Establishment and Application of a Fluorescent Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) Method for Identifying Porcine, Caprine, and Bovine Meats

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A method of fluorescent Polymerase Chain Reaction–restriction fragment length polymorphism (PCR-RFLP) was applied as an analytical and quantitative tool for meat identification. Following alignments of the nucleotide sequences, an oligonucleotide primer pair was designed to amplify the partial sequences within the 12S ribosomal RNA (12S rRNA) gene of mitochondrial DNA from porcine, caprine, and bovine meats. No fragment can be amplified from dog, cat, fish, duck, goose, turkey, and chicken DNA with the primer pair. Using fluorescence sensor capillary electrophoresis, the species-specific DNA fingerprints of pork, goat, and beef were generated by restriction enzyme digestion following a fluorescence-labeling PCR amplification. Species identification was conducted on the meat mixtures. The reliably semiquantitative levels were below 1% for binary mixtures of pork, goat, and beef. Cooking and autoclaving of meats did not influence the generation of the PCR-RFLP profiles or the analytical accuracy.

KEYWORDS: Meat identification; PCR-RFLP; mitochondrial DNA; 12S ribosomal RNA gene

INTRODUCTION

Identifying the species of origin of meat represents a considerable problem for food and animal feed analysts, game law enforcement authorities, and also those seeking to comply with certain religious regulations. The food analyst is confronted with providing proof of fraudulent substitution of more expensive meats with cheaper meats. Identification of the meat species is a prerequisite for the regulatory control of such products. Moreover, recent consumer concern relating to bovine spongiform encephalopathy (BSE) has increased awareness regarding the composition of food products and animal feed.

The past decade has seen DNA replace protein in species identification due to its stability at high temperatures and the fact that its structure is conserved within all tissues of an individual (*1*). DNA-based methods are based on the presence of species-specific sequences of DNA in meat and the possibility of detecting such sequences specifically. This has resulted in the development of species-specific DNA probes (i.e., DNA hybridization) (*2−5*), Polymerase Chain Reaction (PCR) assays (*6−9*), random amplified polymorphic DNA (RAPD) (*10−13*), and PCR−restriction fragment length polymorphism (RFLP) (*7, 14−16*).

PCR-RFLP allows the amplification of a conserved region of DNA sequence using PCR and the detection of genetic variation among species by digestion of the amplified fragment with restriction enzymes. This technique has been used for speciation by exploiting DNA sequence variation within the mitochondrial DNA (mtDNA) including the D-loop region (*17*) and cytochrome *b* (Cytb) gene (*14, 16*). Meyer et al. (*14*) have applied the PCR-RFLP analytical method based on mitochondrial Cytb to the detection of pork in both fresh and heated mixtures of pork and beef at sensitivity levels of <1%. A similar method using Cytb PCR-RFLP was also applied to the identification of both cooked and uncooked meats but was unsuitable for analyzing meat mixtures (*16*).

As a technical improvement, this study developed and applied a method of fluorescent PCR-RFLP based on the mitochondrial 12S ribosomal RNA (rRNA) gene as an analytical and quantitative tool for meat identification. The method can accurately identify the species of meat from commercially important species, including pigs, goats, and cows, and can be potentially developed to reliably quantify meat mixtures of these three domestic species.

MATERIALS AND METHODS

Meat Samples. Samples of raw meats (fresh or frozen) of whole muscle products for investigation were purchased from local supermarkets. A total of six samples each of pork, goat, and beef were used,

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along with two to four individuals of reference species, including dog, cat, fish, duck, goose, turkey, and chicken in this study.

Meat samples for heat or autoclaving treatments were cut into slices ~2 mm thick and packed in sealed plastic bags. Meat samples were heated at 100 °C in boiling water for 30 min or autoclaved at 121 °C and 15 lb/cm^2 for 30 min.

Preparation of Genomic DNA. Total cellular DNA was extracted from meat using the Wizard Genomic DNA purification kit (Promega, Madison, WI). For each DNA preparation, 1 g of meat was washed with sterile water and cut into small pieces. The tissue was homogenized for 1 min with a Waring blender; 0.1 g of homogenate was then transferred to 2 mL of centrifuge Eppendorf, washed again with sterile water, and centrifuged at 6000*g* for 5 min. The pellets derived from meat homogenates were suspended in 0.6 mL of nuclei lysis buffer (Promega), and DNA extraction was carried out according to the manufacturer's protocol for tissue samples.

