

# Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of Mitochondrial *cytb* Gene from Species of Dairy Interest

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**Species identification plays an important role in food allergy prevention and food substitution detection that can reduce the commercial value of a product. For these reasons, many molecular methods have been developed to determine species origin; among them, polymerase chain reaction (PCR)-based methods were successfully applied to processed or unprocessed foodstuffs. An updated PCR-RFLP (restriction fragment length polymorphism) method of the *cytb* gene was developed for the identification of the 4 species of main interest in the dairy industry (*Bos*, *Ovis*, *Capra*, *Bubalus*). The comparative analysis of the 92 *cytb* sequences available in the database belonging to the 4 species allowed identification of 2 highly conserved regions, which were used to design 2 oligonucleotides for the PCR amplification of a 275 base-pair (bp) *cytb* fragment. The *in silico* analysis allowed identification of a set of species-specific restriction endonucleases (*HaeIII*, *TaqI*, and *MwoI*), which generated easily analyzable species-specific restriction profiles of the 275 bp *cytb* DNA fragment. The system was developed for both purified DNA and DNA extracted from meat or dairy products and finally tested on mixed samples, indicating its applicability to foodstuffs.**

Food adulteration is a common problem in the dairy industry because it can be easily applied to dairy products. Substitutions not clearly labeled cannot only weigh upon the commercial value of a product, but can also be responsible for the rise in allergic reactions, resulting in adverse clinical symptoms. Allergenicity of cow milk proteins (1), as well as their residual allergenic potential after

heat treatments or sophistication (2) have long been investigated. In addition, some allergenic cross-reactions between milk proteins from other species have been documented (3–5). However, because some children who are allergic to cow's milk can tolerate goat derivatives (6), this problem might be bypassed by the use of individually based diets (7). Moreover, commercial rules require a correct labeling in order to protect and ensure the quality of the product and its peculiar characteristics.

For these reasons in the past few years many molecular methods, based on proteins or nucleic acids analysis, have been developed and are currently used for species identification in dairy products. However, methods based on the whole-cell protein analysis or immunological analyses have been replaced by DNA-based methods because DNA is more stable at high temperature and its structure is conserved within all tissues of an individual (8 and references therein). Among them, those based on the polymerase chain reaction (PCR) technique have been successfully applied to species identification from untreated meat or dairy products as well as from heat-treated, marinated, or fermented samples (9, 10). In these methodologies, the target sequence is the mitochondrial *cytb* gene, which is ubiquitous and includes both conserved regions that have been used to design universal primers for amplifying, via PCR, a fragment of the same length from all the species considered (11) and variable ones, which differentiate one species from the others.

This *cytb* trait has been exploited to develop a methodology that contemplates the PCR amplification of a *cytb* region followed by the digestion of the PCR products with restriction endonucleases (restriction fragment length polymorphism, RFLP; 9, 10, 12). The PCR-RFLP technique was successfully applied to the identification of different species from dairy and meat samples by using a combination of restriction enzymes (*HaeIII*, *TaqI*, and *HinfI*; 9, 10). Nevertheless, the methodology also led to unexpected restriction profiles or to additional fragments within them that were not disclosed by analysis of the limited number of sequences available at the time the system was developed (10). These intraspecific differences might

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**Table 1. Oligonucleotides used as primers in PCR amplification reactions**

| Primer | Sequence                          | Reference    |
|--------|-----------------------------------|--------------|
| Unifw  | 5'-GGCCTATTYCTAGCAAT-3'           | This work    |
| Unirev | 5'-GATATTTGICCTCATGG-3'           | This work    |
| L14841 | 5'-CCATCCAACATCTCAGCATGATGAAA-3'  | (11)<br>(10) |
| H15149 | 5'-GCCCCCTCAGAATGATATTTGTCCTCA-3' | (11)<br>(10) |

complicate the analysis of restriction patterns and give raise to false positives or false negatives.

The aim of the present work was to analyze all the available *cytb* sequences, focusing the attention on the 4 species of main interest in dairy industry (*Bubalus*, *Capra*, *Bos*, *Ovis*) in order to develop an updated PCR-RFLP method that generates easily analyzable species-specific restriction profiles.

