

Detection of Adulteration and Identification of Cat's, Dog's, Donkey's and Horse's Meat Using Species-Specific PCR and PCR-RFLP Techniques

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Abstract: PCR and PCR-RFLP methods for identification of cat's, dog's, donkey's and horse's meat were developed. DNA from a very small amount of muscles (0.05 g) was extracted to amplify species-specific DNA sequences in cat, dog and horse, in addition to mitochondrial DNA segment (*cytochrome-b* gene) in both donkey and horse. PCR product size of the species-specific region was 672-, 808- and 221-bp in cat, dog and horse, respectively. Using the same horse-specific primer, the same PCR amplification size (221-bp) in donkey was obtained. For discrimination between donkey's and horse's meat, the mitochondrial DNA segment (*cytochrome-b* gene) was amplified. Restriction analysis of PCR-RFLP of the mitochondrial *cytochrome-b* segment (359- bp) showed difference between donkey's and horse's meat. Where, restriction enzyme AluI yielded three fragments in horse's meat; 189-, 96- and 74-bp, whereas no fragments were obtained in donkey's meat. The use of these species-specific primers allowed a direct and rapid identification and detection of adulteration of cat's, dog's, donkey's and horse's meat even after homogenizing.

Key words: Meat Species-Specific, Identification, *Cytochrome-b* gene, PCR; PCR-RFLP

INTRODUCTION

The identification of the species origin of meat could be achieved using many methods such as immunodiffusion tests, enzyme-linked immunosorbent assays, sensory analysis, anatomical differences, histological differentiation of the hair that may possibly exist in the meat, properties of tissue fat, and level of glycogen in muscle tissue, as well as electrophoresis and DNA hybridization (Abdulmawjood *et al.*, 2003; Ilhak and Arslan, 2007). Most of these methods have been reported to have limitations in use due to problems in specificity, complexity, high cost and some requirements for baseline data about the differences in protein compositions. Therefore, there is a need for the development of a more accurate, fast, and easy-to-use method due to the limitations of the existing methods mentioned above. Developments in molecular biology have facilitated identification of plant, bacteria, and animal species with high accuracy. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) techniques have been frequently used for identification of meat species-specific (Meyer *et al.*, 1996 and Alves *et al.*, 2002).

However, consumers are concerned by a variety of issues, such as food authenticity and adulteration. The identity of the ingredients in processed or composite mixtures is not always readily apparent and verification that the components are authentic and from sources acceptable to the consumers maybe required (Lockley and Bardsley, 2000 and Aida *et al.*, 2005). This opens the possibility of fraudulent adulteration and substitution of the expected species with others of less value. To safeguard consumer rights, the legislation of each country should therefore impose a labeling of food products declaring the species used in their manufacture and food laboratories need to have available techniques to ascertain the species used in the manufacture of those products (Malmheden and Emanuelsson, 1998 and Ahmed *et al.*, 2007). Therefore, in this study, PCR and PCR-RFLP techniques were developed for detection of adulteration and identification of cat's, dog's, donkey's and horse's meat using species-specific oligonucleotide primers.

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MATERIALS AND METHODS

DNA Extraction:

According to Baradakci and Skibinski (1994) with some modifications, genomic DNA included mitochondrial DNA (mt-DNA) was extracted from cat's, dog's, donkey's and horse's muscle samples. Into small pieces, 50 mg of the tissue was cut and suspended in 500 μ L STE (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8). After adding 30 μ L 10% SDS and 30 μ L proteinase K (10 mg mL⁻¹), the mixture was incubated at 37 °C overnight. DNA was extracted by equal volumes of phenol–chloroform–isoamylalcohol (25:24:1) and chloroform–soamylalcohol (24:1), successively. DNA was precipitated by adding two equal volumes of chilled ethanol (95%) in the presence of a high concentration of salts (10% 3 M sodium acetate). The pellet was washed with 70% ethanol, air-dried and subsequently dissolved in an appropriate volume (75 μ L, approximately) of double distilled water (ddH₂O).

Species-specific Primers and PCR Amplification:

Species-specific DNA segments in cat, dog, donkey and horse, in addition to mitochondrial DNA segment (*cytochrome-b* gene) in both donkey and horse were amplified with the use of primers sequences as can be seen in Table 1 (Abdulmawjood *et al.*, 2003 and Lenstra *et al.*, 2001). PCR was performed in a reaction volume of 50 μ L using 25 ng of genomic DNA of each specie, 25 pmol of each primer, 10X Taq DNA polymerase buffer including MgCl₂, 0.2 mM dNTPs and 5 unit/ μ L Taq DNA polymerase (Finnzymes). Thermal cycling (Autorisierter Thermocycler and Mastercycler Gradient) was carried out by initial denaturation at 94°C for 4 min, followed by 35 cycles each at 94°C for 60s, annealing temperature at 52-58 for 60s (Table 1), polymerization temperature at 72°C for 60s and final extension at 72°C for 10 min., then the samples were held at 4°C. The amplified DNA fragments were separated on 2-3% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation system (Alpha Imager M1220, Documentation and Analysis System, Canada).

Table 1: Primer sequences of species-specific DNA regions and their annealing temperatures

Species	Primer sequence 5' to 3'	Annealing temperature
Cat's SSR	CTCATTTCATCGATCTACCCA	52 °C
	GTGAGTGTAAACTAGTACTAGAAGA	
Dog's SSR	GGAGTATGCTTGATTCTACAG	52 °C
	AGAAGTGGAATGAATGCC	
Donkey's and Horse's SSR	TTCTGCTCTGGGTGTGCTACTT	55 °C
	CTACTCAGCCAGATCAGGC	
Donkey's and Horse's <i>cytochrome-b</i>	CCATCCAACATCTCAGCATGATGAAA	58 °C
	GCCCCTCAGAATGATATTGTCTCA	

Restriction of mt-DNA PCR Product:

For restriction analysis, digestion of 10 μ L of PCR product (359 bases of mitochondrial *cytochrome-b* gene) in donkey and horse was accomplished with 10 units *AluI* restriction enzyme for one hour at 37 °C. Digested DNA was separated on 3% agarose gels in IX TBE buffer, stained with ethidium bromide, visualized under UV light and photographed.