The concentration of DNA in the samples was measured by UV absorption spectrophotometry at a wavelength of 260 nm, at which wavelength DNA absorption of OD_{260} peaks at a concentration of ~40 μg/mL of duplex DNA.

Primers. The oligonucleotide primers of PCR were designed from published sequences of porcine (*18*, *19*; GenBank accession no. AF034253), caprine (GenBank accession no. M55541), and bovine (GenBank accession no. J01394) mitochondrial 12S rRNA genes with the following sequences:

Mt12S-6F (17 mers): 5′-GCCAGCCACCGCGGTCA-3′

Mt12S-6R (20 mers): 5′-CTTACCTTGTTACGACTTGC-3′

Oligonucleotides were synthesized by the β-phosphoramidite method with Acy-clone plus DNA synthesizer (ABI 391, Perkin-Elmer, Boston, MA) and purified on an Oligo-Pak column. The expected lengths of the amplified fragment of DNA for pork, goat, and beef are all ~0.7 kb.

PCR. Each PCR amplification reaction was set in a volume of 25 μL containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, 100 mM of each dATP, dCTP, dGTP, and dTTP, 0.5 unit of *Taq* DNA polymerase (HT Biotechnology, Cambridge, U.K.), 0.1 μM of each primer, and 100 ng of template DNA. The reaction mixtures were preheated to 94 °C for 5 min to denature the template DNA completely, and then 36 cycles of amplification were run using a 9600 DNA amplifer (Perkin-Elmer) as follows: denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s.

For the fluorescent capillary electrophoresis, the PCR amplification was the same as the above conditions except the number of PCR cycles was reduced to 22 and 1 μM of fluorescent [R6G]dCTP (Applied Biosystems, Foster City, CA) was added to the PCR reaction mixture.

RFLP Analysis. Eight microliters of each PCR amplicon was digested with 1 unit of restriction enzymes *Alu*I, *Dde*I, or *Rsa*I (Promega) in a 10 μL reaction volume at 37 °C for 1 h. Subsequently, 1 μL of each PCR-RFLP reaction product was combined with 0.2 μL of ROX-1000 DNA marker (Applied Biosystems), and then the mixture was subjected onto a 3% GeneScan polymer gel using an ABI Prizm 310 genetic analyzer (Applied Biosystems). The results were collected and analyzed using GeneScan analysis software (Applied Biosystems).

Sequencing. The PCR product was sequenced to confirm the analyses. Meanwhile, double-stranded DNA for cycle sequencing was obtained from the PCR fragments by purification with DNA spin columns (Qiagen). Both strands of the PCR fragments were sequenced using a Taq DyeDeoxy Terminator Cycle sequencing kit and 373A DNA sequencer (Applied Biosystems).

Preparation of Meat Mixtures. Meat samples were prepared that were 1, 2, 5, 10, and 50% binary mixtures of pork, goat, and beef. The mixing process was as follows. The meats were sampled and weighed on the basis of the expected percentage. A total of 20 g of binary mixture of meats was ground and well mixed, and then 1 g was weighed of each mixture for DNA extraction. The total cellular DNA of each mixture was extracted and applied to the fluorescent PCR-RFLP analysis.
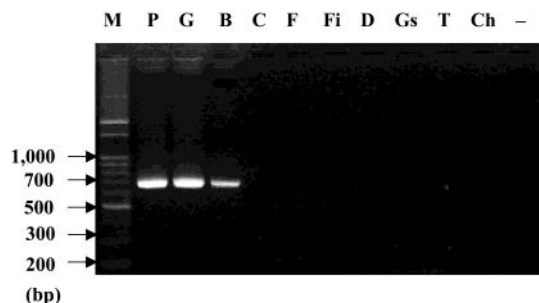

Figure 1. Agrose gel electrophoresis of the PCR products amplified from nine species: P, pork; G, goat; B, beef; C, canine; F, feline; Fi, fish; D, duck; Gs, goose; T, turkey; Ch, chicken. Lane (–) is a PCR product amplified from a reaction solution without any template DNA. M is a molecular marker, and the sizes are as indicated.