## Experimental

### Analysis of Sequence Data

The *cytb* nucleotide sequences were retrieved from databases by using both the BLAST program (13) and the Entrez system from the National Center of Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/Entrez/>). Pairwise and multiple alignments of nucleotide sequences were performed using the program ClustalW, <http://www.ebi.ac.uk/clustalw> (14). The *in silico* simulation of restriction patterns of amplified fragments was performed using the program Webcutter, <http://www.firstmarket.com/cutter/cut2.html>.

### Purified DNA, Meat, and Milk Product Samples

Purified DNA from buffalo, goat, cow, and sheep (hereinafter standard DNAs) were obtained from Biotoools B&M laboratories (Madrid, Spain); the DNA samples were stored at 4°C and used at a final concentration of 10 ng/μL. Meat and milk samples, including milk derivatives, were authenticated by a veterinarian during manufacturing and were frozen at -20°C until use.

### DNA Extraction

Total DNA was extracted from 0.2 g aliquots of frozen meat minced with sterile surgical blades or 0.2 mL frozen milk samples using the Wizard Magnetic DNA Purification System kit (Promega Corp., Madison, WI) according to the supplier's instructions. The concentration and purity of DNA were determined spectrophotometrically at 240, 260, and 280 nm, and DNA was diluted in sterile distilled water at a final concentration of 10 ng/μL.

### PCR Amplification of *cytb* Gene

The amplification of a 275 base pair (bp) *cytb* fragment from standard, meat, and milk DNA was performed in a final volume of 50 μL 1X reaction buffer (Fynnzymes, OY, Espoo, Finland) containing 100 ng DNA, 1mM MgCl<sub>2</sub>, 200 μM of each dNTP, 0.3 μM of each primer Unifw and Unirev (Table 1), and 1.5 units of DYNAzyme II DNA polymerase (Fynnzymes).

Primers L14841 and H15149 (11) were used for amplifying a 359 bp *cytb* fragment at a final concentration of 0.5 μM in a 50 μL PCR mixture containing 100 ng DNA, 1X reaction buffer (10mM Tris HCl, pH 8.8; 50mM KCl; 0.1% Triton X-100; Fynnzymes), 2mM MgCl<sub>2</sub>, 2 μg/mL bovine serum albumin (Promega Corp., Milan, Italy), 200 μM of each dNTP, and 2 units of DYNAzyme II DNA polymerase (Fynnzymes).

All PCR were performed in a PCR Sprint Hybrid thermal cycler (Ashford, Middlesex, UK). For the amplification of the 275 bp *cytb* DNA fragment, the following thermal profile was used: an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 20 s, 44°C for 30 s, 72°C for 25 s; and a final extension at 72°C for 5 min. The amplification of the 359 bp fragment was performed at a first denaturation temperature of 94°C for 1 min; 35 cycles of 94°C for 5 s, 50°C for 30 s, 72°C for 40 s, and finally, 72°C for 3 min, as previously described (10).

Aliquots of 5 μL of the amplification product were examined by electrophoresis on a 2% agarose gel in 1X TRIS-EDTA-acetate (TAE; Gibco BRL, Life Technologies Ltd, Paisley, Scotland).

### Restriction Endonuclease Analysis

Aliquots of 10 μL of the amplification product were treated with 6 units of each enzyme (New England Biolabs Inc., Hitchin, Herts, UK; Table 2) in a final volume of 30 μL, according to the supplier's instructions. The restriction fragments were examined on ethidium bromide-stained 3% agarose gel in 1X TAE, and their size was estimated by comparison with molecular weight ladders (100 bp ladder, Gibco; and Gene Ruler DNA Ladder Mix, Genenco, M-Medical, Florence, Italy).

