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New Method for the Simultaneous Identification of Cow, Sheep, Goat, and Water Buffalo in Dairy Products by Analysis of Short Species-Specific Mitochondrial DNA Targets

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Supporting Information

ABSTRACT: A novel method is presented here as an analytical tool for food control and authentication of dairy products manufactured from the milk of cow, sheep, goat, and buffalo. The method is based on multiplex polymerase chain reaction (PCR) of species-specific mitochondrial DNA (mtDNA) targets followed by fragment size analysis by capillary electrophoresis. The method includes (a) simultaneous detection of four species, (b) internal control for DNA extraction and PCR, (c) mtDNA as a target for PCR, (d) amplicons of <200 bp, and (e) flexibility in the electrophoresis and fragment size detection method. Species identification proved to be straightforward, efficient, sensitive, and robust. The method is sensitive to an at least 1% (v/v) relative proportion of milk in binary mixtures. A survey of commercial products showed that 12.5% failed to conform to the description of the contents, by either the introduction or absence of listed species, thus demonstrating the relevance of this type of testing.

KEYWORDS: *species identification, food authentication, multiplex PCR, mtDNA, dairy products*

INTRODUCTION

Inaccurate description of the contents of food products has become an increasingly important issue, as the protection of the consumer and the right to make informed choices is guaranteed by national and international regulations in most developed countries. Premium products, such as cheese labeled with designation of origin, are the most vulnerable to adulteration by substitution or omission of one or more high-value ingredients in the production process. This leads to substandard quality, loss of product identity, and unfair competition by producers who take economic advantage of fraudulent labeling of food composition. Therefore, it is pertinent to control the quality of dairy products in terms of biological composition and verify the conformity with compulsory production specifications, such as those defined for traditional products by the European PDO (Protected Denomination of Origin), PGI (Protected Geographic Indication), and TSG (Traditional Specialty Guaranteed) standards. The identification of the species that have contributed to the composition of the product is an important aspect of authenticity testing. In fact, previous studies showed that milk and derived dairy products available for retail in Italy, Spain, Portugal, the Czech Republic, Poland, Croatia, Egypt, Taiwan, China, India, and Pakistan presented nonconformity with the alleged composition in terms of species, showing that the problem is global and widespread.[1−12]

DNA-based methods for species identification are particularly appropriate for the analysis of commercial dairy products because (a) DNA derived from somatic animal cells is present in all dairy products,[13] (b) DNA is stable and retrievable for polymerase chain reaction (PCR) analysis even after thermal treatment and other types of processing (e.g., pasteurization, ultra-high-temperature (UHT) treatment, rennet and acid coagulation, dehydration, fermentation, ripening, smoking), as long as an adequate extraction method is used,[14] (c) dairy products are widely manufactured from the milk of goat, sheep, cow, and water buffalo and it is unlikely that milk from other species is incorporated in the production, thus restricting the targets for genetic testing, and (d) genomic sequences for the selection of DNA targets or markers and the design of species-specific PCR primers are publicly available for these species. Although milk and derived products are expected to contain low concentrations of DNA in suboptimal conditions, the use of mitochondrial DNA (mtDNA) as a PCR target allows for the minimization of the problem because of its relative abundance compared to that of nuclear DNA. Furthermore, the high mutation rate of mtDNA allows for discrimination among closely related species.[15−18]

In the past decade, several works have proposed methods based on species-specific PCR followed by electrophoresis for investigating the composition of dairy products in terms of the contributing species, with an important part focusing on the detection of cow in buffalo milk and cheese.[1,9,10,19−22] The detection of cow in dairy products presumably produced from goat and sheep has also been the subject of active research,[3,4,6,8,12,23] as well as the detection of goat in sheep's milk products.[5] These methods have the main disadvantage of not allowing for the simultaneous detection of cow, sheep, goat, and buffalo. This problem has been addressed by Reale et al.,[11] but