RESULTS AND DISCUSSION

The amplification of species-specific DNA segments in cat, dog, horse and donkey yielded PCR products with sizes of; 672-bp in cat, 808-bp in dog and 221-bp in both donkey and horse (Fig. 1). On the other hand, the amplification of mitochondrial DNA segment (*cytochrome-b* gene) in both donkey and horse yielded the same amplicon with a size of 359-bp (Fig. 2). As can be seen in Fig. 1 and 2, the PCR amplification size and the position of the PCR-SSR and the mitochondrial DNA segment (221- and 359-bp) with both donkey and horse are exactly the same corresponding to the fragments of the molecular weight marker (100-bp BLUE eXtended DNA ladder).

For differentiation between donkey's and horse's meat, PCR–RFLP technique for the two PCR products (221- and 359-bp) was used. However, 221-bp PCR product size was treated with numerous restriction enzymes, but without success (no genetic variation was found). The amplified region of the gene encoding *cytochrome-b* (359-bp) in both donkey and horse was also treated with many restriction enzymes. Eventually, three different patterns only in horse were generated after the *AluI* restriction enzyme digestion with sizes; 189-96- and 74-bp, while in donkey no digestion was obtained (359-bp) allowing an identification of donkey's and horse's meat (see Fig. 2).

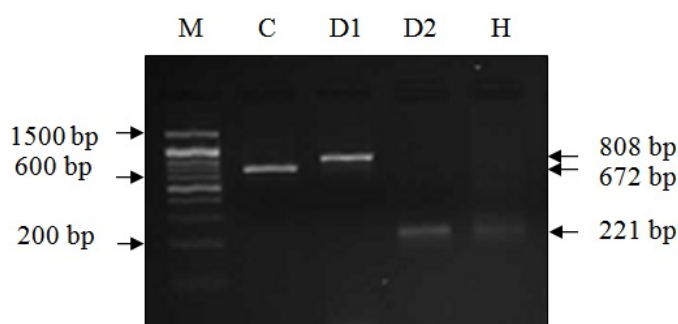


Fig. 1: PCR products (672-, 808- and 221-bp) generated by primers species-specific oligonucleotide. Lane C is cat, lane D1 is dog, lane D2 is donkey, lane H is horse and lane M is a molecular weight marker (100-bp BLUE eXtended DNA).

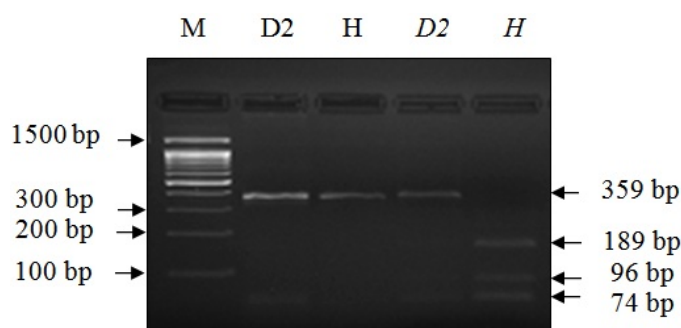


Fig. 2: Agarose gel electrophoresis of amplified *cytochrome-b* gene (lane D2: donkey and lane H: horse) following digestion with *AluI* generated three fragments with sizes of 189-, 96- and 74-bp (lane D2: donkey and lane H: horse). Lane M is a molecular weight marker (100-bp BLUE eXtended DNA).

The common fraudulent practice found in the meat products line is the use of a less costly type of meat in substitution of more expensive or authenticated ones. To avoid unfair competition and to assure consumers of accurate labeling, it was necessary to develop sensitive and rapid techniques for identification and detection of adulteration of meat species-specific, such as PCR and PCR-RFLP techniques. However, the results of these two techniques showed good evidence for molecular markers linked to genetic identification of cat's, dog's, donkey's and horse's meat. Where, PCR amplification of the species-specific regions generated three different PCR fragments; 672-bp in cat, 808-bp in dog and 221-bp in both donkey and horse (Fig. 1). For discrimination between donkey's and horse's meat, using *AluI* restriction enzyme, three fragments (189-, 96- and 74-bp) from the amplified gene encoding *cytochrome-b* gene (359 bp) were obtained in horse, whereas in donkey no fragments were obtained (Fig. 2). This finding allowed us a direct and rapid identification and detection of adulteration of cat's, dog's, donkey's and horse's meat in meat mixtures even after homogenizing.

In previous studies for detection of adulteration and determining species of meat, many different methods such as immunodiffusion, immunoelectrophoresis, isoelectric focusing, DNA hybridization, PCR and PCR-RFLP were developed. For example; pork's meat (0.5-1.0%) in beef's meat, chicken's meat (1%) in lamb's meat and 0.01% soy protein in processed meat products were detected using the PCR, duplex PCR, RFLP and nested-PCR techniques (Baradakci and Skibinski, 1994; Meyer *et al.*, 1995; Meyer *et al.*, 1996; Hopwood *et al.* 1999 and Partis *et al.*, 2000). Other previous studies concentrated mainly on direct and rapid identification of many species of meat using PCR species-specific regions and PCR-RFLP analysis for *cytochrome-b* gene (Abdulmawjood *et al.*, 2003; Ahmed *et al.*, 2007 and Ilhak and Arslan, 2007).

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