RESULTS

Specificity of the PCR. Using the Mt12S-6F/Mt12S-6R primer pair, a single PCR amplicon corresponding in size to the predicted 0.7 kb was observed following electrophoresis for the total cellular DNAs extracted from raw, cooked, or autoclaved samples of pork, goat, and beef. In contrast, PCR amplifications using dog, cat, fish, duck, goose, turkey, and chicken DNA all failed to generate PCR amplicons of any size (**Figure 1**).

The PCR product was sequenced to confirm the analyses. The nucleotide sequences of the porcine, caprine, and bovine species tested in this study were 693, 691, and 692 bp, respectively. The alignment of these sequences displays similarities ranging from 76.6 to 88.4% (**Figure 2A**).

Fluorescent PCR-RFLP Analysis. According to the alignment and restriction mapping of 12S rRNA amplicons among porcine, caprine, and bovine species, the restriction enzymes *Alu*I, *Dde*I, and *Rsa*I were selected to generate the PCR-RFLP profiles (DNA fingerprints) for meat identification (**Figure 2B**). **Figure 3** shows such electrophoretic profiles on a 2% agarose gel, but DNA fragments of <50 bp were hard to justify on the gel. **Table 1** lists the organization of the PCR-RFLP profiles.

PCR amplification was also performed using fluorescent [R6G]dCTP labeling. The PCR products were digested with *Dde*I and subjected onto a 3% GeneScan polymer gel to resolve the RFLP profiles. **Figure 4** displays the *Dde*I profiles of pork, goat, and beef meat with a higher resolution than that obtained by separation onto an agarose gel as shown in **Figure 3**. The fluorescent PCR-RFLP analysis of the three domestic animals showed the same profile for each species as **Table 1**. From the fluorescent profiles, the *Dde*I fragments had sizes of 95 and 598 bp in the porcine amplicons; 48, 76, 167, and 351 bp in the caprine amplicons; and 74, 93, 125, and 351 bp in the bovine amplicons. The profiles excluded fragments of <40 bp and DNA marker with trace signals. In conclusion, the fragment sizes for pork, goat, and beef clearly demonstrate that these three commercial species can be identified using fluorescent PCR-RFLP of the mitochondrial 12S rRNA sequence.

Analysis of Meat Mixtures. Binary mixtures of the cooked and autoclaved pork, goat, and beef at levels of 1, 2, 5, 10, and 50% were tested. A significant fluorescent signal was obtained at the minimum admixture level employed, 1% beef in pork, thus demonstrating a sensitivity of <1% based on the presence of the 351 bp of *Dde*I fragment of beef (**Figure 5**). In fact, each species in the binary mixtures could be detected to a level of 1% tested in this study (data not shown). On the basis of the presence of the 598 bp *Dde*I fragment, pork was detectable in mixtures with either goat or beef. Meanwhile, on the basis of

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gccagccaccgcggtca tacgattaacccaaattaatagatccacggcgtaaagagtgtttaagaaaaaaaatcacaata  80
..............................g.gc...c..gaa...........ac......-..agc.ct.c....-....
..............................gc...c..gagt...........ac......-..agc.cc.t.c..-....

gagttaaattataactaagctgtaaaaagccctagttaaaataaaa-taacccacgaaagtgactctaataatcctgaca 160
..........c...t...a..............a..a...c..c....a...gtg........a..c.g.cagcag......
.g........c....................a.ga...........a...atg...........c...ca.tag.c...g

cacgatagctaggacccaaactgggattagatacccactatgcctagccctaaacccaaatagttacataa-caaaact 240
...t.......a.............................t......t............ta.....a.....a..a.....t.
...t.......a.............................t............a.g...a........a.....t.

attcgccagagtactactcgcaactgcctaaaactcaaaggacttggcggtgcttcacatccacctagaggagcctgttc 320
.................ca.....a...c........................t.t.c..tt...............
....................a....a........................t.t....tt...............