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Table 1. Summarized List of 96 Commercial Dairy Products Analyzed in This Study with Species Labeled in the Product, Type of Product, Number of Samples, Number of Conformities and Nonconformities with the Species Labeled, and Observations Regarding the Nonconformity

species labeled	type of product	no. of samples	no. of conformities	no. of nonconformities	observations
buffalo	butter	1	1	0	
buffalo	cheese	2	2	0	
cow	cheese	43	43	0	
cow	cottage cheese	1	1	0	
cow	cream	1	1	0	
cow	milk (UHT)	3	3	0	
cow	powdered milk	2	2	0	
cow + goat	cheese	2	2	0	
cow + sheep	cheese	7	3	4	absence of sheep in four samples
cow + sheep + goat	cheese	6	2	4	absence of sheep in three samples; absence of goat in one sample
goat	cheese	5	4	1	presence of cow in one sample
goat	milk (UHT)	1	1	0	
goat	yoghurt	1	1	0	
sheep	cheese	17	15	2	presence of cow in one sample; presence of cow and goat in one sample
sheep	cottage cheese	2	1	1	presence of cow in one sample
sheep	fresh milk	1	1	0	
sheep	yogurt	1	1	0	
total		96	84	12	

Table 2. List of Nine Primer Pairs Used in Multiplex PCR for the Simultaneous Amplification of Two Different mtDNA Fragments for Each Species (Cow, Sheep, Goat, and Buffalo) and One Conserved Fragment Common to All Species (Internal Positive Control)[a]

fragment	mtDNA coordinates	reference genome	GenBank ID	primer	primer sequence (5′−3′)	fragment size (bp)	label	concn in multiplex primer mix (μM)
Bos1	15255−15407	*B. taurus*	AF492351	Bos1_F	GCCGGCACAATCGAAAACAAAT	153	6-FAM	2
				Bos1_R	CTTCAGCTTTGGGGGTTGATG			2
Bos2	5117−5277	*B. taurus*	AF492351	Bos2_F	GTTAACAGCTAAACACCCTAGCT	169	6-FAM	18
				Bos2_R	[GACTGACT] AGGTTTGACTCCTCTTTTTACCAA			18
Bubalus1	14314−14488	*Bu. bubalis*	NC_006295	Bubalus1_F	CCAAAATTTAACACAATCCCGCAA	175	6-FAM	2
				Bubalus1_R	CATTGGTCGTGGTTGAATTCCA			2
Bubalus2	2989−3185	*Bu. bubalis*	NC_006295	Bubalus2_F	GAATTTATCTCAATTAGTAACGCAAC	197	6-FAM	18
				Bubalus2_R	CTACTAATGTGAGGAATGCCACT			18
Capra1	13935−14052	*C. hircus*	GU295658	Capra1_F	CACCAAAATTCAACACAATACCACAT	118	6-FAM	2
				Capra1_R	AGCGTTATCTTTGTAATAGGTTTTGT			2
Capra2	7869−7995	*C. hircus*	GU295658	Capra2_F	CTACCACAACCCAGAATTAACAG	127	6-FAM	2
				Capra2_R	TAAGGGTAACAAGGGGGAGG			2
Ovis1	13943−14100	*O. aries*	AF010406	Ovis1_F	CCAAAATTCAACACAATACCACAC	158	6-FAM	2
				Ovis1_R	TTCCATGTGAGAATGATGATGACA			2
Ovis2	9347−9503	*O. aries*	AF010406	Ovis2_F	CGTAGATGTAGTATGACTTTTCCT	181	6-FAM	18
				Ovis2_R	[GACTGACTGACTGACTGACTGACT] GTGAAGTTAGTTAGGAGAGTAATTATA			18
CF	2911−3049	*B. taurus*	AY526085	Conserved_F	TCCCAGTACGAAAGGACAAGA	139	6-FAM	2
				Conserved_R	CAATTACCGGGCTCTGCCA			2

[a]Sequences between brackets are DNA tails added to optimize fragment size differences and electrophoretic separations. mtDNA coordinates are given relative to the reference genome referenced in the same line.

the authors' proposal based on minisequencing of SNPs (single nucleotide polymorphisms) in a nuclear gene (k-casein) still constitutes an important disadvantage in the context of dairy samples that may contain low-quantity/quality DNA.

Here we present a method that allows for the simultaneous detection of cow, sheep, goat, and buffalo based on single multiplex PCR targeting short species-specific mtDNA regions, followed by capillary electrophoretic separation with or without automatic fragment size detection. This method also includes an internal positive control for DNA extraction and PCR.

MATERIALS AND METHODS

The method described here allows for the identification of four species (cow, goat, sheep, and water buffalo) in a simple three-step procedure: (a) amplification of nine mtDNA target regions (two fixed-size species-specific fragments for each species and one mammalian conserved fragment as an internal positive control) in multiplex PCR,

(b) electrophoretic separation of PCR products in capillary electrophoresis with or without automatic fragment size detection, and (c) determination of fragment sizes by comparison with an allelic ladder.

