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Identification of Meat Species Using PCR-RFLP Marker of Cytochrome *b* Gene

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Abstract

Food labeling regulations require that the meat species in various meat products are accurately declared to the consumer. Substitution or adulteration of costly meat with a cheaper one is one of the most common problems in the meat industry. In this study, PCR-restriction fragment length polymorphism(RFLP) method of the mitochondrial cytochrome *b* (mt cyt *b*) gene has been applied for identification of the origin of six mammalian meat species(beef, pork, horse, goat, mutton and deer) and three poultry meat species(chicken, turkey and duck) as raw materials for meat products. PCR was used to amplify a variable region of mt cyt *b* gene. Meat species differentiation was determined by digestion of the amplified products with a 359 bp fragment using *Hae*III and *Hinf*I restriction enzymes, which generated species-specific RFLP patterns. This PCR-RFLP DNA marker of mt cyt *b* gene could be very useful for the accurate and reliable identification and discrimination of animal meat species in routine analysis.

Key words : cytochrome *b* gene, PCR-RFLP, meat species identification

INTRODUCTION

The identification of species in food is becoming a very important issue concerning the assessment of food composition, which is necessary to provide consumers accurate information about the products they purchase. Consumers demand higher protection from falsely labeled meats and meat products for a variety of economic, religious and health reasons including the fraudulent substitution of cheaper meats in place of more expensive species (Rodriguez e*t al.*, 2005). However, fraudulent or unintentional mislabeling still exits and may not be detected, resulting in a poor-quality products. This has led to the need for reliable and specific methods of meat species determination in a variety of products where meat may be mixed with other ingredients and processed.

So far, many different analytical approaches that rely on protein analysis have been developed to identify animal species such as electrophoresis techniques, liquid chromatography and immunoassays in order to protect consumers from fraud and adulteration. These techniques are, however, not reliable for resolving mixtures of meat species, or identifying species in highly processed meat products because heat treatments denature proteins, destroying the profile of water soluble proteins (Hird *et al.*, 2003). Furthermore, proteins lose their biological activity after slaughter and their presence and characteristic depend on cell types (Calvo *et al.*, 2001).

More recently, molecular techniques have been applied for species differentiation and have proved to be reliable, sensitive and fast. Today, DNA-based assays using the polymerase chain reaction (PCR) are the most widely used as molecular technique for the identification of the species of origin in food, especially in meat products (Aida *et al.*, 2004; Dooley *et al.*, 2004; Girish *et al.*, 2005; Koh *et al.*, 1998; Matsunaga *et al.*, 1999; Rodriguez *et al.*, 2003). Both nuclear and mitochondrial genes have been targeted for species identification by PCR techniques. The detection of nuclear DNA sequences has been useful in this regard, but it might be limited as a result of the generally low copy number of these sequences (Meyer *et al.*, 1994). The advantage of mitochondrial-based

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DNA analyses derives from the fact that there are many mitochondria per cell and many mitochondrial DNA molecules within each mitochondrion, making mitochondrial DNA as a naturally amplified source of genetic variation (Rodriguez *et al.*, 2005). In this paper, we described an application of the PCR-RFLP technique using mitochondrial cytochrome *b* (mt cyt *b*) gene for the identification of species origin of animal meat samples.

MATERIALS AND METHODS

Meat Samples

Nine meat species from beef (*Bos taurus*), pork (*Sus scrofa*), horse (*Equus caballus*), goat (*Capra hircus*), mutton (*Ovis aries*), deer (*Cervus nippon*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and duck (*Anas boschas*) were used in this study. All meat samples were obtained from commercial sources. Raw samples were transported to the laboratory under refrigeration and they were processed immediately or stored frozen at −85℃ until used.

