Identification of Species in Animal Feedstuffs by Polymerase Chain Reaction−Restriction Fragment Length Polymorphism Analysis of Mitochondrial DNA

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Restriction site analysis of Polymerase Chain Reaction (PCR) products of cytochrome *b* mitochondrial DNA was applied to identify species in meat meal and animal feedstuffs. PCR was used to amplify a variable region of cytochrome *b* mitochondrial DNA gene. Species differentiation was determined by digestion of the obtained 359 bp amplicon with restriction enzymes, which generated species-specific electrophoresis patterns; the sequencing of PCR products was used as confirming analysis. PCR-RFLP analysis revealed the presence of meat meal in animal feedstuffs and distinguished species of interest. The results supported the application of the method in control measures which should be adopted for meat-meal-based animal feed, as suggested by EU law. As a technical improvement, to simplify the analysis, the number of enzymes presented in this study for the detection of different species was smaller than others described in the literature; discrimination between ruminant and nonruminant species and between mammalian and poultry species was possible with few digestions.

Keywords: *Species identification; meat meal; feedstuffs; PCR; cytochrome b gene*

INTRODUCTION

The risk of occurrence of bovine spongiform encephalopathy (BSE) results not only from infected or incubating animals but also from the use of concentrated feed or its incriminating ingredient, mammalian meat and bone meal, contaminated with the causal agent.

The end products in the rendering process—conversion of animal waste to meat and bone meal—are a lipid fraction and a protein fraction, which may be the carrier of any infectious agent present in raw material that was not destroyed by the heating process (*1*).

This is why feeding ruminant proteins to ruminants has been prohibited since 1988; later Commission Decision 94/381/EC (*2*) banned in Member States the feeding of protein derived from mammalian tissues to ruminant species. However, with the same decision, the Commission may authorize the feeding of proteins from species other than ruminants to ruminants in Member States where a method to distinguish between mammalian protein derived from ruminant and nonruminant species has been enforced.

Cross-contamination of feedstuffs for ruminant with mammalian meat meal used in animal feed for other species is one of the main problems for feed manufacturing industries, which are very interested in adopting a control process program to monitor this processing cross-contamination.

Traditionally, species identification had been established through different methods, which may be chosen for test applications depending on the purpose. Mainly they are based on the analysis of certain biomolecules, such as proteins—electrophoretic, isoelectric focusing, immunochemical, and HPLC methods—(*3*−*8*), fatty acids (*9*), and DNA (*10*−*13*), or they are based on specific microscopic structure elements determination (*14*).

Polymerase Chain Reaction (PCR)-based methods have given better results in responding to critical samples in which most of the DNA has been degraded. Recent analyses of genetic material using DNA techniques such as PCR (*10*, *11*, *13*, *15*, *16*), direct DNA hybridization (*12*, *17*−*19*) or sequencing (*15*, *20*) have been developed.

Besides coding sequences, the genome contains a large amount of repetitive noncoding sequences—interspersed nuclear and satellite elements—which provide excellent markers for DNA-based species identification techniques (*17*, *21*, *22*). Probes for hybridization protocol can be designed on species-specific satellite DNA (*12*, *17*, *19*).

Mammalian wide interspersed repeat elements (MIR) have been demonstrated to be suitable for the identification of species. PCR amplification with MIR specific primers and patterns obtained by polyacrylamide gel electrophoresis appeared to be species-specific and phylogenetically informative (*21*).

In previous studies restriction fragment length polymorphism (RFLP) analysis was described as a promising test for authentication of species in processed human food (*11*, *15*, *16*, *23*).

Here we report on the suitability of the PCR-RFLP technique for species identification in rendered animal material and commercial animal feedstuffs. Sequence identification of the PCR products was produced as confirming analysis.