Samples. Commercial dairy products (standard cheese, PDO cheese, cottage cheese, powdered milk, UHT milk, fresh milk, yogurt, cream, and butter were purchased in the local food retail market (Table 1). Samples for analysis were collected from internal sections of the solid dairy products. Commercial dehydrated animal rennet used in the production of cheese was obtained from industrial suppliers (Danisco, France; Lusocoalho Lda, Portugal). Reference blood samples from cow, sheep, water buffalo, and goat were collected on FTA cards. DNA was extracted from all samples using a standard phenol–chloroform method.[24]

PCR Primer Design. Species-specific PCR primers were designed in the coding region of the mitochondrial genome based on the alignment of the GenBank reference sequences for *Bos taurus* (AY526085), *Bubalus bubalis* (NC_006295), *Capra hircus* (GU295658) and *Ovis aries* (AF010406). The following criteria were used for the selection of the target regions: (a) amplicon size shorter than 200 bp, (b) forward and reverse primers located in different contiguous genes to impair the amplification of mtDNA sequences from other sources (e.g., fungal mtDNA, whose gene order is substantially different from that of mammals), and (c) significant size differences among amplicons for unambiguous electrophoretic separation. The specificity of the PCR primers was guaranteed by their location in mtDNA regions unique to each species (preferentially at the 3′ position of each primer). Tails were added to two of the primers [$(AGTC)_6$ and $(AGTC)_2$ to Bos2 and Ovis2 fragments, respectively]. These tails serve the purpose of increasing the length of the amplicon to optimize electrophoretic separations but do not affect primer annealing.

Intraspecific sequence polymorphism that could compromise primer annealing and/or originate variable sequence sizes was assessed by manual inspection of the alignments of publicly available complete mtDNA sequences for cow (n = 145), sheep (n = 3), goat (n = 2), and water buffalo (n = 4).

To identify a conserved region for use as an internal positive control, 237 mammalian mtDNA reference sequences were also aligned for the design of a primer pair [here named a "conserved fragment" (CF)] that would amplify the highest number of species, according to the criteria for primer location and amplicon size described above.

All sequences used were retrieved from GenBank (www.ncbi.nih.gov) and aligned using the Muscle tool implemented in Geneious Pro v5.3 software (Biomatters Ltd., Auckland, New Zealand). All primers were tested for potential formation of hairpin structures and primer–primer interactions using the OligoCalc software.[25] Potential reactions among all primer pairs in the multiplex PCR were tested using AutoDimer software.[26] Primers used for automated fragment detection fluorescent capillary electrophoresis in the ABI 3130 xl sequencer (Applied Biosystems, Foster City, CA) were labeled with a fluorescent dye (Table 2). Unlabeled primers were used for capillary electrophoresis in the QIAxcel system (QIAGEN GmbH, Germany).

PCR. Amplification reactions were prepared in a total volume of 10 μL as follows: 5 μL of Multiplex PCR Master Mix (QIAGEN), 1 μL of primer mix, and 5–10 ng of DNA. The thermocycler program for posterior analysis in capillary electrophoresis in the ABI 3130 xl sequencer consisted of polymerase activation at 95 °C for 15 min, 26 amplification cycles at 94 °C for 30 s, 61 °C for 1.50 min, and 72 °C for 1 min, and a final extension at 72 °C for 60 min. PCR amplifications for posterior electrophoretic separation in the QIAxcel system were adapted from the method described above by increasing the number of amplification cycles to 30. PCR protocols were optimized in a 2720 ThermoCycler (Applied Biosystems).

Allelic Ladder. An allelic ladder for fragment size detection was produced by mixing DNAs extracted from the reference blood samples of the four species in approximately equal concentrations (5–10 ng/μL). Labeled primers were used to produce the allelic ladder for ABI 3130 xl sequencer electrophoretic separations. Unlabeled primers were used for producing the allelic ladder for QIAxcel system separations.