DNA Extraction from Meat Samples

DNA samples were extracted from 2 g of lean meat tissue using a CTAB extraction method previously described by Dooley *et al.* (2004). Briefly, minced meat mixed with 10 mL CTAB-lysis buffer, pH 8.0 (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM EDTA) and incubated at 65℃ with agitation for 1 hour. Proteinase K (10 μL of a 20 mM/mL solution) was added and the sample incubated at 65℃, with agitation overnight. An aliquot (1 mL) was transferred to a 1.5 mL Eppendorf tube and centrifuged for 10 min at 13,000 g before 800 μL of the supernatant was transferred to a fresh 1.5 mL tube. Chroloform (600 μL) was added and the sample vortexed. Following centrifugation at 13,000 g for 10 min, 600 μL of the aqueous layer was removed and mixed with 500 μL of absolute isopropanol in a fresh 1.5 mL tube. After 30 min at room temperature, the DNA was recovered, cleaned and redissolved in 50 μL of sterile distilled water. Recorved DNA concentration was determined by a spectrophotometer at 260 nm.

PCR Amplification

The universal primers used were shown as complementary to conserved regions of mt cyt *b* gene in vertebrata (Kocher *et al.*, 1989). The primer sequences were as follows: CYTb F 5′- CCA TCC AAC ATC TCA GCA TGA TGA AA-3′ and CYT*b* R 5′- GCC CCT CAG AAT GAT ATT TGT CCT CA-3′. PCR amplifications were carried out in a final volume of 20 μL containing 50 ng template DNA, 0.1 μM of each primer, 250 μM of each dNTP, 1.0 U of Taq DNA polymerase and 2 μL of 10 X PCR buffer. The PCR reaction was performed with a Perkin-Elmer (GeneAmp PCR system 9700, PE Biosystem, Foster City, CA) thermal cycler according to the following PCR step-cycle program: pre-denaturation of 94℃ for 1 min, followed by 30 cycles of denaturation at 94℃ for 20 s, annealing at 55℃ for 30 s and extension at 72℃ for 40 s, and final extension at 72℃ for 2min.

RFLP Analysis

PCR amplicons of the mt cyt *b* gene were subjected to restriction enzyme digestion with *Hae*III and *Hinf*I (New England Biolabs, Beverly, USA) restriction enzymes according to the suppliers' instructions, respectively. Briefly, five units of each enzyme were applied to 5 μL of amplified DNA in a final volume of 10 μL digestion mixture and were incubated at 37℃ for 3 hr. Digested products were analyzed by electrophoresis (100 constant voltage) on 4% NuSieve GTG agarose gel (FMC) for about 4 hr in 1 X TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) and then stained with ethidium bromide. The size of restriction fragments was estimated by 100 bp DNA ladder molecular weight markers (Promega, Madison, USA) as size reference.

RESULTS

In this study, the mt cyt *b* gene was used as a molecular marker for the identification of meat species using PCR-RFLP method. To amplify a DNA fragment from the mt cyt *b* gene of the six mammalian species (cattle, pig, horse, dear, sheep and goat) and three poultry species (chicken, turkey and duck), a specific universal primer pair was designed and synthesized based on sequences available in the GenBank/EMBL database for several animal species. PCR amplified products of the mt cyt *b* gene are shown in Fig. 1. The size of the amplified product was approximately 359 bp in all species.

Based on analysis of the restriction map of sequences *Hae*

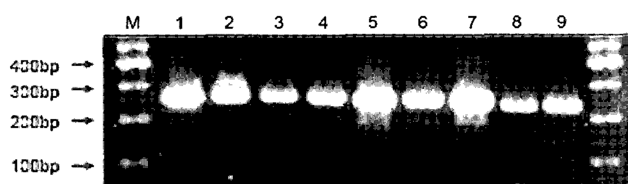

Fig. 1. PCR products of cyt *b* gene in nine animal species. Lane 1, sheep; lane 2, pig; lane 3, cattle; lane 4, deer; lane 5, horse; lane 6, goat; lane 7, chicken; lane 8, turkey; lane 9, duck; M, size marker of 100 bp DNA ladder.