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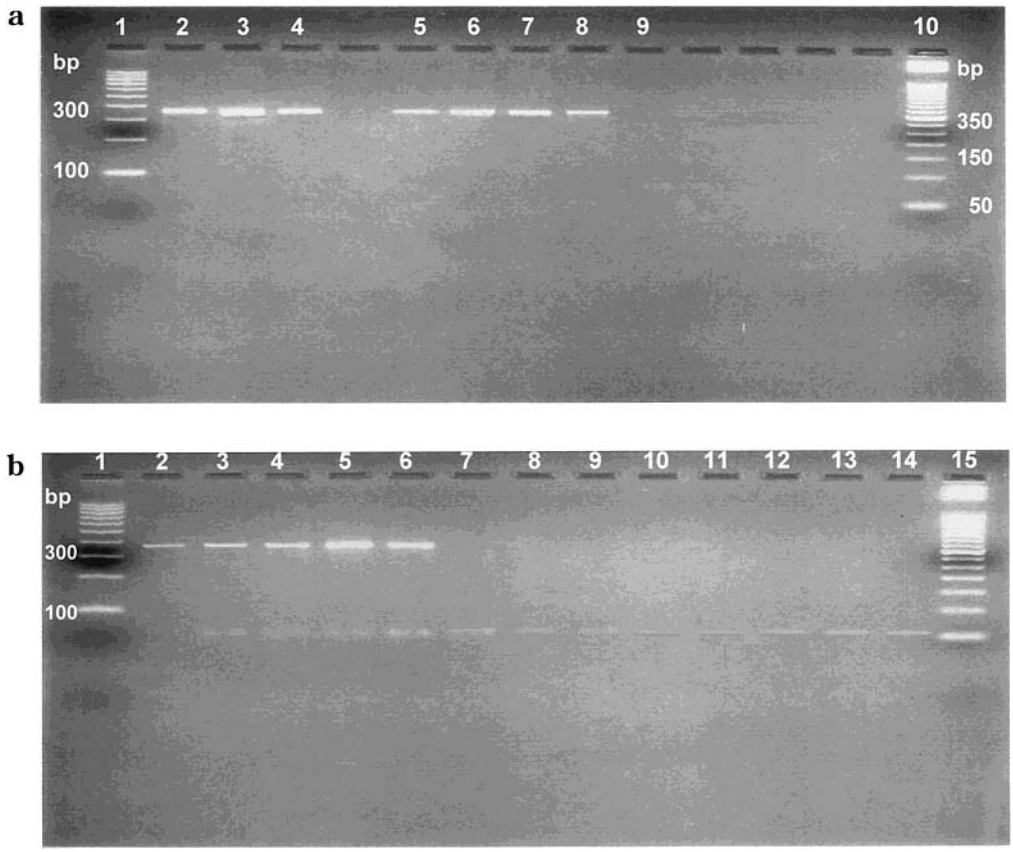

Figure 1. Electrophoretic analysis of cyt *b* PCR products: (a) samples (lane 1) 100 bp ladder, (lanes 2−4) meat meals, (lanes 5 and 6) feedstuffs with 0.5 and 1% of poultry meat, respectively, (lanes 7 and 8) feedstuffs with 0.5 and 1% of meat meal, respectively, (lane 9) control feedstuffs without meat meal, and (lane 10) ladder 50 bp; (b) samples (lane 1) ladder 100 bp, (lanes 2−6) feedstuffs with meat meal, (lanes 7−11) feedstuffs without meat meals, (lane 12) maize meal, (lane 13) soya meal, (lane 14) sunflower meal, and (lane 15) ladder 50 bp.

MATERIALS AND METHODS

Samples. *Reference Feedstuffs.* Reference feedstuffs had been purposely prepared by an experimental plant (Università Cattolica del Sacro Cuore, Facoltà di Agraria, Piacenza, Italy) and supplemented with poultry or meat meal at different percentages (0.5 or 1% w/w). A reference feedstuff except of any meat meal was included as a control negative sample.

Test Feedstuffs and Raw Materials. Feedstuff samples and raw materials (maize, sunflower, and soy meals) were supplied by a feed manufacturing industry (ASA srl, Verona, Italy).

Meat Meals. Meat meal samples were obtained on the market from different rendering industries.

Control DNA. Genomic DNA samples isolated from peripheral blood of different species (*Bos taurus*, *Bubalis bubalis*, *Ovis aries*, *Capra hircus*, *Sus scrofa*, *Equus caballus*, *Gallus gallus*, and *Meleagris gallopavo*) were used as control templates.