Sequencing. The nine fragments included in the multiplex (Bos1, Bos2, Bubalus1, Bubalus2, Ovis1, Ovis2, Capra1, Capra2, and CF) were sequenced in both directions in the reference samples to confirm the size and sequence of the predicted amplicon. In some cases, internal PCR and sequencing primers were designed for the survey of the complete fragment (Supplementary Table 1, Supporting Information). Singleplex PCRs were performed as described in the previous section. PCR products were purified using ExoSAP-IT (USB Corp.) according to the manufacturer's instructions. Sequencing reactions of purified PCR products were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's recommendations. Sequencing reaction products were purified using Sephadex G-50 fine DNA grade columns (GE Healthcare). Sequencing was performed in the ABI 3130xl sequencer according to the manufacturer's recommendations. Sequence analysis was performed using SeqScape software (Applied Biosystems).

Electrophoresis. Two alternative electrophoresis methods were tested for the separation of PCR products: (1) capillary electrophoresis with fluorescently labeled primers (ABI 3130xl sequencer) [PCR products (0.5 μL) were combined with 12 μL of LIZ 500 Mix; LIZ 500 Mix was produced with 960 μL of HI-DI formamide (Applichem) and 40 μL of GeneScan-500 LIZ Size Standard (Applied Biosystems); electrophoresis was performed according to the manufacturer's instructions; fragment size determination was performed using GeneMapper v4.0 (Applied Biosystems)] and (2) capillary electrophoresis without fluorescently labeled primers (QIAxcel system) [PCR products (10–100 ng/μL) were run using the DNA High Resolution Gel Cartridge (QIAGEN) under method OM700.mtd for 10 s at 5 kV voltage for sample injection and 700 s and 3 kV voltage for fragment separation; the results were analyzed using QX Biocalculator Fast Analysis Sofware (QIAGEN)].

Specificity and Cross-Reactivity. PCR primer specificity and cross-reactivity were tested in singleplex PCR using different mixtures of DNA from the reference species. Specificity tests were also performed in *Homo sapiens*, *Canis familiariz*, *Felis catus*, *Equus caballus*, *Mus musculus*, *Sus scrofa*, and a fungus (*Aspergillus fumigatus*) reference samples in multiplex PCR.

Sensitivity. The sensitivity of the multiplex was preliminarily assessed by testing binary mixtures of sheep's, cow's, and goat's milk. Binary milk mixtures were produced for all combinations of species, with one of them at 1% (v/v) (Supplementary Table 2, Supporting Information). Commercial rennets used in the production of cheese were also tested to assess the potential contribution of residual DNA to the genetic profile of the product. Dehydrated rennet samples were diluted in water to the same concentration used in the industrial application.

RESULTS AND DISCUSSION

This study was aimed at the development of an optimized multiplex PCR and electrophoresis test for the simultaneous detection of *B. taurus* (cow), *C. hircus* (goat), *O. aries* (sheep), and *Bu. bubalis* (water buffalo) in dairy products based on the detection of species-specific mtDNA target regions. The PCR multiplex is composed by nine primer pairs optimized for coamplification in a single reaction, eight of which target species-specific fragments of fixed size (two for each species). One primer pair that targets a phylogenetically conserved fragment in mammals (CF) was also included as an internal positive control for DNA extraction and PCR. The work flow consists of DNA extraction and multiplex PCR amplification followed by electrophoresis and fragment size detection. The method was designed to allow for the detection of fragment size by comparison to a DNA ladder in two alternative electrophoresis methods: capillary electrophoresis with fluorescently labeled primers on an ABI 3130xl sequencer and capillary electrophoresis without fluorescence on a QIAxcel system.

Amplicon Sequence and Size. All sequenced PCR products of reference samples (total blood, milk, and cheese) had the expected sequence. The sizes of the DNA targets were

