



Use of PCR-restriction fragment length polymorphism analysis for identification of yeast species isolated from bovine intramammary infection

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ABSTRACT

This study reports a rapid PCR-based technique using a one-enzyme RFLP for discrimination of yeasts isolated from bovine clinical and subclinical mastitis milk samples. We analyzed a total of 1,486 milk samples collected over 1 yr in south Sardinia and northern Italy, and 142 yeast strains were preliminarily grouped based on their cultural morphology and physiological characteristics. Assimilation tests were conducted using the identification kit API ID 32C and APILAB Plus software (bioMérieux, Marcy l'Etoile, France). For PCR-RFLP analysis, the 18S-ITS1-5.8S ribosomal(r) DNA region was amplified and then digested with *Hae*III, and dendrogram analysis of RFLP fragments was carried out. Furthermore, within each of the groups identified by the API or PCR-RFLP methods, the identification of isolates was confirmed by sequencing of the D1/D2 region using an ABI Prism 310 automatic sequencer (Applied Biosystems, Foster City, CA). The combined phenotypic and molecular approach enabled the identification of 17 yeast species belonging to the genera *Candida* (47.9%), *Cryptococcus* (21.1%), *Trichosporon* (19.7%), *Geotrichum* (7.1%), and *Rhodotorula* (4.2%). All *Candida* species were correctly identified by the API test and their identification confirmed by sequencing. All strains identified with the API system as *Geotrichum candidum*, *Cryptococcus uniguttulatus*, and *Rhodotorula glutinis* also produced characteristic restriction patterns and were confirmed as *Galactomyces geotrichum* (a teleomorph of *G. candidum*), *Filobasidium uniguttulatum* (teleomorph of *Crypt. uniguttulatus*), and *R. glutinis*, respectively, by D1/D2 rDNA sequencing. With regard to the genus *Trichosporon*, preliminary identification by API was problematic, whereas the

RFLP technique used in this study gave characteristic restriction profiles for each species. Moreover, sequencing of the D1/D2 region allowed not only successful identification of *Trichosporon gracile* where API could not, but also correct identification of misidentified isolates. In conclusion, the 18S-ITS1-5.8S region appears to be useful in detecting genetic variability among yeast species, which is valuable for taxonomic purposes and for species identification. We have established an RFLP database for yeast species identified in milk samples using the software GelCompar II and the RFLP database constitutes an initial method for veterinary yeast identification.

Key words: yeasts, PCR-RFLP, bovine milk, molecular diagnosis

INTRODUCTION

Mastitis, inflammation of the mammary gland, is a disease that causes enormous losses to the dairy cattle industry, resulting in reductions in the quantity and quality of the milk (Schukken et al., 2009). Bacterial agents are the major cause, but several other groups of microorganisms, including yeasts, fungi, and algae such as *Prototheca*, have become more frequent (Möller et al., 2007; Seker, 2010). Although yeasts are considered opportunistic pathogens, they have been associated with IMI in dairy cattle, commonly related to treatment directed against other pathogens using contaminated syringes, cannulas, or contaminated antibiotic preparations (Spanamberg et al., 2008; Scaccabarozzi et al., 2011). The most frequently isolated organisms among mastitis-causing yeasts are *Candida* species, but other yeast genera such as *Trichosporon*, *Cryptococcus*, and *Geotrichum* have also been isolated from clinical cases with low frequencies (Chahota et al., 2001; Spanamberg et al., 2008). The susceptibilities of different species to antifungal agents may be different; therefore, no clear evidence exists of the effectiveness of this therapy (Segundo Zaragoza et al. 2011).

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Traditionally, yeasts are identified by morphological and physiological criteria (Kurtzman and Fell, 2000), but these methods are strongly influenced by culture conditions and can give uncertain results. One of the most successful methods for yeast identification is PCR-RFLP analysis, based on the differentiation of microorganisms by comparison of the restriction patterns obtained by digestion of a chosen target DNA with restriction endonucleases. Here, we report a rapid PCR-based technique, using a one-enzyme RFLP, for discrimination of yeast strains isolated from bovine milk samples with IMI.