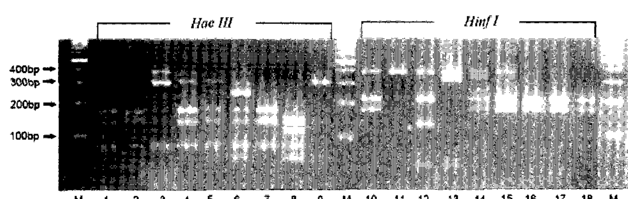

Fig. 2. DNA profiles of PCR products of cyt *b* gene digested with *Hae*Ⅲ and *Hinf*Ⅰ restriction enzymes in animal species, respectively. Sheep (lanes 1 and 10), pig (lanes 2 and 11), cattle (lanes 3 and 12), deer (lanes 4 and 13), horse (lanes 5 and 14), goat (lanes 6 and 15), chicken (lanes 7 and 16), turkey (lanes 8 and 17), duck (lanes 9 and 18), M: size marker (100 bp DNA ladder).

Ⅲ and *HinfI* enzymes were chosen for RFLP analysis, so as to identify and differentiate meat species. The PCR products digested with *Hae*Ⅲ and *HinfI* enzymes showed species-specific RFLP patterns according to their restriction profiles, respectively, as shown in Fig. 2. In mammalian species, *Hae* Ⅲ enzyme generated fragments of 78, 281 and 359 bp in cattle, 74, 132 and 153 bp fragments in Pig, 78, 126, 159, 241 and 281 bp fragments in horse, 74, 126, 159, 285 and 359 bp fragments in deer, 55, 74, 126, 159, 241 and 281 bp fragments in goat and 69, 124 and 166 bp fragments in sheep. In poultry species, *Hae*Ⅲ enzyme yielded 74, 126 and 159 bp fragments in chicken, 55, 70, 108 and 126 bp fragments in turkey and 78 and 281 bp fragments in duck (Table 1). On the other hand, in mammalian species, *HinfI* enzyme generated 55, 106, 198 and 359 bp fragments in cattle, a 359 bp fragment in pig, 161, 198, 241, 320 and 359 bp fragments in horse, 55, 304 and 359 bp fragments in deer, 161, 198 and 359 bp fragments in goat and 163, 196 and 359 bp fragments in sheep. In poultry species, 10, 161 and 188 bp fragments in chicken, 161 and 198 bp fragments in turkey and 161 and 198 bp fragments in duck (Table 1).

The same results were obtained from three separate experiments with different samples. When the amplified PCR products were digested with *HinfI* restriction enzyme, turkey and duck showed very similar DNA band patterns, but they can be clearly differentiated by digestion with restriction enzyme *Hae*Ⅲ(Fig. 3). As a result, the mt cyt *b* PCR-RFLP typing method by using *Hae*Ⅲ and *HinfI* restriction enzymes allowed the discrimination of economically important animal species

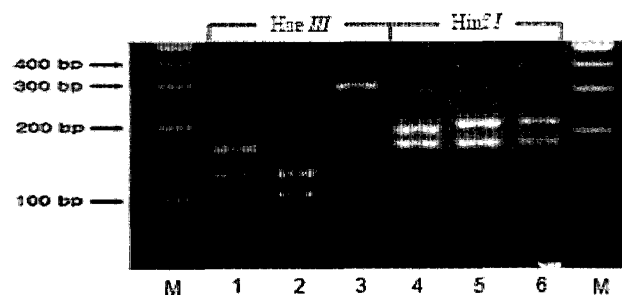

Fig. 3. DNA banding patterns of PCR products of cyt *b* gene digested with *Hae*Ⅲ and *Hinf*Ⅰ restriction enzymes in three poultry species, respectively. Chicken (lanes 1 and 4), turkey (lanes 2 and 5), duck (lanes 3 and 6). M, 100 bp DNA ladder.

Table 1. Restriction fragment sizes following PCR-RFLP analysis of the cyt b gene with *Hae*III and *Hinf*I restriction enzymes in nine animal species

Species	*Hae*Ⅲ fragment size (bp)	*Hinf*Ⅰ fragment size (bp)
Sheep	69, 124, 166	163, 196, 359
Pig	74, 132, 153	359
Cattle	78, 281, 359	55, 106, 198, 359
Deer	74, 126, 159, 285, 359	55, 304, 359
Horse	78, 126, 159, 241, 281	161, 198, 241, 320, 359
Goat	55, 74, 126, 159, 241, 281	161, 198, 359
Chicken	74, 126, 159	10, 161, 188
Turkey	55, 70, 108, 126	161, 198
Chicken	78, 281	161, 198

including cattle, pig, horse, deer, goat and sheep as mammalian species and chicken, turkey and duck as poultry species.