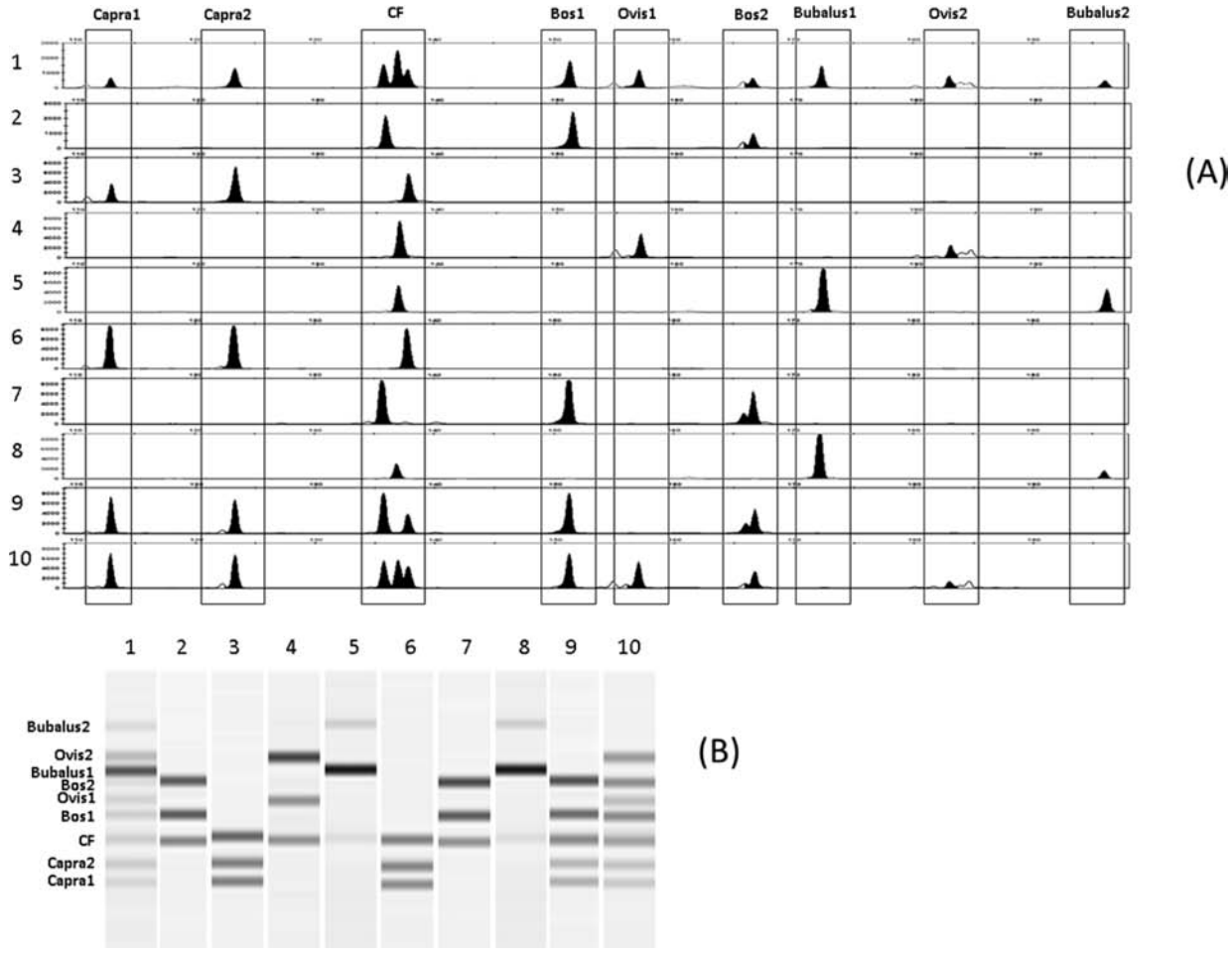

Figure 1. Electrophoretic profiles representative of reference samples for the allelic ladder (1), cow (2), goat (3), sheep (4), and water buffalo (5) and commercial samples of goat yogurt (6), powdered cow milk (7), buffalo mozzarella cheese with PDO (8), mixture cheese (9), and "Flor de Guadamur" cheese (10), as obtained using the (A) ABI 3130 xl sequencer and (B) QIAxcel system.

between 118 and 197 bp: Bos1 (153 bp), Bos2 (169 bp), Capra1 (118 bp), Capra2 (127 bp), Ovis1 (158 bp), Ovis2 (181 bp), Bubalus1 (175 bp), and Bubalus2 (197 bp). The CF had a small size variation among the species surveyed (*B. taurus*, 139 bp; *Bu. bubalis* and *O. aries*, 140 bp; *C. hircus*, 141 bp) due to interspecific indel polymorphisms (Supplementary Figure 1, Supporting Information).

Small-sized DNA targets are an important aspect because this method is destined to the analysis of a great variety of dairy products, many of which may have suffered some degree of processing. In such cases, it is recommendable to target small regions to overcome amplification problems due to fragmented DNA. The length differences in the CF do not hamper the correct species identification because it is strictly interspecific, i.e., the size of the fragment remains constant for a particular species. Amplification of the CF with a different size from cow, goat, sheep, or buffalo may indicate contamination of the sample by another mammal such as rat, mouse, or human.