MATERIALS AND METHODS

Sample Collection

A total of 1,486 milk samples from clinical and sub-clinical mastitis were collected over 1 yr (from May 2011 to April 2012) in south Sardinia (822) and northern Italy (664). For yeast isolation, the milk samples were cultured on potato dextrose agar supplemented with chloramphenicol (0.01%; Microbiol, Cagliari, Italy), incubated at 30°C, and observed daily for growth for up to 1 wk.

Identification of Strains

Colonies appearing as yeast-like in morphology were examined by the Dalmau technique (Odds et al., 1997) for microscopic characteristics. A total of 142 (9.5%) yeast strains (98 in south Sardinia and 44 in northern Italy) were preliminarily grouped based on their culture morphology and physiological characteristics. Assimilation tests were conducted using the identification kit API ID 32C and APILAB Plus software (bioMérieux, Marcy l'Etoile, France).

PCR-RFLP Analysis

Genomic DNA was extracted as previously described (Fadda et al., 2010). The primers NS1 (5'-GTAGT-CATATGCTTGTCTC-3') and ITS2 (5'-GCTGC-GTTCTTCATCGATGC-3') were used to amplify most of the 18S gene, the internal transcribed spacer (ITS)1 segment, and most of the 5.8S gene in ribosomal (r) DNA.

Each reaction mixture contained 5 μ L of DNA template (20 ng/ μ L), 5 μ L of 10 \times Taq buffer (Eppendorf, Hamburg, Germany), 1 μ L of 50 mM MgCl₂ (Eppendorf), 1 μ L of 10 mM deoxynucleotide triphosphate (dNTP) mix (Eppendorf), 2.5 μ L each of 10 μ M primers (NS1 and ITS2 (Sigma, St. Louis, MO), 0.5 μ L of 5 U/ μ L Taq DNA polymerase (Eppendorf) in 50

μ L of final volume. All amplifications were performed in a Mastercycler gradient 5331 (Eppendorf) under the following conditions: initial denaturation of 95°C for 3 min followed by 35 cycles of 60 s at 95°C, 60 s at 55°C, and 90 s at 72°C, and a final extension step of 5 min at 72°C. A 6- μ L aliquot of PCR product was digested without further purification with 5 U of *Hae*III (Sigma) in a final volume of 25 μ L at 37°C for 1.5 h. Amplified products and their restriction fragments were separated on 1.8 and 2.5% agarose gels, respectively, in 1 \times Tris-borate-EDTA buffer. Gels were stained with 0.5 μ g/mL ethidium bromide, visualized by using a UV-light transilluminator, and photographed under UV light.

Dendrogram analysis of RFLP fragments was carried out with GelComparII software package (version 6.5; Applied Maths, Kortrijk, Belgium), using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and Dice similarity coefficient. Restriction fragments shorter than 100 bp were not considered in the analysis.

DNA Sequencing

The D1/D2 domain of the 26S rRNA gene was amplified with the following primer set: NL1 (5'-GCATAT-CAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTC-CGTGTTTCAAGACG-3'). Amplification was performed according to the following program sequence: 95°C for 4 min, followed by 30 cycles consisting of 94°C for 1 min, 55°C for 2.5 min, and 72°C for 2.5 min, with a final extension at 72°C for 10 min. The amplified products were purified with PCRExtract Mini Kit (5 Prime, Hamburg, Germany) and were subjected to direct sequencing with an ABI Prism 310 automatic sequencer (Applied Biosystems, Foster City, CA). The results were edited and analyzed by the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). Within each of the species, no more than 2 isolates were analyzed. For the *Trichosporon* group, the ambiguous results obtained with morphological and biochemical identification led us to perform sequencing of all strains.

RESULTS

From 1,486 milk samples, we isolated 142 yeast colonies that, based on morphological aspect and assimilation profiles, were identified as indicated in Table 1. Preliminary classification of 68 *Candida* strains, by the presence of blastoconidia, pseudohyphae, or both, was confirmed by API system and 8 different species were found at an acceptable to excellent level of identification, according to the APILAB Plus software.

