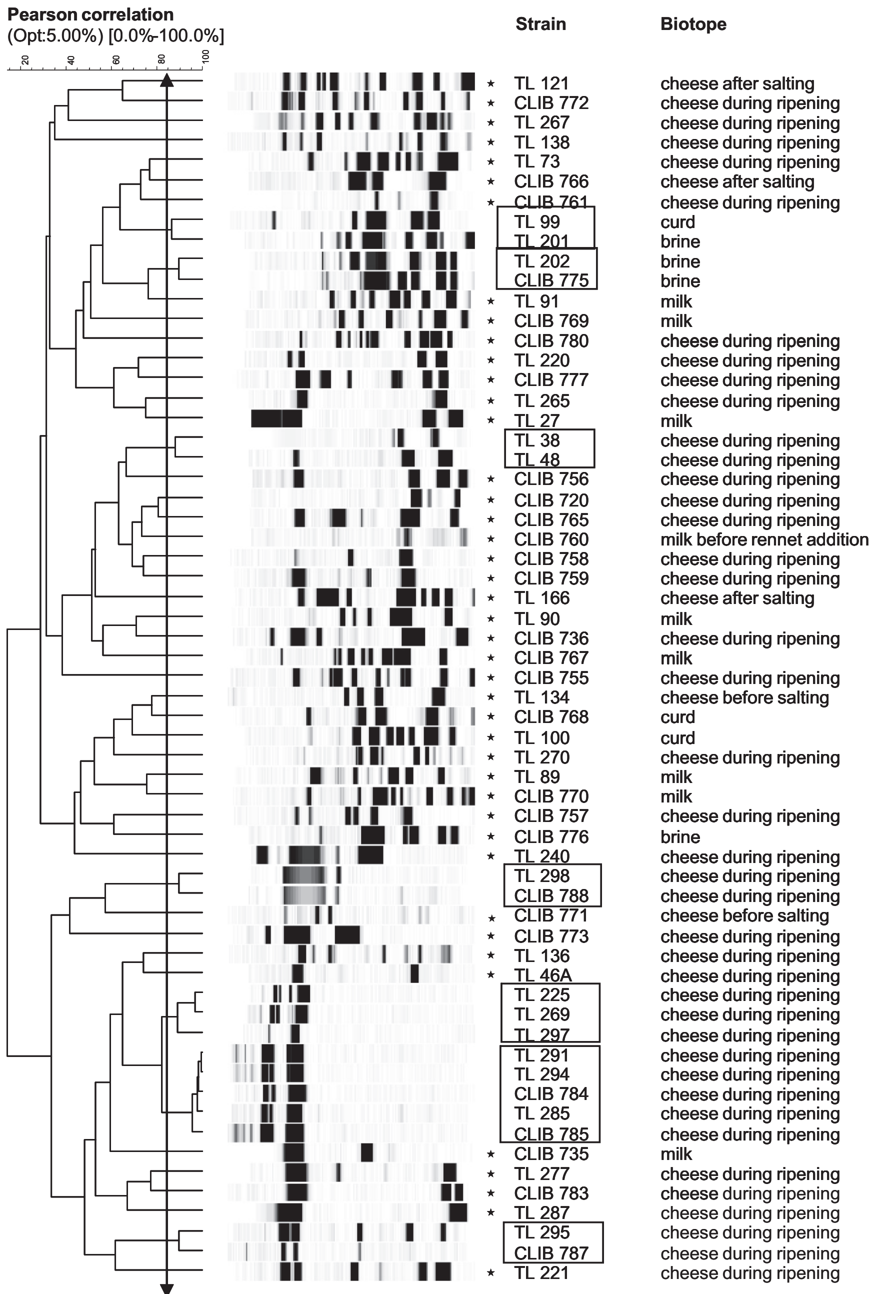

Figure 2. *K. marxianus* strains inter-LTR fingerprinting. Origin and biotope of isolation of the 61 studied *K. marxianus* strains is indicated. Stars indicate strains displaying a unique profile. Strains with similar profiles are boxed. The vertical bar indicates the 85% similarity cut-off.

bands ranged from 400 to 1300 bp for the genomic fingerprints of *D. hansenii* strains and from 300 to 1500 bp for the genomic fingerprints of all *K. marxianus* strains. The patterns of the various strains differed in fragment number, size and intensity.