Nucleic Acids Extraction. *Blood Samples.* Blood samples (0.7 mL) were transferred into a 1.5 mL Eppendorf tube, and 0.8 mL of lysis buffer (150 mM NaCl; 15 mM sodium citrate·$2H_2O$; pH 7.0) were added. The tube contents were gently mixed and centrifuged at 12000*g* for 1 min, at ambient temperature. The leucocytes pellet was washed with 1 mL of lysis buffer and incubated for 2 h at 56 °C in 0.40 mL of extraction buffer (200 mM sodium acetate, pH 7.0; 1% sodium dodecyl sulfate), after the addition of 10 μL of a proteinase K solution (1% w/v). Total DNA was isolated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v, pH 8.0) and ethanol precipitation. The dried DNA pellet was dissolved in 0.1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Meat Meal Samples. Meat meal samples (10 g) were defatted as described by Bligh and Dyer (*24*), and then DNA was extracted from 200 mg of each meat sample, following the protocol described by Ausubel and co-workers (*25*).

Feedstuff Samples and Raw Materials. From defatted feedstuff samples (20 g) and raw materials (10 g) were taken 100 mg, and DNA was isolated using a commercial kit (QIAamp, QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocol.

DNA Quality/Quantity Analysis. The quality of the extracted DNA samples was examined by electrophoresis analysis through a 1% agarose gel (Ultrapure DNA grade agarose, Bio-Rad, Hercules, CA). DNA concentration was estimated by means of a spectrophotomers.

PCR Conditions. The universal primers used were shown as complementary to conserved regions of mitochondrial cytochrome *b* gene in vertebrata (*26*); the primer sequences used were as follows: F 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA and R 5′-GCC CCT CAG AAT GAT ATT TGT CCT CA (Life-Technologies, Gibco). PCR reaction yielded a 359 bp fragment, which included a 307 variable and informative region (*26*). Amplifications were carried out in a final volume of 50 μL containing 100−200 ng of extracted DNA, RedTaq 1× reaction buffer, 0.8 mM of dNTPs (Sigma, St. Louis, MO); 15 pmol of each primer, and 2.5 units of RedTaq polymerase (Sigma). The PCR reaction was performed with a Perkin-Elmer (GeneAmp PCR system 2400, PE Biosystem, Foster City, CA) thermal cycler, and the conditions were as follows: preheating step, 1 min/94 °C; cycling parameters, 5 s/94 °C, 30 s/50−55 °C, 40 s/72 °C, 20−30 cycles; final extension step, 2 min/72 °C.

Negative control (no DNA added) was also included in parallel with each amplification set.

Endonuclease Digestion and Restriction Fragment Length Analysis. An endonuclease screening on the amplified region of the cytocrome *b* gene was performed. PCR products (5−10 μL) were subjected to restriction endonuclease, digestions with 5 units of each enzyme (*Rsa*I, *Taq*I, *Bst*U I, *Bsa*J I,