Table 1. Identification of yeast species by morphological and biochemical tests

Genus (morphological identification)	Species (API identification ¹)	Strains, no.	
<i>Candida</i>	<i>Can. famata</i> (acceptable/excellent)	36	
	<i>Can. krusei</i> (excellent/good)	11	
	<i>Can. parapsilosis</i> (excellent/good)	5	
	<i>Can. catenulata</i> (excellent)	5	
	<i>Can. rugosa</i> (excellent)	4	
	<i>Can. utilis</i> (excellent)	3	
	<i>Can. inconspicua</i> (good)	3	
	<i>Can. sake</i> (excellent)	1	
	<i>Trichosporon</i>	<i>Cryptococcus curvatus</i> (doubtful/excellent) ²	15
		<i>Trichosporon mucoides</i> (low discrimination)	15
<i>Cryptococcus laurentii</i> (excellent)		6	
<i>Trichosporon asahii</i> (good)		3	
<i>Trichosporon inkin</i> (unacceptable profile)		2	
Unacceptable profile		2	
<i>G. candidum</i> (excellent/good)		10	
<i>Geotrichum</i>			
<i>Cryptococcus</i>	<i>C. uniguttulatus</i> (excellent)	15	
Pink yeasts	<i>Rhodotorula glutinis</i> (low discrimination/doubtful)	6	

¹API system (bioMérieux, Marcy l'Etoile, France).

²The high variability observed for *Cryptococcus* strains identification using the API system can be attributed to the variability of some phenotypic traits among the strains, which is a problem often encountered with phenotypic test-based systems.

The genus *Trichosporon* is phenotypically characterized by the ability to form blastoconidia, true mycelia, and, above all, arthroconidia, asexual spores that disarticulate from true hyphae. Arthroconidia and true mycelia are also present in species of the *Geotrichum* genus but differences in the ability to hydrolyze urea allowed us to differentiate between the 2 genera. Morphological identification of 10 *Geotrichum* genera was confirmed by API system with excellent to good conformity as *Geotrichum candidum*. Preliminary identification of 43 *Trichosporon* strains, based on arthroconidia presence and positive urease test, was less accurate. In fact, 21 *Trichosporon* strains were classified as *Cryptococcus laurentii* (6 strains) and *Cryptococcus curvatus* (15 strains) with excellent identification and doubtful to excellent identification, respectively (the high variability observed for *Cryptococcus* strains identification using the API system can be attributed to the variability of some phenotypic traits among the strains, which is a problem often encountered with phenotypic test-based systems). The identification of only 3 *Trichosporon asahii* was good, whereas 15 *Trichosporon mucoides* and 2 *Trichosporon inkin* strains were identified with low level of discrimination and unacceptable profiles, respectively. Furthermore, 2 strains could not be identified. Budding yeast cells without hyphae and pseudohyphae, preliminarily attributed to *Cryptococcus*, were identified as *Cryptococcus uniguttulatus* with a doubtful profile. Pink yeasts included the single species *Rhodotorula glutinis*.

In an attempt to overcome the ambiguity of the results obtained by taxonomic methods and to develop a rapid and accurate yeast identification technique, a molecular typing method based on PCR-RFLP of the

18S-ITS1-5.8S rDNA region digested with *Hae*III was applied in our laboratory to identify yeasts isolated from milk. Furthermore, within each of the groups identified by API or PCR-RFLP method, the identification of isolates was confirmed by sequencing of the D1/D2 region. Table 2 summarizes the results of phenotypic and molecular identification. The small-subunit rRNA-coding DNA with the neighboring ITS region was amplified in all isolates with the exception of 4 strains identified as *Candida rugosa*, which did not give PCR products under the amplification conditions used. The size of the amplified PCR products ranged from 1.8 to 2.2 kbp, not differing sufficiently to distinguish among genera or among species within a genus, but digestion of amplicons by the restriction enzyme *Hae*III gave very discriminant results. With the exception of *Can. rugosa*, the RFLP patterns for all *Candida* species were completely specific, so none of the species examined was mistaken for another; moreover, D1/D2 sequencing confirmed the API identification at the species level, and homologies from 99 to 100% were obtained with sequences in GenBank.

All strains identified by the API system as *G. candidum*, *Crypto. uniguttulatus*, and *R. glutinis* also produced characteristic restriction patterns and were confirmed as *Galactomyces geotrichum* (teleomorph of *G. candidum*), *Filobasidium uniguttulatum* (teleomorph of *Crypto. uniguttulatus*), and *R. glutinis*, respectively, by D1/D2 rDNA sequencing, with 99 to 100% nucleic acid sequence identity.