**Table 2. Restriction endonucleases used in this work**

| Restriction endonuclease | Recognition sequence | Incubation temperature, °C |
|--------------------------|----------------------|----------------------------|
| <i>Hae</i> III           | GG/CC                | 37                         |
| <i>Taq</i> I             | T/CGA                | 65                         |
| <i>Mwo</i> I             | GCNNNNN/NNGC         | 60                         |
| <i>Hin</i> FI            | G/ANTC               | 37                         |

**Table 3. Accession numbers of the *cytb* sequences retrieved from databases**

| Species                                | Accession No.                                                                                                                                                                                     | No. of sequences |
|----------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| Water buffalo                          |                                                                                                                                                                                                   | 22               |
| <i>Bubalus bubalis</i>                 | D88629, D88628, D82893, D88632, D88983, D88630, D88627, D82892, D88631, D34638, D32193, D88634, D82894, D88636, D88633, D88635, D34637, D88637, D88638, X84906, X78960, AY079132                  | 22               |
| Goat                                   |                                                                                                                                                                                                   | 14               |
| <i>Capra hircus</i>                    | D84201, X56289, AB004073, AB004070, AB004072, AB004071, AB004075, AB004074, AJ231404, AJ231402, AF217254, AB044307, AB044308, AJ231403                                                            | 14               |
| Cow                                    |                                                                                                                                                                                                   | 39               |
| <i>Bos taurus</i>                      | D34635, J01394, V00654, AJ401081, NC_001567, AF490528, AB090987, AB090986, AB090984, AB090983, AB090982, AF493542, AB090980, AB090985, AB090981, AF172364, AF493541, AF490529, AF371961, AF281085 | 20               |
| <i>Bos grunniens</i>                   | AF091631, AY079129                                                                                                                                                                                | 2                |
| <i>Bos indicus</i>                     | Y16064, AF348597, AY079130, AB090979, AF419237                                                                                                                                                    | 5                |
| <i>Bos javanicus</i>                   | D82889, D34636, Y16058, Y16059, AY079131                                                                                                                                                          | 5                |
| <i>Bos frontalis</i>                   | AY079128                                                                                                                                                                                          | 1                |
| <i>Bos gaurus</i>                      | Y16057, AY079127, AF348596, AF348593                                                                                                                                                              | 4                |
| <i>Bos grunniens mutus</i>             | Y16063                                                                                                                                                                                            | 1                |
| <i>Bos indicus</i> x <i>Bos taurus</i> | AF376008                                                                                                                                                                                          | 1                |
| Sheep                                  |                                                                                                                                                                                                   | 17               |
| <i>Ovis aries</i>                      | AF034730, D84203, NC_001941, AF010406, D84205, X56284, AB006800, AF172365, AJ401083, S49215, AF217257                                                                                             | 11               |
| <i>Ovis ammon darwini</i>              | AF034727, AF242350                                                                                                                                                                                | 2                |
| <i>Ovis ammon ammon</i>                | AF242349                                                                                                                                                                                          | 1                |
| <i>Ovis dalli dalli</i>                | AF034728                                                                                                                                                                                          | 1                |
| <i>Ovis vignei</i>                     | AF034729                                                                                                                                                                                          | 1                |
| <i>Ovis dalli</i>                      | U17860                                                                                                                                                                                            | 1                |

## Results and Discussion

### Retrieval and Analysis of *Bubalus*, *Capra*, *Bos*, and *Ovis cytb* Sequences

The nucleotide sequence search system Entrez (*see Experimental*) and the BLASTn option of the BLAST program (13) were used to retrieve all the available *Bubalus*, *Capra*, *Bos*, and *Ovis cytb* gene sequences. In this way, an inventory of 92 *cytb* sequences was obtained (Table 3). Pairwise and multiple alignments of these sequences, performed by using the program ClustalW (14), allowed identification of 2 conserved regions of 17 bp and separated by a 241 bp region, including several polymorphic positions. The 2 conserved regions were used to design 2 universal oligonucleotides (referred to as Unifw and Unirev, *see Experimental*; Figure 1) that allowed PCR amplification of a 275 bp fragment of the *cytb* gene.

The entire region was then analyzed using the program Webcutter (*see Experimental*) to check the presence of recognition sites of each known restriction endonuclease in order to identify one or a combination of enzymes that could