Electrophoretic Profiles of Reference Samples (Single Species and Mixtures). The electrophoretic profiles of samples containing a single species were consistent with the expected size for the two species-specific fragments and the CF. It was also possible to identify correctly and unambiguously the species present in DNA mixtures (two-, three-, and four-species). The results were 100% consistent and reproducible in six independent singleplex and multiplex PCR amplifications and electrophoretic runs for each sample, independently of the electrophoretic separation and fragment detection method (Figure 1). Electrophoretic separation and fragment size determination are possible using these two different methods because the size difference between all fragments is at least 5 bp. Within this range of fragment sizes (118−197 bp), it is possible to safely achieve unambiguous identification of electrophoretic peaks.

Specificity. The specificity tests performed in human (*H. sapiens*), dog (*Can. familiariz*), cat (*F. catus*), horse (*E. caballus*), mouse (*M. musculus*), pig (*S. scrofa*), and fungus (*A. fumigatus*) reference samples in multiplex PCR produced no amplification for the eight species-specific fragments (data not shown).

Sensitivity. Sensitivity tests performed in two-species milk mixtures showed that at least 1% (v/v) species-specific milk is detectable by capillary electrophoresis using the ABI 3130 xl sequencer the QIAxel system (Figure 2). Sensitivity tests in mixtures including water buffalo were not performed because this milk was not available for the production of controlled mixtures. However, it was possible to detect specific water buffalo DNA in a commercial sample of butter ("Burro di Buffala") using the three different electrophoretic methods even though this sample presumably contained a very low proportion of DNA compared to other dairy products such as cheese.

Survey of Commercial Products. To obtain a preliminary assessment of the conformity of dairy products with the