For the *Trichosporon* group, 6 different RFLP profiles characterized the 6 profiles recognized by biochemical methods. Sequencing of the D1/D2 domain confirmed

Table 2. Molecular characteristics of yeast isolates

API identification ¹	Size of RFLP products, bp	Identification by sequencing of D1/D2 region ²
<i>Candida famata</i>	190, 250, 270, 500	<i>Can. famata</i>
<i>Candida krusei</i>	170, 190, 230, 260, 530	<i>Can. krusei</i>
<i>Candida parapsilosis</i>	190, 260, 390, 500	<i>Can. parapsilosis</i>
<i>Candida catenulata</i>	160, 180, 220, 280, 410, 490	<i>Can. catenulata</i>
<i>Candida rugosa</i>	Not amplified	<i>Can. rugosa</i>
<i>Candida utilis</i>	150, 190, 320, 490	<i>Can. utilis</i>
<i>Candida inconspicua</i>	160, 190, 220, 450, 500	<i>Can. inconspicua</i>
<i>Candida sake</i>	160, 180, 320, 450, 500	<i>Can. sake</i>
<i>Trichosporon mucoides</i>	270, 320, 600	<i>T. mucoides</i>
<i>Cryptococcus laurentii</i>	200, 270, 320, 600	<i>Trichosporon cutaneum</i>
<i>Trichosporon asahii</i>	270, 950	<i>Trichosporon aquatile</i>
<i>Trichosporon inkin</i>	270, 390, 600	<i>Trichosporon lactis</i>
Unacceptable profile	220, 270, 320, 650	<i>Trichosporon gracile</i>
<i>Cryptococcus curvatus</i>	230, 320, 530, 590	<i>Crypto. curvatus</i>
<i>Cryptococcus uniguttulatus</i>	190, 215, 220, 250, 270, 290, 380	<i>Filobasidium uniguttulatum</i>
<i>Geotrichum candidum</i>	190, 290, 480, 500	<i>Galactomyces geotrichum</i>
<i>Rhodotorula glutinis</i>	190, 230, 240, 350, 390, 500	<i>R. glutinis</i>

¹API system (bioMérieux, Marcy l'Etoile, France).

²Confirmatory identification by sequencing D1/D2 region of 26S rRNA gene; D1/D2 sequencing confirmed the API identification at the species level, and homologies from 99 to 100% were obtained with sequences in GenBank.

the limited ability of the API ID 32C method to provide reliable identification of isolates of *Trichosporon* species, as previously reported (Colombo et al., 2011). Only *Trichosporon mucoides* and *Crypto. curvatus* were correctly identified by biochemical tests. All isolates belonging to the *Trichosporon cutaneum*, *Trichosporon lactis*, and *Trichosporon aquatile* species were misclassified as *Crypto. laurentii*, *Trichosporon inkin*, and *Trichosporon asahii*, respectively. Two isolates of *Trichosporon gracile* could not be identified.

Dendrogram analysis of RFLP fragments was carried out using the unweighted pair-group method with arithmetic mean algorithm and Dice similarity coefficient, and a database of yeast 18S-ITS1-5.8S RFLP patterns was constructed. Figure 1 shows the RFLP-based dendrogram with several strains of each species identified. Each strain of the same species showed similar digestion profiles with 90 to 100% similarity coefficients.

DISCUSSION

Commercial yeast identification methods based on morphological and physiological characteristics were designed to shorten the identification time of yeast isolates in the clinical laboratory, but their use for identification of environmental and wild yeasts is often inconclusive. The use of these systems, as well as geographical variations, could explain the discrepancy in the distribution of species reported in several surveys regarding yeasts isolated from bovine mastitic milk. *Candida*, *Pichia*, *Cryptococcus*, and *Rhodotorula* were the most frequent genera isolated from milk of

cows with mastitis in southern Brazil (Spanamberg et al. 2008), whereas the genera *Candida*, *Trichosporon*, *Cryptococcus*, and *Saccharomyces* were isolated from subclinical mastitic cows in Turkey (Türkyilmaz and Kaynarca, 2010). Bourtzis-Hatzopoulou et al. (2003) also reported that the yeasts isolated as a causative agent of bovine mastitis in Greece belonged to the *Candida*, *Geotrichum*, and *Rhodotorula* genera. Moreover, the distribution of yeast species is diverse in surveys from different countries (Seker, 2010; Türkyilmaz and Kaynarca, 2010; Segundo Zaragoza et al., 2011), and only few data, to our knowledge, are reported for Italy (Moretti et al., 1998; Scaccabarozzi et al., 2011).