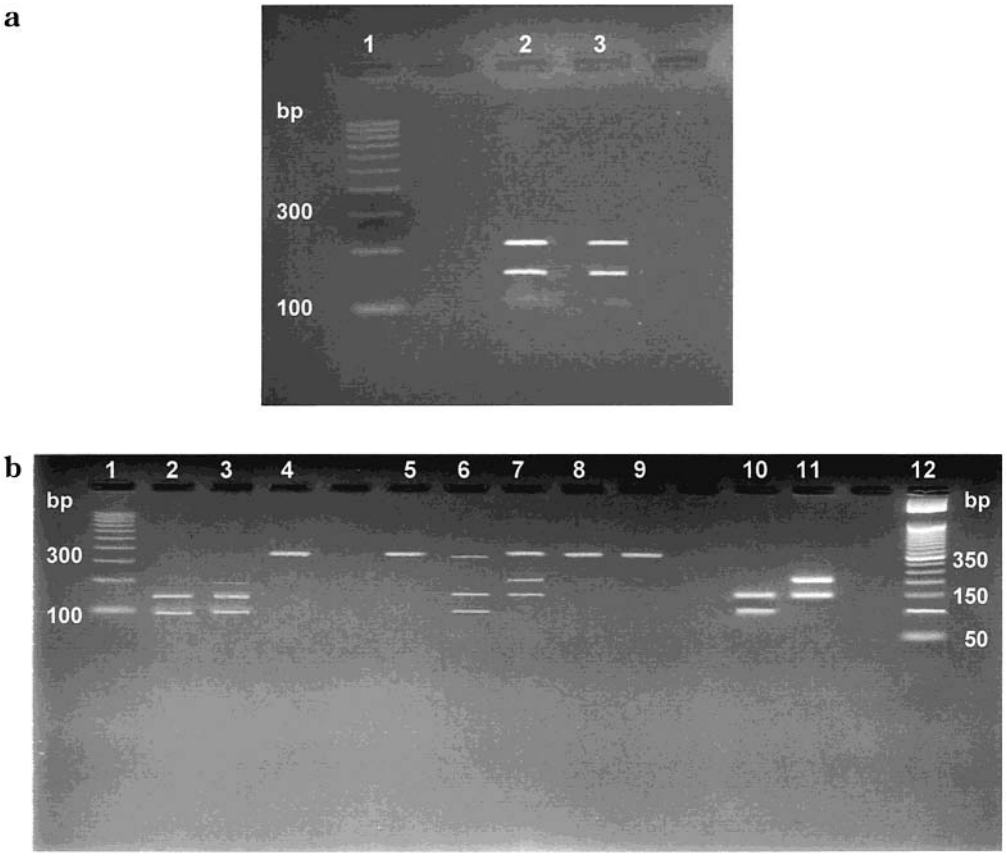

Figure 2. Restriction profiles of cyt *b* PCR amplicons digested with *Rsa*I (a) samples (lane 1) 100 bp ladder, (lanes 2 and 3) reference feedstuffs with 0.5 and 1% of poultry meat, respectively; (b) samples (lane 1) 100 bp ladder, (lanes 2−4) meat meals, (lanes 5−9) feedstuffs, (lane 10) turkey control DNA, (lane 11) chicken control DNA, and (lane 12) 50 bp ladder.

and *Bst*N I, New England Biolabs, Hertfordshire, U.K.) in a final reaction volume of 50 μL. Incubation temperature and time of reaction were chosen according to the manufacture's protocol. Digested samples (15−20 μL) were electrophoresed through 4% agarose gels [3% w/w, NuSieve (FMC, ME) and 1% w/w, Ultrapure DNA grade agarose Bio-Rad] in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5). The size of restriction fragments was estimated by 100 and 50 bp DNA ladder molecular weight markers (Sigma).

Sequencing of the Amplicons of the Cytochrome *b* Gene. Amplified fragments were excised by a sterilized scalpel blade and extracted from the gel using a commercial kit (QIAquick gel extraction kit, QIAGEN); 8 μL of the cleaned up DNAs was sequenced with the forward primer (3.2 pmol) described above under PCR Conditions in the presence of the ABI PRISM ready reaction big-dye terminator cycle sequencing kit (PE, Biosystem), following the manufacture's manual.

RESULTS AND DISCUSSION

PCR-RFLP Analysis. Among DNA-based methods, the hybridization technique has been tested on high-temperature-treated samples, giving species-specific identification results (*27*). In our experience this method was not suitable for our purposes (unpublished data); the low sensitivity obtained in our samples was due to the drastic thermal treatment adopted in the rendering process, which seriously compromised DNA quality.

Compared to other techniques for species identification by DNA-based methods, PCR-RFLP of mitochondrial DNA has offered the greatest advantage when applied to the analysis of short fragments of degraded DNA.

PCR products obtained from meat meals and reference feedstuff samples are shown in Figure 1a; amplicons produced from test and raw material samples PCR are presented in Figure 1b. PCR yielded the expected 359 bp amplicon in reference and test feedstuff (Figure 1a, lanes 5−8; Figure 1b, lanes 2−6) samples. The positive amplifications obtained in these feedstuff samples agreed with the declared presence of animal meat meal in their labels and supported the potential application of the PCR-based method in the detection of meat meal-based animal feed. Because the commercial kit used for the DNA extraction from feedstuff samples purified reasonably also DNA of plant origin, we tested the PCR on DNA extracted from raw material (soy, maize, and sunflower meals) that did not produce any characteristic fragments (Figure 1b, lanes 12−14). The absence of any PCR amplicons from raw material was otherwise expected because specificity of the primers used for the vertebrata DNA had already been reported (*20*, *26*).