DISCUSSION

In recent years, species identification of animal products has become an important issue with regard to food authentication (Sawyer *et al.*, 2003). Conventional techniques are based on the electrophoretic or immunochemical detection of species-specific proteins. Recently, the DNA-based methods applied to species identification in animal products have received particular attention. These molecular techniques are promising and can be overcome the drawbacks of many conventional methods. As each cell contains about one thousand copies of mitochondrial DNA, PCR assays based on its amplification were shown to be more sensitive as compared to single or low copy nuclear DNA targets (Partis *et al.*, 2000). Since the quantity of PCR products generated corresponds to the copy number of the target DNA sequence, a higher copy number of mitochondrial DNA ensures a sufficiently high quantity of PCR product, even when small amounts of fresh or processed meat samples are used (Girish *et al.*, 2005). Because mitochondrial DNA has a relatively high mutation rate compared with nuclear DNA, they contain a greater accumulation of point mutations that can be used to better define species differences (Kocher *et al.*, 1989). Therefore, the mitochondrial encoded gene for cyt *b* was selected in this study as the target sequence for species identification. Mitochondrial DNA sequence is highly conserved in different species of animals (Antoinette *et al.*, 1995). Sequence comparison of mt cyt *b* gene available in the GenBank/EMBL database for several animal species permitted the design of 2 conserved primers, CYT*b* F and CYT*b* R, which amplified a DNA fragment of approximately 359 bp from nine meat species examined in this study. Nucleotide substitutions within and between mt cyt *b* gene sequences are sufficient for species identification of different biological samples (Prakash *et al.*, 2000). Sufficient restriction sites were also found for interspecific differentiation of meat species (Meyer *et al.*, 1995).

The analysis of PCR-RFLP of cyt *b* fragments has already been successfully applied for species differentiation in heated and processed meat products e.g. sausages (Meyer *et al.*, 1995). Chikuni *et al.* (1994) also used mt cyt *b* gene as molecular marker for meat identification. In another report, meats from buffalo, emu and crocodile were identified by PCR amplification and sequencing of mt cyt *b* gene (Forrset and Carnegie, 1994). Recently, Aida *et al.* (2005) reported a method for detection of pig meat and fat from other animal species for Halal authentication using PCR-RFLP of cyt *b* gene. Comparison between cyt *b* sequence is suitable for differentiation between closely related species because of rapid evolution on the mitochondrial DNA. In this study, the closely related species such as sheep and goat or chicken and turkey could also be differentiated by PCR-RFLP of mt cyt *b* gene. Similarly, Red deer meats can be differentiated from the Sika deer meats using PCR-RFLP (Matsunaga *et al.*, 1999). In a previous study, Min *et al.* (1996) reported identification of 4 meat species (Korean cattle beef, dear, sheep and goat meats) using PCR-random amplified polymorphic DNA (RAPD) method. However, main disadvantage of this RAPD method is that the reproducibility of the DNA banding patterns are difficult to achieve due to various factors such as PCR conditions or intra-species polymorphisms. Thus, PCR-RAPD analysis is less or not suitable for species identification compared to PCR-RFLP method (Koh *et al.*, 1998). The PCR-RFLP analytical method is simpler and less time-consuming than sequencing analysis, and exotic animals (sequences not known) can be analyzed quickly (Meyer *et al.*, 2005).

It can be concluded that PCR-RFLP analysis of mt cyt *b* gene described in this study represents a powerful, sensitive and highly reliable method for the routine meat species identification in the meat industry. This DNA-based assay can therefore be applied for food inspection services to detect fraudulent manipulations such as the use of cheaper meat in the manufacture of various meat products.

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