3.2. Genetic diversity within *D. hansenii* strains in French cheese

For *D. hansenii,* grouping of the profiles led to seven clusters and 25 unique profiles with a similarity coefficient of 75%. Two observations can be made from the obtained inter-LTR profile dendrogram (Fig. 1). A number of strains share very similar profiles such as CLIB 665, CLIB 690, CLIB 664, CLIB 692, CLIB 698 and CLIB 617. These strains were isolated from the surface of Chevrotin des Aravis in the same batch between 17 and 25 days after the start of the ripening process; their classification into the same cluster is therefore not surprising (Figs. 1 and 3). This is also true for the cluster including CLIB 607, CLIB 608, CLIB 684, CLIB 685 and CLIB 702; these strains were isolated from a Camembert at different times during the first steps of the cheese-making process or even in the dairy factory atmosphere (Fig. 1). These sets of strains are thus associated to a dairy factory and a batch.

The second observation is that, for most of the *D. hansenii* strains studied, a wide genetic diversity was observed no matter what geographical region the strains were from, the type of cheese analyzed or the process step. Overall, the 56 inter-LTR profiles obtained corresponded to seven clusters and 25 individual patterns (Fig. 1). This is highlighted in Figure 3 by the clustering of the patterns obtained with 33 strains isolated from the surface of Chevrotin des Aravis between 17 and 25 days after the start of ripening. These strains, isolated from Haute-Savoie, exhibited four clusters and 14 unique patterns. Although some of

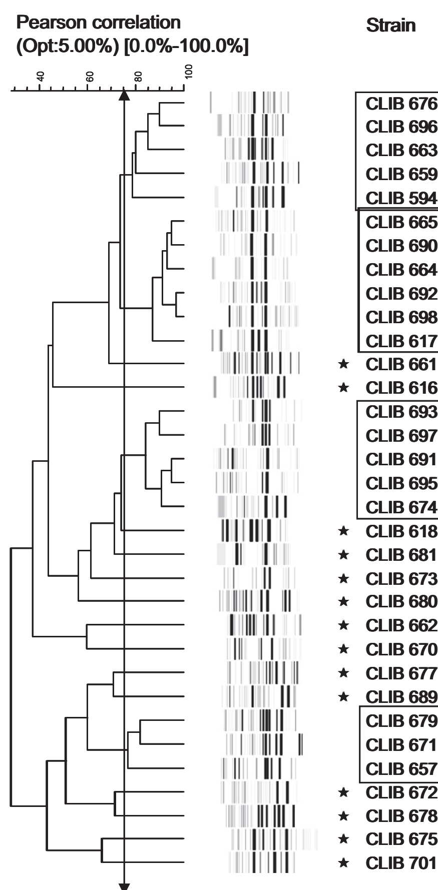

Figure 3. Clustering of the *D. hansenii* strain patterns from the same geographical origin. The inter-LTR fingerprinting profiles of a total of 33 strains originating from the Haute-Savoie area and isolated from cheese during ripening are compared. Stars indicate strains displaying a unique profile. Strains with similar profiles are boxed. The vertical bar indicates the 75% similarity cut-off.

the strains were clearly related if not identical (CLIB 691 and CLIB 695, or the already mentioned clusters CLIB 676, CLIB 696, CLIB 663, CLIB 659, CLIB 594 and CLIB 665, CLIB 690, CLIB 664, CLIB 692, CLIB 698 and CLIB 617), these results showed an important genetic diversity

among the *D. hansenii* strains isolated from the same batch. Furthermore, we found that eight strains isolated from the Alençon area generated a cluster of five strains constituted of CLIB 607, CLIB 608, CLIB 684, CLIB 685 and CLIB 702 and three individual patterns (CLIB 609, CLIB 656 and CLIB 686) (Fig. 1), indicating that strains isolated from the processing environment were genetically closely related to the strains isolated from the cheese of this region.

3.3. Genetic diversity within *K. marxianus* strains in French cheese

Inter-LTR PCR was performed for 61 strains of *K. marxianus* isolated from dairy environment and cheese and camembert from several dairy factories in Normandy [2, 20]. Data analysis revealed an important genetic diversity among the studied strains seven clusters and 43 unique patterns with a similarity coefficient of 85% (Fig. 2). Some strains grouped in clusters of two, exhibiting similar profiles such as the groups TL 298/CLIB 788 and TL 202/ CLIB 775. Two larger clusters of eight strains (TL 225, TL 269, TL 297, TL 291, TL 294, CLIB 784, TL 285 and CLIB 785), consisted of strains all isolated from the same batch few days after the start of ripening. These *K. marxianus* strains are very likely identical or closely related. However, the overall diversity was really high. This is illustrated in Figure 4 in which clustering of the inter-LTR genetic profiles of 38 strains of the Alençon area showed the existence of three clusters and 32 unique patterns.