tataatcgataaaccccgatagaccttaccaaccccttgccaattcagcctatataccgccatcttcagcaaaccctaaaa 400
......t..............a.........gt......t...a...t........t...............
.....................a....c.....tt.....t...a...t........t...............

aggaacaatagtaagcacaatcatagcacataaaaacgttaggtcaaggtgtagcttatgggttggaaagaaatgggcta 480
.....t..a.......t.....g...........g.................a.c.....aa...g............
.....a..a.......gt...t...at.........................a.c....aaa...g............

cattttctacat-aagaatatccaccacacgaaagtttttatgaaactaaaaaccaaaggaggatttagcagtaaatcaa 560
..........c.c.....a..t..--.t..a....cca...........tg..................t......ct..
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gaatagagtgcttgattgaataaggccatgaagcacgcacacaccgcccgtcaccctcctcaagcatgtagtaataaaaa 640
........................cc.........................................g.t.ga.-ac...gc.ct
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taacctatattcaattacac-aaccatgcaagaaga g gcaagtcgtaacaaggtaag  693    Pig
c..a.c-......cacg...c.gt..catg...g.....................  691    Goat
.....c-....t..acg...t.g...catg...g.....................  692    Cattle
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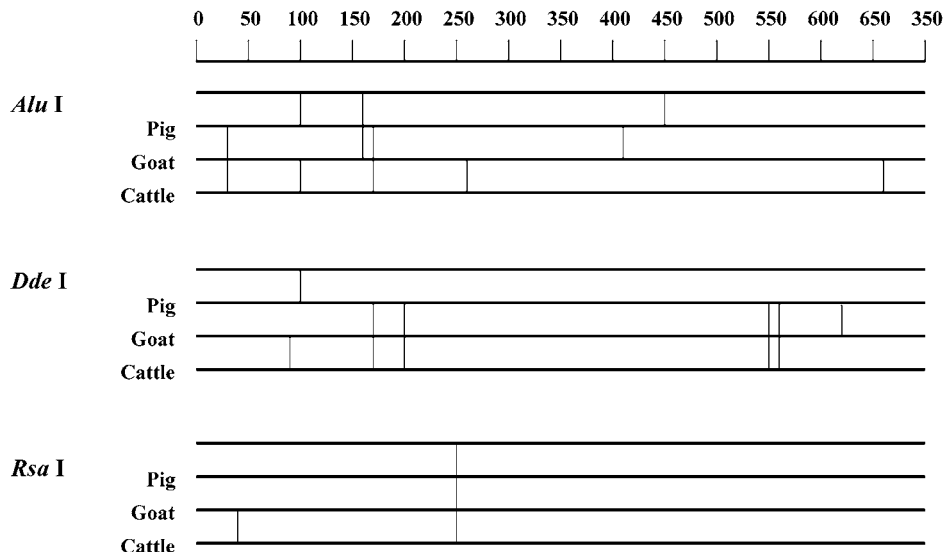

Figure 2. Nucleotide sequences and restriction maps of the PCR products amplified from pork, goat, and beef: (A, top) alignment of the sequence of mitochondrial 12S rRNA (open boxes represent the forward primer and complementary sequences of reverse primer, and the dots are goat and bovine nucleotides that are identical to porcine nucleotides, respectively); (B, bottom) restriction maps of *Alu*I, *Dde*I, and *Rsa*I, where the relative length of each restriction fragment is drawn.

the presence of both 351 and 167 bp *Dde*I fragments, goat was detectable in mixtures of pork or beef. The result of fluorescent PCR-RFLP generated with *Alu*I or *Rsa*I digestion also can be shown by such profiles for the three domestic animals except

for the *Rsa*I profile in the pork and beef mixture (data not shown).