generate species-specific restriction profiles. In this way, 3 endonucleases (*Hae*III, *Taq*I, and *Mwo*I) generating the theoretical fragments (Table 4) were selected for further RFLP experiments. The analysis of data revealed that: (1) none of the enzymes by itself allowed discrimination of the 4 species; (2) *Hae*III generated a 230 bp fragment peculiar to the goat *cytb*; (3) the water buffalo and goat amplicons yielded specific *Taq*I fragments of 179 and 206 bp, respectively, which can be used for their recognition; (4) the presence of cow DNA in food products can be easily established by the presence of a 160 bp fragment in the *cytb*/*Mwo*I profile; (5) the discrimination of sheep from cow DNA is obtained by comparing the *Hae*III and *Mwo*I restriction profiles: the identification of sheep can be determined when the 46 bp *Mwo*I fragment and 159-114 bp *Hae*III fragments are present but the 160 bp *Mwo*I fragment is absent; (6) the sequence AY079131 (*Bos javanicus*), showing an alternative *Taq*I restriction site that yielded to fragments identical to the buffalo profile, was no longer taken into account because of the world distribution (Thailand, Malaysia, Java, and Borneo) and natural habitat (forest) of this species; (7) the combination

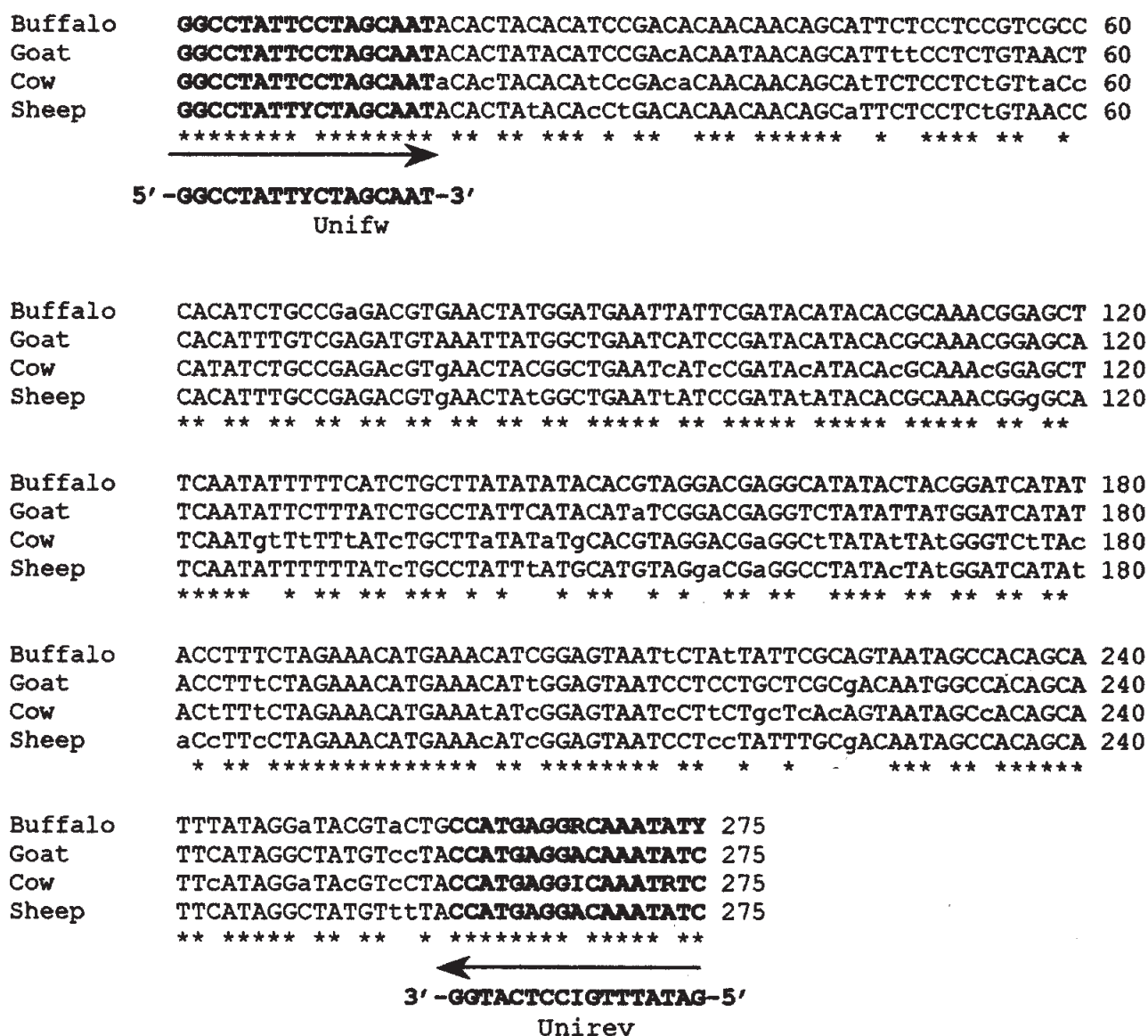


Figure 1. ClustalW multi-alignment of *cytb* sequences of the consensus sequence of each species in the primer regions (*Bubalus*, *Capra*, *Bos*, and *Ovis*, respectively). Asterisks correspond to conserved nucleotides in all sequences, whereas lowercase letters in the representative sequences correspond to not-conserved nucleotides in a given species.

of selected restriction enzymes allowed identification of each species in any 2-compound mixed samples, detecting food origin from 2 different sources (Table 5).

#### PCR Amplification and Restriction Analysis of the *cytb* Gene

The experimental system was first set up on standard DNA. For this purpose, the PCR parameters were varied in order to find the optimal ones. The amplification of the *cytb* fragment performed at an annealing temperature ( $T_a$ ) of 44°C and 1mM  $Mg^{2+}$  allowed amplification of a single fragment of 275 bp from standard DNA (Figure 2). Variations of the  $T_a$

and/or in  $Mg^{++}$  concentration may result in the amplification of additional bands.

The reproducibility of the PCR amplification was tested by performing the same PCR 3 times on 3 different sets of standard DNA. In all tests, we obtained the amplification of a single fragment of 275 bp (data not shown).

In order to exclude the accidental amplification of DNA from human operators, the system was also tested using DNA extracted from human blood cells as negative control. No amplification fragment was detected (data not shown), according to the low annealing ratio observed by comparing