To develop a PCR-RFLP method for improving the sensitivity of species detection, sequence analyses on the amplified portion of the cytocrome *b* gene were compared to define restriction endonucleases generating species-specific fragments. *Rsa*I enzyme produced characteristic PCR fragments only in avian species (chicken and turkey); *Rsa*I RFLP analysis was used to distinguish chicken and turkey from mammalian species. Chicken-specific fragments (149−210 bp) were detected in two reference feedstuffs with different amounts of poultry meat added (0.5 and 1% w/w) (Figure 2a); Figure 2b showed *Rsa*I RFLP electrophoretic patterns obtained in meat meals and test feedstuff samples. The enzyme digestion revealed the presence of species-specific fragments for turkey (109−149 bp) and chicken in meat

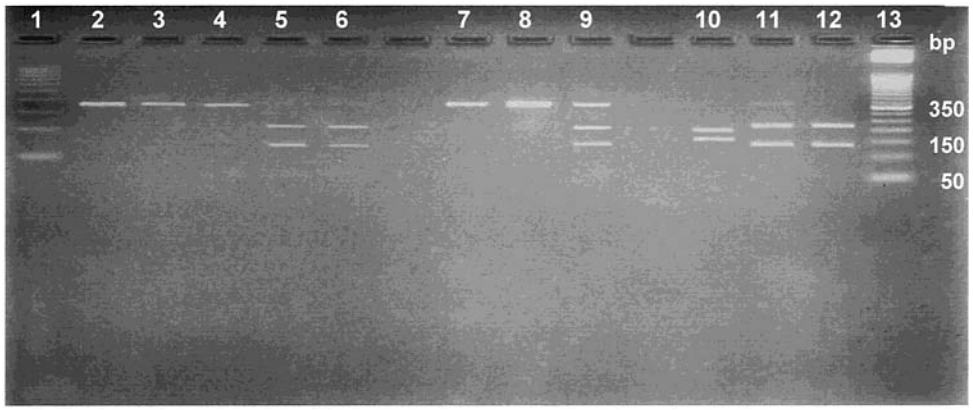

Figure 3. Restriction profiles of cyt *b* PCR amplicons digested with *Taq*I: samples (lane 1) ladder 100 bp, (lanes 2−6) feedstuffs, (lanes 7−9) meat meals, (lane 10) buffalo control DNA, (lane 11) goat control DNA, (lane 12) pig control DNA, and (lane 13) 50 bp ladder.

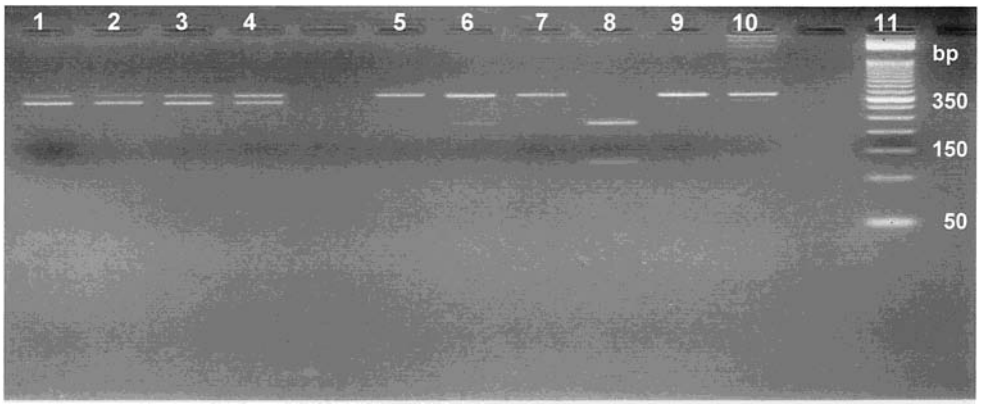

Figure 4. Restriction profiles of cyt *b* PCR amplicons digested with *Bsa*J I: samples (lane 1) cattle DNA, (lane 2) buffalo DNA, (lane 3) sheep DNA, (lane 4) goat DNA, (lane 5) chicken DNA, (lane 6) turkey DNA, (lane 7) horse DNA, (lane 8) pig DNA, (lanes 9 and 10) meat meals, and (lane 11) 50 bp ladder.