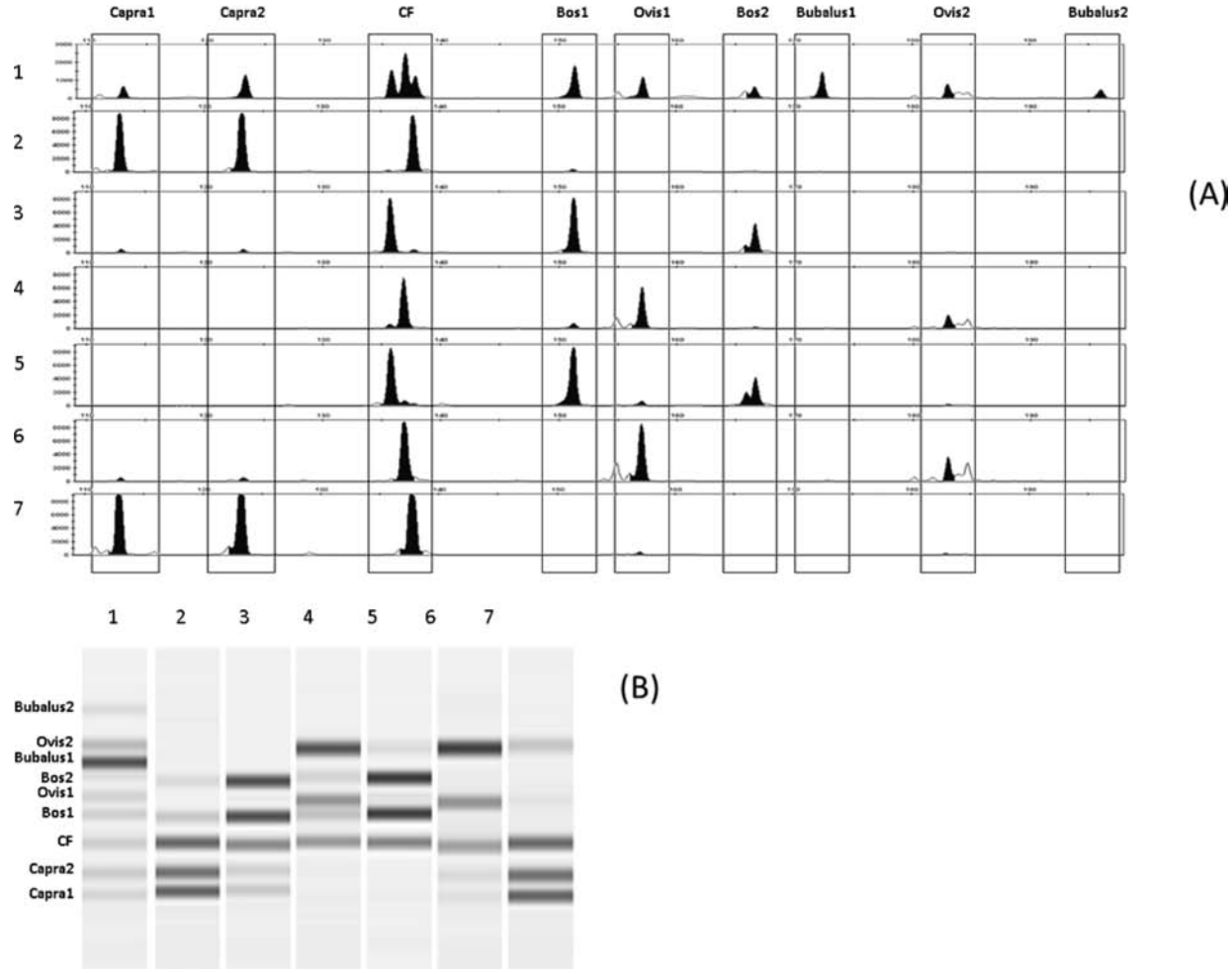

Figure 2. Electrophoretic profiles representative of allelic ladder (1) and binary milk mixtures [1% cow + 99% goat (2), 99% cow + 1% goat (3), 1% cow + 99% sheep (4), 99% cow + 1% sheep (5), 1% goat + 99% sheep (6), and 99% goat + 1% sheep (7)] for the assessment of the sensitivity of the methods in the (A) ABI 3130 xl sequencer and (B) QIAXcel system.

description of contents stated in their labels, we used this method to test 96 products available in the local food retail market. This survey of commercial dairy products showed that 12.5% of the samples were not compatible with the information presented in the label. Nonconformities consisted of the presence of one unmentioned species in four cases and the absence of one mentioned species in eight cases. Also, two unmentioned species (cow and goat) were detected in one sample described as containing exclusively sheep (Table 1). These results are consistent with the hypothesis of deliberate adulteration of the products aiming at an economic gain either by the nondeclared addition of low-value milk (i.e., cow) or by the absence of high-value milk (i.e., sheep). This is particularly evident in products that should contain cow and sheep, where the omission of sheep was found in almost half of the samples. Also, the addition of cow to cheese described as pure sheep occurred in four samples.

All PDO products analyzed here were in conformity with their legal specifications. However, two samples of cheese with PDO contained only sheep, while both labels indicated sheep and goat in their composition. Although this represents a nonconformity relative to the label, it does not represent a nonconformity with regard to the PDO specifications because these traditional products are legally allowed to contain either both species or just one of them, depending on the availability of sheep' and goat's milk throughout the year.

In conclusion, our DNA-based analysis of commercial dairy products has shown a relevant occurrence of nonconformity between the declared and the actual compositions in terms of the contributing species. This discrepancy is not acceptable in most developed countries where national and international regulations mandate a clear description of food composition.

Compared to previously described methods, we emphasize the following advantages: (a) four species can be detected simultaneously in a single multiplex PCR, (b) the multiplex comprises an internal control for DNA extraction and PCR, (c) the target for PCR is the high-copy-number mtDNA, (d) all amplicons are shorter than 200 bp, (e) there is flexibility in the electrophoresis and fragment size detection method, and (f) the method has shown a sensitivity of at least 1% (v/v) milk mixtures in both alternative electrophoretic methods for fragment size detection.

The method proved to be straightforward, robust, and reproducible, and it can be easily implemented in a standard DNA laboratory equipped with capillary electrophoretic platforms, as demonstrated here for the ABI 3130 xl sequencer and the QIAxcel system.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Figure 1 showing the alignment of conserved fragments amplified and sequenced in reference samples of

B. taurus, *Bu. bubalis*, *O. aries*, and *C. hircus* highlighting indel polymorphisms responsible for interspecific fragment size differences (1−2 bp) in the conserved fragment detected in capillary electrophoresis separations, Supplementary Table 1 listing the primers used for amplification and sequencing (in both directions) for the assessment of sequence polymorphism and size determination (bp) of the mtDNA fragments included in the described method for the simultaneous identification of cow, sheep, goat, and buffalo in dairy products (mtDNA coordinates are given relative to the reference genome listed in the same line), and Supplementary Table 2 listing the binary milk mixtures (volume/volume) produced in the laboratory for preliminary testing of the described method's sensitivity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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