**Table 4. Theoretical DNA fragments generated after treatment of 275 bp *cytb* amplified DNA with *Hae*III (H), *Taq*I (T), or *Mwo*I (M)<sup>a</sup>**

| Restriction enzyme | Restriction fragment | Water buffalo | Goat     | Cow      | Sheep    |
|--------------------|----------------------|---------------|----------|----------|----------|
| T                  | 275                  |               |          | o        | x        |
| H                  | 273                  | x             |          | o        |          |
| H                  | 230                  |               | <b>x</b> |          |          |
| T                  | 206                  |               | <b>x</b> |          |          |
| T                  | 179                  | <b>x</b>      |          |          |          |
| M                  | 160                  |               |          | <b>x</b> |          |
| H                  | 159                  |               |          | o        | <b>x</b> |
| M                  | 115                  | x             | x        | x        | x        |
| H                  | 114                  |               |          | o        | <b>x</b> |
| M                  | 114                  | x             | x        |          | x        |
| T                  | 96                   | <b>x</b>      |          |          |          |
| T                  | 69                   |               | <b>x</b> |          |          |
| M                  | 46                   | o             | x        |          | x        |
| H                  | 43                   | x             | o        |          |          |
| M                  | 35                   | o             |          |          |          |
| M                  | 11                   | o             |          |          |          |
| H                  | 2                    | x             | x        | x        | x        |

<sup>a</sup> Species-specific restriction fragments are represented in bold, whereas fragments generated in only 1 or few representatives of a given species are represented by o.

primers Unifw/Unirev with all the available human *cytb* sequences.

The amplified *cytb* DNA from the 4 species of interest was then digested with *Hae*III, *Taq*I, and *Mwo*I, and the restriction products were analyzed by agarose gel electrophoresis. Figure 3A shows the restriction patterns obtained, which are completely in agreement with the expected ones: *Taq*I yielded 179 and 96 bp fragments for water buffalo and produced fragments of 206 and 69 bp for goat; *Mwo*I originated a 160 bp fragment when cattle DNA was present, and *Hae*III allowed specific recognition of sheep (159 and 114 bp bands).

When the same experiments were performed on DNA extracted from meat or dairy products, they gave identical results (not shown), suggesting that this experimental procedure might be successfully applied to species identification in dairy products.