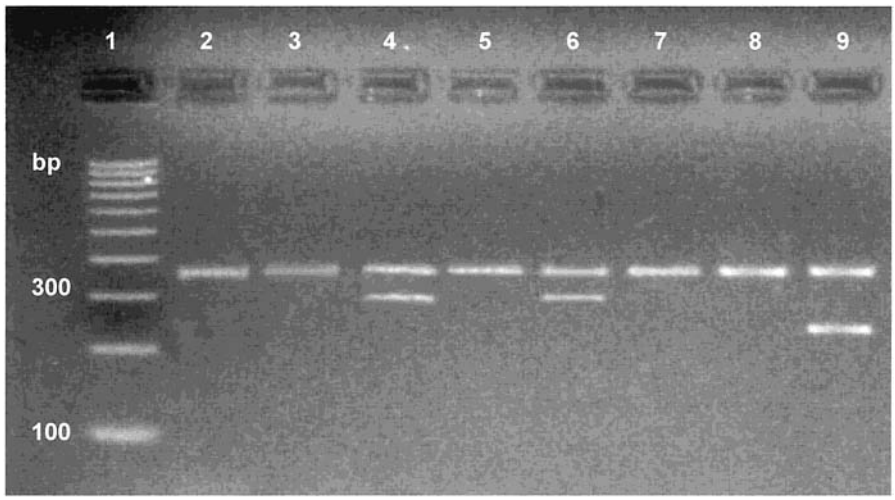

Figure 5. Restriction profiles of cyt *b* PCR amplicons digested with *Bst*U I: samples (lane 1) 100 bp ladder, (lane 2) cattle DNA, (lane 3) buffalo DNA, (lane 4) goat DNA, (lane 5) ship DNA, (lane 6) goat DNA, (lane 7) pig DNA, (lane 8) chicken DNA, and (lane 9) turkey DNA.

meals (Figure 2b, lanes 2 and 3) and in test feedstuff samples (Figure 2b, lanes 6 and 7), when the amplified PCR fragment was not completely digested. The failed and partial digestion shown in Figure 2b supported the presence of mammalian species in samples for which the PCR 359 bp products could be clearly distinguished.

Electrophoretic analysis of *Taq*I endonuclease digested amplicons showed the expected fragments for buffalo (168−191 bp), goat (139−220 bp), and pig (141−218 bp) as shown in Figure 3. From the *Taq*I patterns obtained from feedstuffs and meat meals we deduced the presence of pig and/or goat DNA in some of these samples (Figure 3, lanes 5, 6, and 9). *Taq*I digestion failed to accurately discriminate between goat and pig because the size of digested fragments are very similar (141−218 and 139−220 bp, respectively).