This study investigated the possibilities for semiquantitation of species in a mixture. **Figure 5** shows the fluorescent signals
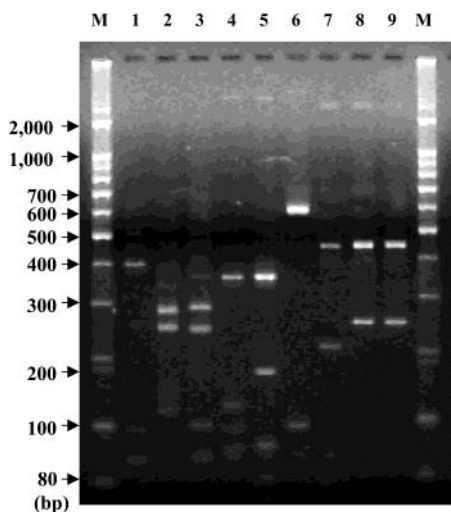
Figure 3. Electrophoresis of agarose gel displays the *Alu*I, *Dde*I, and *Rsa*I digestion of the PCR products amplified from pork, goat, and beef: (lanes 1–3) *Alu*I treatment; (lanes 4–6) *Dde*I treatment; (lanes 7–9) *Rsa*I treatment. Lanes 1, 4, and 7 are the PCR products amplified from beef; lanes 2, 5, and 8 are goat; lanes 3, 6, and 9 are pork. M is a molecular marker, and the sizes are as indicated.

Table 1. Expected Restriction Fragment Sizes Following PCR-RFLP Analysis of the Partial 12S rRNA Gene Applied with the Mt12S-6F/Mt12S-6R Primer Pair

species	uncut (bp)	*Alu*I fragment size (bp)	*Dde*I fragment size (bp)	*Rsa*I fragment size (bp)
pig	693	68, 99, 241, 285	95, 598	250, 443
goat	691	14, 32, 120, 247, 278	14, 35, 48, 76, 167, 351	250, 441
cattle	692	32, 34, 65, 69, 98, 394	14, 35, 74, 93, 125, 351	43, 207, 442

of PCR-RFLP from a significant percentage of beef in pork mixtures and displayed integral measurements of the fluorescent intensity of each signal. Similarly, **Figure 6** shows the fluorescent intensities of various percentages of beef in pork produced from three independent analyses. This result revealed that the major signal of the 596 bp *Dde*I fragment of pork is not influenced by the percentages of beef in the mixture and that the major signal of the 351 bp *Dde*I fragment of beef is positively correlated with the percentage of beef in a pork mixture. The data gathered herein show that the fluorescent PCR-RFLP assay can produce a calibration graph containing semiquantitative data.

DISCUSSION

This study aimed to develop a sensitive and semiquantitative method for simultaneously identifying multiple commercially important meat species, including pork, goat, and beef. Consequently, a fluorescent PCR-RFLP based on the sequence of the mitochondrial 12S rRNA gene using a single primer pair was developed for application as an analytical tool for meat identification.

Generally, thousands of mitochondria existed in cells, and the number of mtDNAs per mitochondrion has been estimated to vary between 2 and 10 in different tissues (*20, 21*). Consequently, the high abundance of mtDNA in total cellular DNA can be prepared and allows amplification of PCR to be more effective than those of nuclear sequences. MtDNA is inherited maternally and is thought to be useful for phylogenetic studies, because in mammals its evolution is more diversified than that of nuclear DNA (*22, 23*). Therefore, RFLP analysis
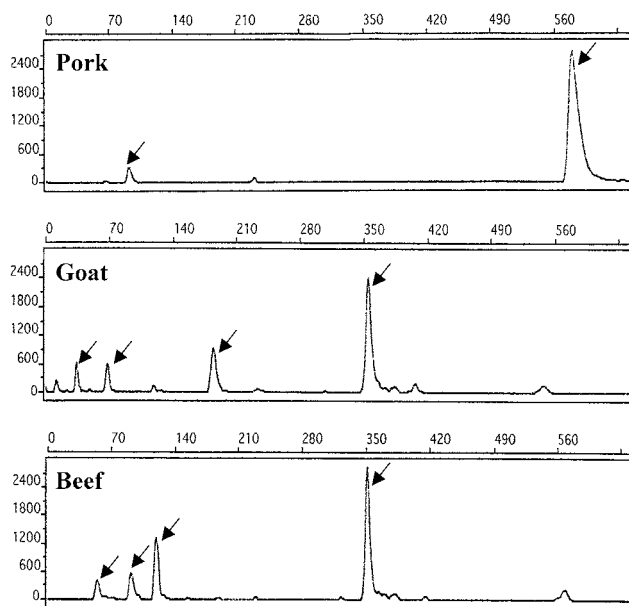