In order to check the reliability of the system on mixtures of DNA from different species, the DNA from the 4 species were mixed in the most common pairwise combinations (Figure 4). A single 275 bp PCR amplification fragment was obtained by using primers Unifw/Unirev (data not shown). Then, 10 µL of each amplification product was digested with *Hae*III, *Taq*I, and *Mwo*I and analyzed by agarose gel electrophoresis. Data obtained are reported in Figure 4 and revealed that in all mixed samples each species can be easily identified by its specific restriction fragment. No additional or unexpected fragment was observed.

As reported above, the *cytb* analysis represents a very powerful tool in species identification from food products.

Conserved primers for the amplification of a fragment of the mitochondrial *cytb* gene were identified and applied to species identification by sequencing (11, 15–17) or PCR-RFLP techniques (9, 10, 12). The latter ones, which are simpler and less time-consuming than sequencing, allowed quick analysis

**Table 5. Restriction DNA fragments observed on agarose gel after digestion of 275 bp *cytb* amplified DNA with *Hae*III, *Taq*I, or *Mwo*I**

| Species       | Size of restriction fragments (bp) generated after digestion |              |              |
|---------------|--------------------------------------------------------------|--------------|--------------|
|               | <i>Hae</i> III                                               | <i>Taq</i> I | <i>Mwo</i> I |
| Water buffalo | 273                                                          | 179          | 115          |
|               | 2                                                            | 96           | 114          |
|               |                                                              |              | 46           |
| Goat          | 230                                                          | 206          | 115          |
|               | 43                                                           | 69           | 114          |
|               | 2                                                            |              | 46           |
| Cow           | 273                                                          | 275          | 160          |
|               | 2                                                            |              | 115          |
| Sheep         | 159                                                          | 275          | 115          |
|               | 114                                                          |              | 114          |
|               | 2                                                            |              | 46           |

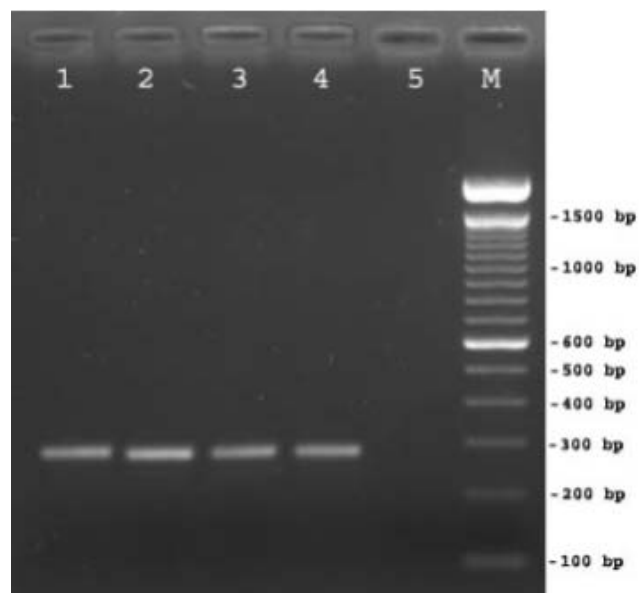


**Figure 2.** Agarose gel electrophoresis of the 275 bp *cytb* DNA fragment from water buffalo (lane 1), goat (lane 2), cow (lane 3), and sheep (lane 4), amplified via PCR by using the Unifw/Unirev primers pair. A negative control is included in lane 5. M: Molecular weight ladder (100 bp ladder, Gibco BRL).

of many vertebrate species and were used mostly to determine species origin.

The aim of the present work was to develop a more clear and reliable PCR-RFLP method than those currently available, specific for dairy industry needs. To this purpose, all the available *cytb* gene sequences were retrieved from databases in order to check the inter- and intraspecific variations occurring within *cytb* sequences. The analysis of the 92 *cytb* sequences belonging to *Bubalus*, *Capra*, *Bos*, and *Ovis* allowed identification of a pair of universal primers, which then allowed amplification of a 275 bp fragment from both standard DNAs and DNAs extracted from derivatives or meat. Moreover, the efficiency of the amplification with the primers pair Unifw/Unirev (that may vary from species to species depending on the number of mismatches in the annealing region) was comparable in each species, allowing analysis of mixed samples (Figures 1 and 2). The *in silico* simulation of restriction analysis of the 275 bp amplified regions was developed, and 3 endonucleases (*HaeIII*, *TaqI*, and *MwoI*), which generate species-specific restriction patterns, were selected for the identification of mixed double-compound samples. After an amplification step, DNAs from different sources were digested with selected endonucleases, and the expected restriction profiles were obtained (Figures 3a and 4).