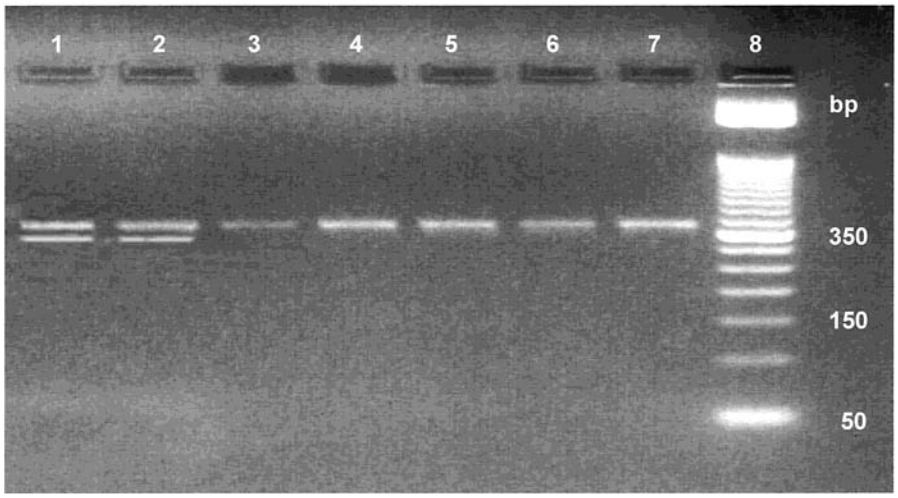

Figure 6. Restriction profiles of cyt *b* PCR amplicons digested with *Bst*N I: samples (lanes 1 and 2) cattle, (lane 3) ship DNA, (lane 4) goat DNA, (lane 5) pig DNA, (lane 6) chicken DNA, (lane 7) turkey DNA, and (lane 8) 50 bp ladder.

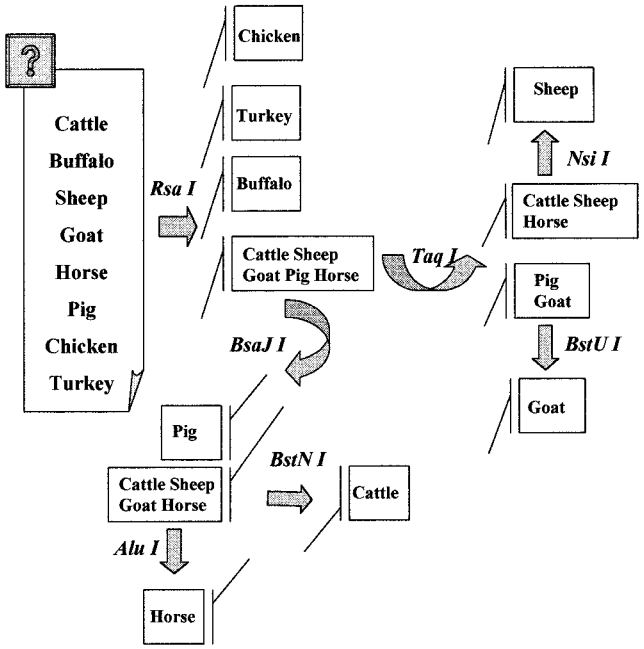

Figure 7. Flowchart of endonucleases digestion.

Discrimination between pig and ruminant species was achieved by *Bsa*J I digestion of PCR products (Figure 4), which generated 131−228 bp (lane 8) and 320 bp (lanes 1−4) fragments, respectively.

Further investigation to identify ruminant species was obtained by digestion with other restriction enzymes (*Bst*U I and *Bst*N I), as shown in Figures 5 and 6.

*Bst*U I digestions allowed the identification of goat DNA, producing a 296 bp species-specific fragment (Figure 5, lanes 4 and 6); furthermore, the enzyme produced a characteristic electrophoretic pattern from turkey DNA (lane 9). *Bst*N I endonuclease digestion produced a 318 bp species-specific fragment for cattle (Figure 6, lanes 1 and 2).

Figure 7 illustrates in summary a species-specific typing approach that successfully discriminates sources of animal meal on the basis of restriction fragment length polymorphisms detected by PCR.

Sequencing Analysis and Species Identification. The combination of PCR and sequencing of amplicons as an identification tool in discriminating species was described by Barlett and Davidson (*20*). As previously reported in the literature, the amount of intraspecific variation of cytochrome *b* is less than between species and does not preclude the identification of even closely related species (*28*).

The sequencing of PCR fragments obtained from meat meal and feedstuff samples was used to confirm our previous results obtained with endonuclease digestion. Sequences were compared against all entries of the nonredundant GeneBank database sequences, by means of the BLASTN program (*29*), available at http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/.

Sequence alignments obtained from meat meal and feedstuff samples are shown in Figure 8. Although in feedstuff samples the sequenced fragments showed many nucleotide ambiguities, related to the thermal treatment adopted in the rendering process, intraspecies polymorphisms of amplified fragments allowed detection of species.