As for *D. hansenii*, no correlation was observed between inter-LTR PCR profiles and batch variety, process time, sampling time or place (surface or inside). It has to be noted that we had no example of strains from different origins like geographical localization, batch type or dairy plant sharing a similar profile. Thus, this high specificity displayed by the patterns we have generated indicates that it may be used in the future to associate the strains to the varieties of cheese to ease PDO labeling or to correlate strain technological properties to the type of cheese.

4. DISCUSSION

Although the availability of sequences of retrotransposons and LTRs is a prerequisite, these elements proved to be of real interest for the design of primers dedicated to yeast strain typing in species other than *S. cerevisiae*. In this study, a PCR-based fingerprint method was developed to assess genetic diversity among *D. hansenii* and *K. marxianus* strains, based on the variability of the insertion of the LTR retrotransposons, Tdh5 and Tkm1, respectively.

Previous works on *D. hansenii* have shown that a large genetic variability existed at the chromosomal structure level as monitored by Pulse Field Gel Electrophoresis (PFGE) [7, 24] and at the DNA sequence level [8, 28]. By comparing, for some strains, the typing results of PFGE and inter-LTR PCR methods, we were able to confirm that strains, which were shown to share similar electrophoretic karyotypes in a previous study [7], were closely related using the proposed approach, i.e. CLIB 626, CLIB 627 and CLIB 628 (data not shown). Although transposition monitoring cannot reflect physiological properties of the strains, it can nevertheless indicate that strains are closely related. It is less true for changes in chromosomal structures which are considered to be due to frequent recombination events between repeated sequences leading to reciprocal translocations [5, 26].

As observed for *D. hansenii*, this work showed the extreme diversity among *K. marxianus* strains. This is certainly linked to the high estimated number of transposons in this species [22], but this diversity at the level of transposon distribution must