The design of the new couple of specific primers described in this work for the 4 species considered overcame some problems of the method previously described by Meyer et al. (10), as revealed by comparing the performance of the 2 methods on the same DNA samples. For this purpose, the PCR amplification of the *cytb* gene was obtained by using

primers Unifw and Unirev or the couple of primers L14841 and H15149 first identified by Kocher et al. (11). As shown in Figures 2 and 5, the set of primers described in this work gave a comparable signal from all species (Figure 2), whereas the PCR performed by using the protocol by Meyer et al. (10) showed a different specificity of the 2 primers in each species (Figure 5), which, in turn, is due to the mismatch ratio in the target sequence of the 4 different species.

The restriction analysis performed using the enzymes *HaeIII*, *TaqI*, and *HinfI* selected by Meyer et al. (10) revealed the presence of additional fragments for goat or sheep amplicons (Figure 3B), which were probably due to a digestion of an additional amplification fragment.

Finally, the *in silico* simulation of restriction analysis revealed that the *HinfI* restriction sites were not conserved among different representatives of the same species (*see*, for instance, the *Ovis* sequences AF034730, AF010406, X56284, AB006800, AF172363, AJ401083), and no *HinfI* restriction sites were present in both sheep (accession D84203, NC\_001941, D84205, S49215, AF034727, AF034729, AF242350, AF242349) and cattle sequences (accession AY079129, AY079131, AY079128, AF348596, Y16057, AF348593, AY079127, AF091631, D82889, D34636, Y16058, Y16059, Y16057, Y16063, Y16064), compromising the identification of cow DNA.

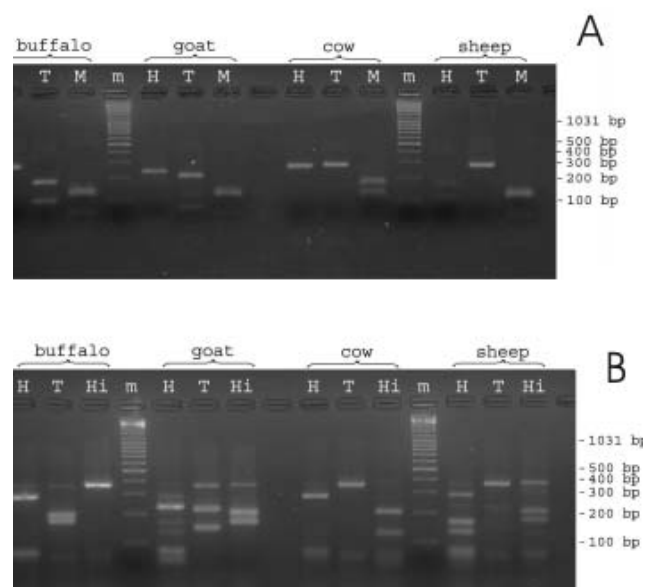


**Figure 3.** (A) Agarose gel electrophoresis (3%, w/v) of the restriction fragments obtained by digestion of the 275 bp amplified DNA with *HaeIII* (H), *TaqI* (T), and *MwoI* (M) of buffalo (b), goat (g), cow (c), and sheep (s) standard DNA. (B) Agarose gel electrophoresis (3%, w/v) of the restriction fragments obtained by digestion of the 359 bp amplified DNA with *HaeIII* (H), *TaqI* (T), and *HinfI* (Hi) of buffalo (b), goat (g), cow (c), and sheep (s) standard DNA. M: Molecular weight ladder (Gene Ruler DNA Ladder Mix, Genenco).