Progress in molecular biology has provided a large number of DNA-based techniques for identifying and characterizing environmental yeast isolates. Restriction enzyme analysis of the 18S rDNA gene was used for identifying yeast strains associated with several beverages (Dlauchy et al. 1999). Recently, RFLP analysis of the 5.8S rRNA gene and the 2 ribosomal ITS has been successfully used to detect and differentiate several yeast species in both clinical and wild environmental strains (Carvalho et al., 2010; Diba et al., 2012). Previous works (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999) suggested the value of using a database containing endonuclease restriction patterns for the 5.8-ITS region for the identification of environmental or wild yeast strains. To our knowledge, this is the first report of PCR-RFLP analysis of 18S-ITS1-5.8S rDNA region digested with the single enzyme *HaeIII* as a rapid and relatively inexpensive molecular procedure for accurate differentiation of most yeast species isolated in bovine mastitic milk samples.

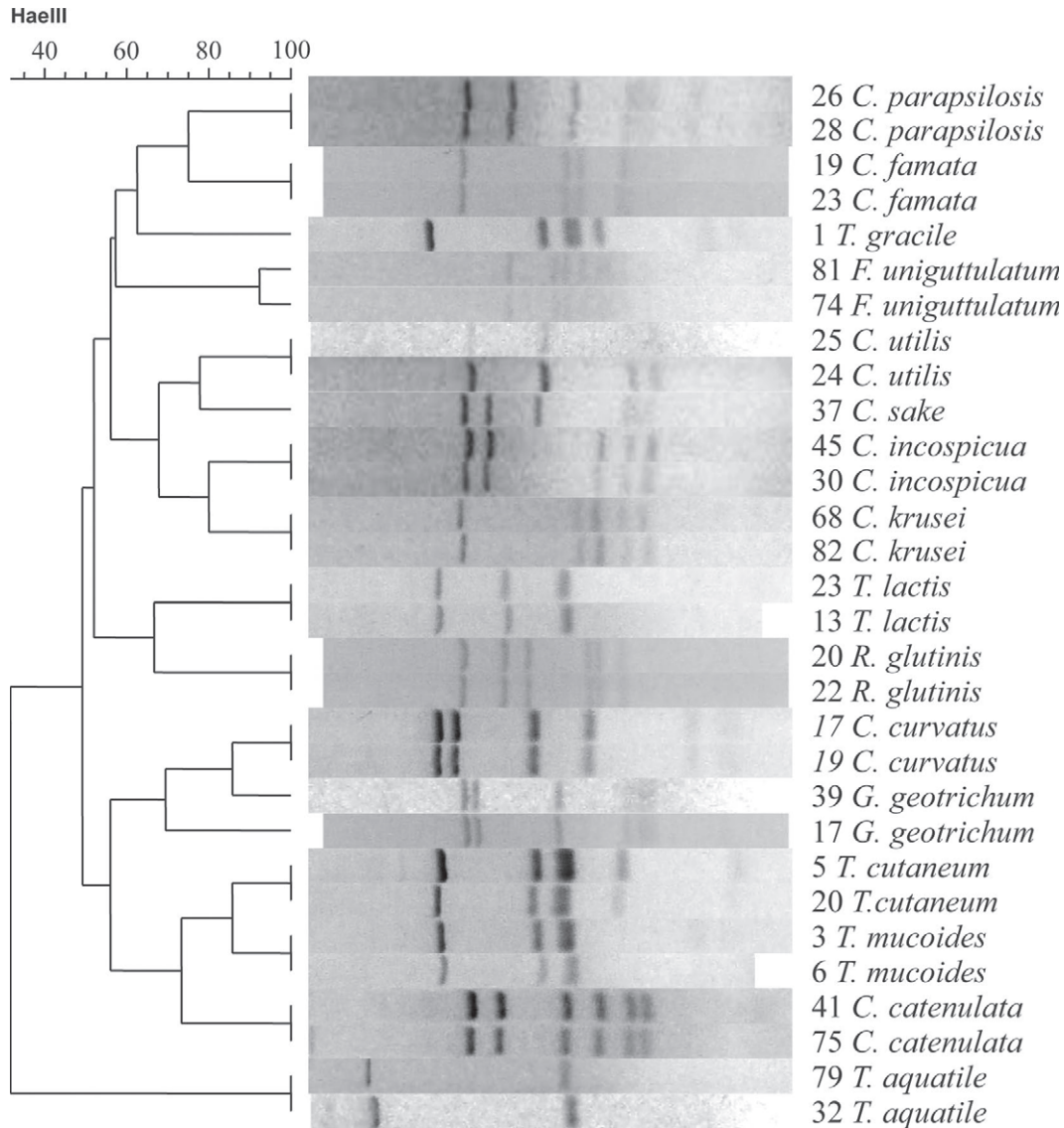


Figure 1. Dendrogram of representative PCR-RFLP patterns from each yeast species identified: *Candida parapsilosis*, *Candida famata*, *Trichosporon gracile*, *Filobasidium uniguttulatum*, *Candida utilis*, *Candida sake*, *Candida inconspicua*, *Candida krusei*, *Trichosporon lactis*, *Rhodotorula glutinis*, *Cryptococcus uniguttulatus*, *Geotrichum candidum*, *Trichosporon cutaneum*, *Trichosporon mucoides*, *Candida catenulata*, and *Trichosporon aquatile*.

In our study, the combined phenotypic and molecular approach enabled identification of 17 yeast species belonging to the genera *Candida* (47.9%), *Cryptococcus* (21.1%), *Trichosporon* (19.7%), *Geotrichum* (7.1%), and *Rhodotorula* (4.2%).

Candida famata (teleomorph *Debaryomyces hanse-nii*) was the predominant species, and the majority of strains (88.9%) were isolated from Sardinian milk samples, confirming that this species is an important component of the microflora of both Sardinian ovine and bovine milk (Cosentino et al., 2001; Fadda et al., 2004). Interestingly, *Candida albicans* was not observed among the species of the prevalent genus, in agree-