Figure 4. Fluorescent capillary electrophoresis showing the *Dde*I profiles of pork, goat, and beef. The PCR products, incorporating a fluorescent [R6G]dCTP, are mitochondrial 12S rRNA gene amplified from pork, goat, and beef, digested by *Dde*I, and then subjected onto a 3% GeneScan polymer gel using an ABI Prizm 310 genetic analyzer. The length of DNA fragments was identified using an ROX-1000 DNA marker, which was simultaneously subjected onto the gel for electrophoresis.

of mtDNA has been commonly used in animal breeding for identifying and monitoring animal stocks (*24–27*).

The PCR-RFLP analytical method as reported by Meyer et al. (*14*) and Partis et al. (*16*) provides a practical approach for detecting genetic variation of the mitochondrial Cytb gene among species. The present study thus evaluated this method to assess its suitability as a routine analytical method for determining the species origin of meat. The cytochrome *b* locus has been well characterized among different vertebrate groups (*28*). These studies have revealed that the level of Cytb gene sequence variation provides a suitable method of addressing general questions on interspecific diversity. Furthermore, mitochondrial rRNA genes have been widely used in investigations of phylogenetic relationships in vertebrates (*29*).

The rates of diversification of the mitochondrial Cytb and rRNA genes among vertebrates are similar, but the rRNA gene contains coexisting highly conserved and diversified regions. The sequence similarity of the complete mitochrondrial Cytb gene among pigs, goats, and cows is 79.1–83.6%, and the similarity of the complete mitochondrial 12S rRNA gene is 76.4–87.4%. Due to the rRNAs maintaining a complex conserved conformation, the sequences located in the loop of the rRNA structure are more diversified and those located in the stem are highly conserved when the vertebrates' sequences are aligned (*30, 31*). The mitochondrial 12S rRNA was selected as a molecular marker for species identification in this study. The unique primer pair, Mt12S-6F/Mt12S-6R, was designed on the basis of the highly conserved stem regions of the 12S rRNA gene, the sequence of which is identical to the complement sequences among porcine, caprine, and bovine mtDNA with the exception of one mismatched bp when Mt12S-6R is compared to the complement sequences of the ovine mtDNA. Therefore, using this primer pair amplified the target sequence of the 12S rRNA gene from the total cellular DNA of pork, goat, and beef with a similar sensitivity of PCR amplification. Although the sequence similarities of the PCR fragments within
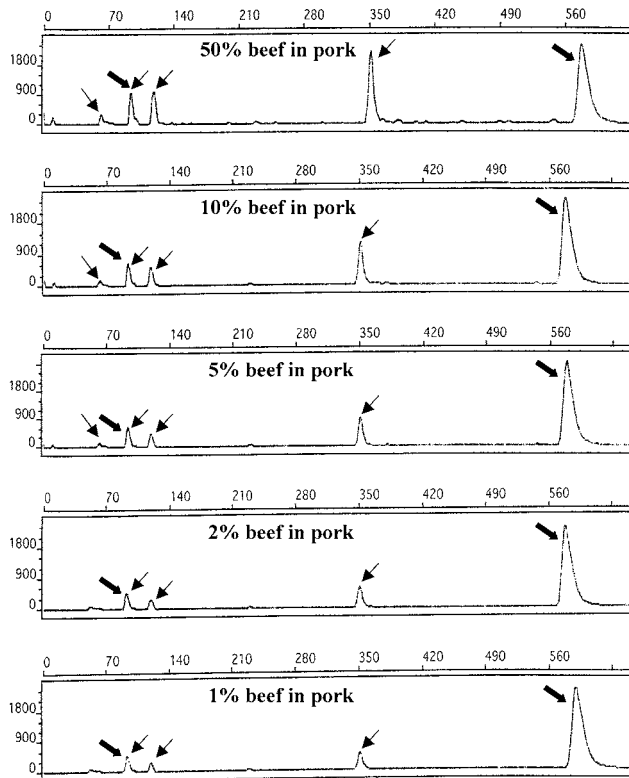
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Figure 5. *Dde*I restriction profiles obtained from fluorescent PCR-RFLP analyses of mixtures of pork and beef. Meat mixtures of 50, 10, 5, 2, and 1% beef in pork were tested. Bold arrows indicate the *Dde*I restriction profiles of pork; thin arrows indicate the *Dde*I profile of beef. Beef could be easily differentiated from pork on the basis of the presence of a 351 bp fragment. Notably, the fluorescent intensity of peaks of ∼95 bp *Dde*I peaks are larger than those of the *Dde*I/125 bp peaks in the 50, 10, 5, 2, and 1% beef in pork mixtures because of the peaks combining with the signals from *Dde*I/93 bp of beef and *Dde*I/95 bp of pork.
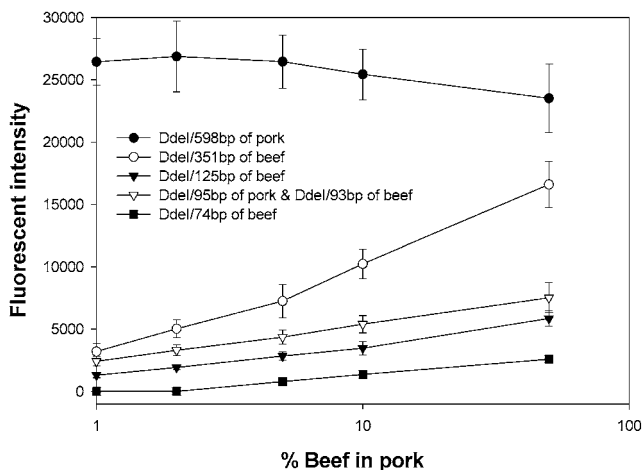