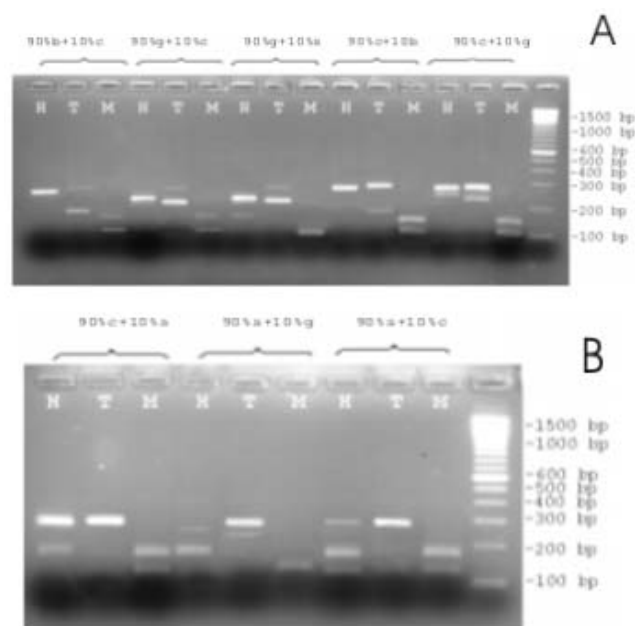


**Figure 4.** (A) Agarose gel electrophoresis (3%, w/v) of the restriction fragments obtained by digestion of the 275 bp amplified DNA with *Hae*III (H), *Taq*I (T), and *Mwo*I (M) of buffalo (b), goat (g), cow (c), and sheep (s) standard DNAs, mixed in a 2-compound sample (90% in 10%). Last lane: molecular weight ladder (100 bp ladder, Gibco BRL). (B) Agarose gel electrophoresis (3%, w/v) of the restriction fragments obtained by digestion of the 275 bp amplified DNA with *Hae*III (H), *Taq*I (T), and *Mwo*I (M) of buffalo (b), goat (g), cow (c), and sheep (s) standard DNAs, mixed in a 2-compound sample (90% in 10%). Last lane: molecular weight ladder (100 bp ladder, Gibco BRL).

The whole body of data obtained suggested that the updated PCR-RFLP method described in this work overcame most of the previous problems, allowing identification of species origin in 2-compound mixed samples, even if, in some cases, it might fail in a 3-compound samples identification. However, the rarity of milk substitutions from 2 additional species (in a 3-compound mixed sample) makes the system suitable for routine analyses for the detection of food substitutions concerning the 4 species taken into account. The amplification of a short DNA fragment (275 bp) allows species identification even when the DNA samples are degraded or fragmented as a result of prolonged heat treatments or intensive homogenization processes; moreover, species-specific restriction patterns are characterized by a low number of fragments, thus simplifying interpretation. Finally, the design of new primers based on the analysis of several available sequences appears to guarantee good control of intraspecific variability that should avoid the possibility of false-positive or false-negative results.

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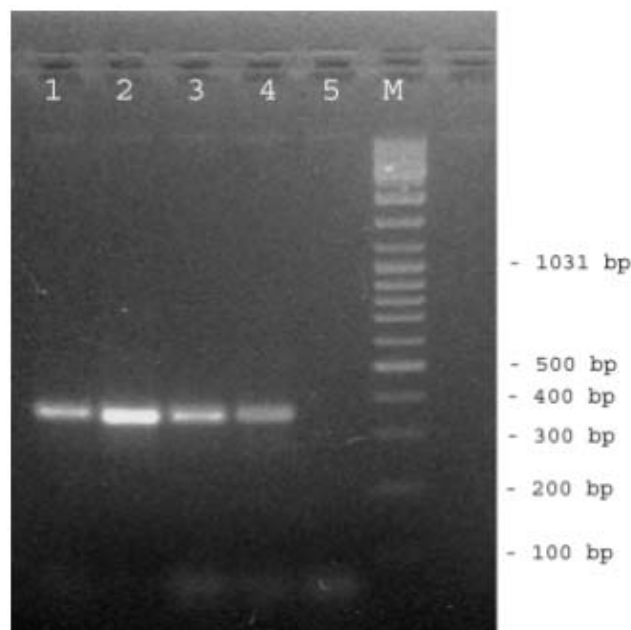


**Figure 5.** Agarose gel electrophoresis of the 359 bp *cytb* DNA fragment from buffalo (lane 1), goat (lane 2), cow (lane 3), and sheep (lane 4), amplified via PCR by using the L14841/H15149. A negative control is included in lane 5. M: Molecular weight ladder (Gene Ruler DNA Ladder Mix, Genenco).

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