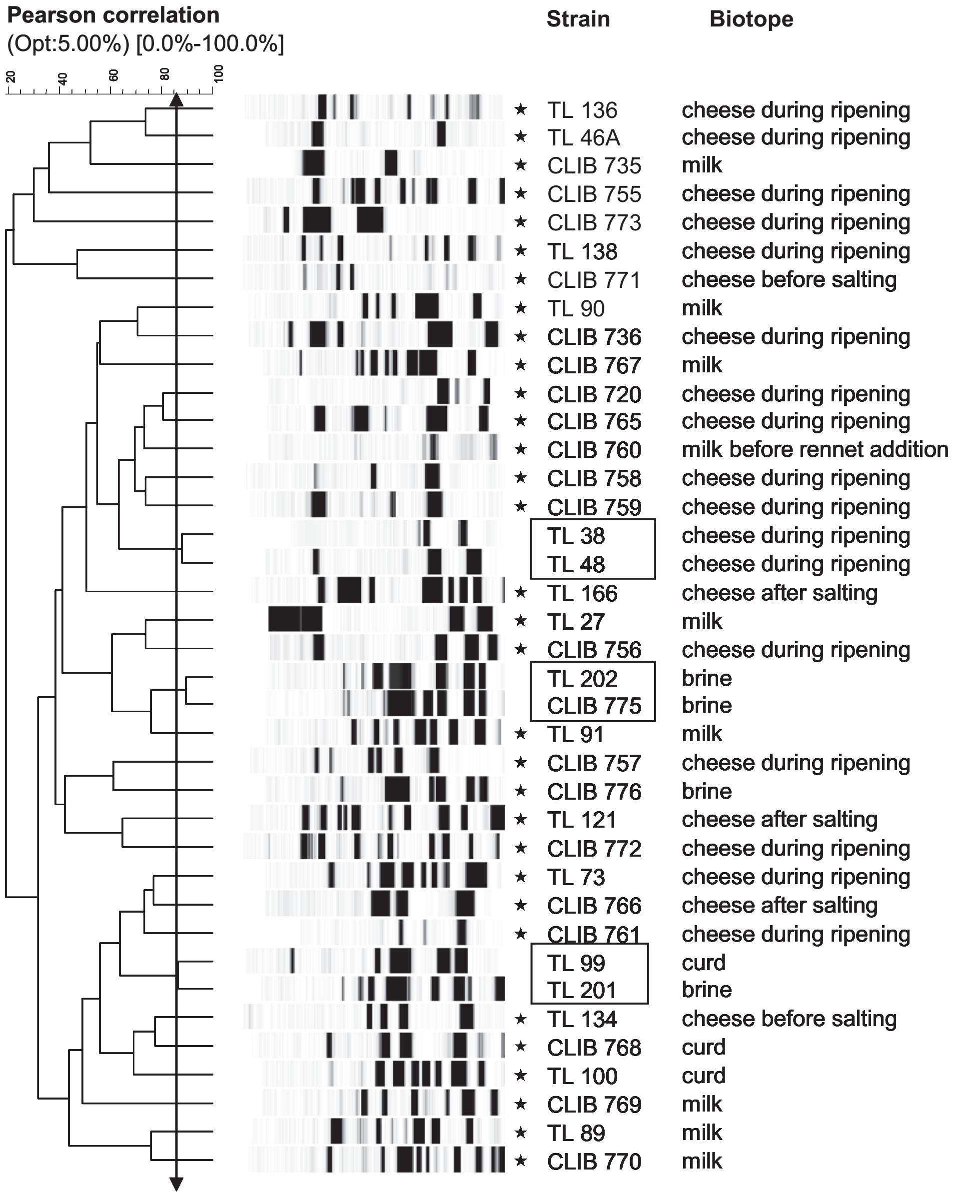

Figure 4. Clustering of the *K. marxianus* strain patterns from the same geographical origin. The inter-LTR fingerprinting profiles of a total of the 38 *K. marxianus* strains originating from the Alençon area are compared. Stars indicate strains displaying a unique profile. Strains with similar profiles are boxed. The vertical bar indicates the 85% similarity cut-off.

clearly reflect intra-specific genetic diversity. This result indicating a probable high transposition activity is interesting, as a very closely related species *K. lactis*, another major yeast in cheese, does not seem to carry any active transposon [11, 22]. This work has to be further carried out to evaluate whether the genetic diversity based on the transposition history of the strains tested and observed in this study is correlated in any way with physiological or technological properties.

The fact that most of the strains of our study were isolated from cheese during the ripening process emphasizes the observed diversity; this is especially true for the *K. marxianus* strains originating from the Normandy Alençon area. A widespread genetic diversity was observed among cheese yeasts isolated from the studied traditional cheese, as previously described for *Y. lipolytica* and *Geotrichum candidum* [19]. The persistence of a high genetic diversity among cheese yeast flora could suggest that traditional cheeses may require the presence of a complex flora for their elaboration.

Although the large majority of the strains displayed a specific profile, we could find a number of groups of two to six strains, sharing a very similar profile. It has to be noted that the strains belonging to these groups were isolated from the same batch or from the same facilities. We found that for *D. hansenii*, strains isolated from the processing environment were genetically closely related to the strains isolated from the cheese of this region. A similar observation was made with strains involved in the processing of a Danish cheese [25]. This type of strains may be prevalent in the dairy factory, as it was found in the atmosphere of the dairy house, in the milk and in the cheese after draining. In agreement, with these observations, a dominant strain was also found during the production of Danish Danbo type of cheese [25]. One can object to this observation that the typing method used in this work, mtDNA RFLP, is not very discriminant (see [28]). A study assessing technological properties of over 20 *K. marxianus* strains from water buffalo mozzarella did not differentiate these strains on the basis of the production of end metabolites such as sulfur dioxide, higher alcohols, ethyl acetate and acetaldehyde [29]. The type of cheese, i.e. of fermentation, may of course be essential with regard to technological properties.

The case of the very close strains of *K. marxianus* TL 202 and CLIB 775 (Fig. 2) is particularly interesting. Both strains were isolated from brine at different moments, suggesting an adaptation of a certain genotype to these environmental conditions. This indicates that the method described here should therefore allow for following a strain during the cheese-making process environment, materials and ingredients.

In conclusion, inter-LTR PCR fingerprinting is easy and rapid to perform and therefore provides a real alternative to more time- and labor-consuming methods (i.e. PFGE) or less discriminating methods (mitochondrial DNA RFLP). In this study, the inter-LTR PCR characterization of *D. hansenii* and *K. marxianus* strains from fermentation and ripening of French cheeses indicates that strains may be specific to traditional cheese type or to an area. These facts are in full agreement with the notions of "terroir" and typicity promoted by the PDO, although further studies are required to evaluate the role of these strains in the cheese typicity and how they can be used by the cheese manufacturers.

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