Figure 6. Graph of varying percentages of beef in pork produced from analysis of the signal intensity of the fluorescent graph. The mixtures analyzed included 1, 2, 5, 10, and 50% beef in pork mixtures, and three independent meat mixture series were tested using the process shown in **Figure 5**. Each value indicates the mean and standard deviation (SD) from the analysis of each peak of the fluorescent PCR-RFLP profiles.

the mitochondrial 12S rRNA gene amplified in this study are 76.6−88.4%, four regions (all of the regions are >20 bp) have a sequence similarity of <50%. From the alignment data of the three species sequences, several diversified regions are poten-

tially able to generate different restriction enzyme cutting sites for PCR-RFLP analysis.

This study used a single restriction enzyme to distinguish the mitochondrial 12S rRNA amplicons from the three domestic species. Additional examples of "profile convergence" will be found as PCR-RFLP profiles are obtained from more individuals with different lines of each species, although this problem can be solved through the careful selection of alternative restriction enzymes. Besides interspecies identification, it is worth emphasizing that the identification of intraspecies mitochondrial 12S rRNA gene is possible in further studies. For example, *Sus scrofa* (wild boar)/*Sus scrofa* domestics (domestic pig) and the sheep species *Ovis ovis*/*Ovis aries* (7) may be routinely identified using the novel fluorescent PCR-RFLP method.

This study set the PCR condition and selected 22 cycles following a series of pilot studies. Production of PCR amplicon of mitochondrial 12S rRNA genes was logarithmically correlated to the number of PCR cycles ranging from 18 to 26 cycles in this study using fluorescent capillary electrophoresis. Therefore, fluorescent PCR-RFLP is a reliable method of semiquantifying the specific meat in a meat mixture. Moreover, cooking and autoclaving of meats did not influence the generation of the PCR-RFLP profiles or the quantitative accuracy. This study suggested that the quality of template DNA does not influence the specificity when the primer pair designed in this study is used to amplify the 12S rRNA fragment from pork, goat, and beef.

CONCLUSION

This study developed a fluorescent PCR-RFLP method for species identification among pork, goat, and beef. The novel method applied PCR amplification of the mitochondrial 12S rRNA gene with a unique primer pair and incorporated a fluorescent-labeling nucleotide with sufficient specificity to detect three commercially important domestic animals. This method can reliably identify pork, goat, and beef and, furthermore, can identify and semiquantify any of these meats when they are present in meat mixture at levels of <1%.

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