ment with recent studies in Turkey (Türkyilmaz and Kaynarca, 2010; Seker, 2010) and Brazil (Spanamberg et al., 2008), although its presence has been reported in other surveys (de Casia dos Santos and Marin, 2005; Segundo Zaragoza et al., 2011).

A limitation of our method was that *Can. rugosa* could not be amplified by the set of primers used, presumably because of a limitation of the forward NS1 primer, as reported by Mannarelli and Kurtzman (1998).

In regard to the genera *Cryptococcus* and *Trichosporon*, the RFLP technique used in this study gave characteristic restriction profiles for each species, and sequencing of the D1/D2 region allowed not only suc-

cessful identification of *Trichosporon gracile* (which API ID 32C could not), but also the correction of misidentified isolates. *Cryptococcus curvatus* and *Crypto. uniguttulatus* (sequence in GenBank is reported as teleomorph *F. uniguttulatum*) were successfully identified with both phenotypic and molecular methods, whereas identification of *Crypto. laurentii* strains at an excellent level by the API system was corrected to *Trichosporon cutaneum* by sequencing. According to the data reported in this and previous studies, it is evident that morphological and biochemical methods are not sufficient for *Trichosporon* species identification (Ahmad et al., 2005; Rodriguez-Tudela et al., 2005). The API ID 32C system is programmed to identify only 3 species of *Trichosporon*: *T. asahii*, *T. inkin*, and *T. mucoides*; consequently, it was unable to identify *T. cutaneum*, *T. lactis*, *T. aquatile*, and *T. gracile* species in the milk samples analyzed.

CONCLUSIONS

The 18S-ITS1-5.8S region appears to be useful in detecting genetic variability among yeast species, which is valuable for taxonomic identification to the species level. We have established a RFLP database for yeast species identified in milk samples using the software GelCompar II, creating an initial method for veterinary yeast identification. Once the database of species has been enlarged, rapid and reliable identification of yeast species from milk will be possible.

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