CONCLUSIONS

The recent European crisis related to mad cow disease has focused attention on the importance of control measures in animal feed content to avoid the risk of BSE diffusion through contaminated meat-based animal feedstuffs.

Compared to other techniques in species identification by DNA analysis, PCR-RFLP methods of identification of meal sources in animal feed appear to be promising.

Despite the considerable DNA degradation that occurs in the rendering process, PCR was able to amplify even the low number of DNA molecules extracted from feedstuffs.

A particular combination of previously designed primers (*26*), complementary to a conserved region of the vertebrate cytochrome *b* gene, allowed the detection of the presence of animal meat meal in feedstuffs, excluding the contamination from nucleic acid of plant origin present in extracted DNA. The amplification of this region of mitochondrial DNA fits our purposes mainly for three reasons: (i) the high number of copies per cell of mt-DNA; (ii) its mutation rate, which induces substantial genetic interspecies variation; and (iii) the length of fragment, which is long enough to distinguish

Sample : meat meal

```
gb|L08381|TKYMTCYTBA Meleagris gallopavo mitochondrion cytochrome b gene,
complete cds.
           Length = 1143

 Score =  410 bits (207), Expect = e-113
 Identities = 222/226 (98%), Gaps = 1/226 (0%)
 Strand = Plus / Minus


Query: 1   atagcctacaaaggctgttgctatgagggtgagnagtaagact-ctcctgtatttcaggt 59
           |||||||||||||||||||||||||||||||| ||||||||| ||||||||||||||||
Sbjct: 396 atagcctacaaaggctgttgctatgagggtgagaagtaagactactcctgtatttcaggt 337


Query: 60  ttctttatataggtacgaaccataatataggccgcgtccaatgtgtaggaagatgcagat 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 336 ttctttatataggtacgaaccataatataggccgcgtccaatgtgtaggaagatgcagat 277


Query: 120 gaagaagaatgaggcgccattcgcatggaggttacggaggagtcaaccgtattgtacgtt 179
           ||||||||||||||| |||||||||||||||| |||||||||||||||||||||||||
Sbjct: 276 gaagaagaatgaggccccattcgcatggaggttatggaggagtcaaccgtattgtacgtt 217


Query: 180 tcggcatgtgtaggccacagaagagaatgcaagagtggtgtctgca 225
           |||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 216 tcggcatgtgtaggccacagaagagaatgcaagagtggtgtctgca 171
```

Sample : feedstuff

```
gb|L08381|TKYMTCYTBA Meleagris gallopavo mitochondrion cytochrome b gene,
complete cds.
           Length = 1143

 Score =  147 bits (74), Expect = 4e-34
 Identities = 80/83 (96%)
 Strand = Plus / Minus


Query: 1   gctatgagngtgagnagnaagactactcctgtatttcaggtttctttatataggtacgaa 60
           |||||||| ||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 377 gctatgagggtgagaagtaagactactcctgtatttcaggtttctttatataggtacgaa 318


Query: 61  ccataatataggccgcgtccaat 83
           |||||||||||||||||||||||
Sbjct: 317 ccataatataggccgcgtccaat 295
```

Figure 8. Alignment of PCR amplicon sequences with sequences of cytochrome *b* gene database (GenBank) using BLASTN.

congeneric organisms and at the same time short enough to allow amplification also from highly degraded DNA.

Although intraspecific variation should be taken into account in the interpretation of RFLP data, the amount of this variation did not preclude the identification of even closely related species. Despite the informative restriction enzymes used in this study being only a few among endonucleases that could be used to characterize all species, this choice was found to be more convenient because with few digestions it is possible to discriminate between ruminant and nonruminant species and between mammalian and poultry species.

The methodological approach described may be carried out in different steps: (i) identification of animal products in feedstuffs; (ii) discrimination between mammalian and nonmammalian species in animal feed; and (iii) identification of different species in feedstuffs.

Nucleotide sequencing of the amplified fragment was used as a confirming analysis.

Further investigation on this assay is being carried out in our laboratory mainly to test which are the most informative and better responding enzymes in discriminating different species. The sensitivity of the proposed approach will be developed toward an improved quantitative analytical assay.

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