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SPECIES IDENTIFICATION USING POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) ANALYSIS OF MEAT AND MEAT MIXTURES

(With 4 Tables and 4 Figures)

By

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الأستعراف على الحيوانات باستخدام تقنية النوع المتخصص للتفاعل
التضاعفى لسلسلة الدى ان ايه فى أنواع اللحوم المختلفة

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تم فى هذه الدراسة استخدام تقنية النوع المتخصص للتفاعل التضاعفى لسلسلة الدى ان ايـه للتفريق بين أنواع اللحوم المختلفة لعشرة أنواع من الحيوانات (الأبقار والجاموس والأغنـام والخنازير والكلاب والخيول والحمير والفئران والجرذان والجمال). وتعتبر هذه التقنية مـن التقنيات عالية التخصص وذات حساسية شديدة فى تحديد نوع العينة وتحديد أصلها ولذلك تم استخدامها فى عدد من المجالات منها الطب الشرعى والزراعة والمحاجر وفى مجال أدوات التجميل. تم أخذ عينات من لحوم الحيوانات المختلفة بصورة نقية وكذلك تم عمل خليط مـن اللحوم لمعرفة مدى قدرة هذه التقنية على تحديد الغش فيها. حيث تم خلط لحوم الأبقار وهى الأكثر انتشارا كمنتجات لحوم مع كل من لحوم الخنازير والحمير والكلاب بنسـبة ٠٠٥ أو ١٠٠ % من وزن العينة. بعد استخلاص الدى ان ايه تم تمريره للحصول على حـزم جيـن السيتوكروم ب. كما تم استخلاص هذه الحزم وهضمها باستخدام أنزيمين قاطعين مختلفـين وهما الهى ٣ والرسا ١ ثم تمريرها مرة أخرى للحصول على الحزم الخاصة بكل نوع مـن الحيوانات. ووجد أن حزم جين السيتوكروم ب والتى تم استخلاصها على الجيلاتين الأول قد ظهرت عند مستوى واحد لكل الحيوانات وهو ٣٥٩ زوج قاعدى. وبعد الهضم والتمريـر أعطت عدد من الحزم الخاصة تختلف فى عددها وأماكن تواجدها تبعا لكل نـوع مـن الحيوانات. وقد أعطى أنزيم الهى ٣ معظم الحزم والتى أعتمد عليها فى التفريق بين أنواع الحيوانات المختلفة. ظهرت حزمة واحدة لكل من الأبقار والجاموس عند ٧٣ زوج قاعدى وتم التفريق بينهم باستخدام الأنزيم الآخر لتظهر حزمة واحدة فى الجاموس ولا تظهـر فـي الآخر. وظهرت حزمة واحدة أيضا فى كل من الكلاب والفئران والجرذان عند ٢٣٢ و ١٤١ و ٢٩٢ زوج قاعدى بينما ظهرت حزمتين لكل من الأغنام والحمير عند مستوى ٧٣ و ٢٣٢ للأغنام و١٩ و ١٥٣ زوج قاعدى للحمير. وظهرت ثلاث حزم للخنازير والخيول عنـد مستوى ٧٣ و ٢٢٧ و ٢٣٢ زوج قاعدى للخنازير و٧٣ و ٢٣٢ و ٣٣٨ زوج قاعدى

للخيول. بينما لم تظهر الجمال أي حزم. أما في حالة خليط اللحوم فقد ظهرت الحـزم الخاصة بكل من الفصيلتين المكونين للخليط معا في نفس عمود الفصل والخاصة بكل نــوع من الحيوانات. من النتائج المتحصل عليها أمكن التفريق بين أنواع اللحوم المختلفة باستخدام هذه التقنية، كذلك يمكن اثبات غش منتجات لحوم الأبقار بلحوم أخرى غير مألوفة ولو بنسب قليلة أما فى حالة لحوم الجمال فتوصى الدراسة باستخدام نوع آخر من أنزيمـات الهضـم الملائمة له.

SUMMARY

In this study we used the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique to differentiate between meat among ten animal species (cattle, buffalo, sheep, pig, dog, horse, donkey, rat, mice and camel). Such discrimination tests may have important applications in the forensic science, agriculture, quarantine and customs fields. Muscle tissue samples were taken from the different animal species (5 each) and kept frozen till used. Meat mixtures were prepared by adding 0.5 or 1.0 % levels of pork, donkey and dog meat to meat samples of cattle (w/w). The 359 bp fragments of cytochrome b (Cytb) gene were generated and distinct digestion patterns of these DNA fragment were observed after HaeIII and RsaI enzymes treatment. The results indicated that PCR amplification yielded 359 bp fragments in each of these species and mixtures. The amplicons were digested with two restriction enzymes resulting in a pattern that could identify and differentiate each of the above species except in camel which need specific restriction enzyme to digest its PCR product. In conclusion, these ten species of animals can be qualitatively identified and differentiated by PCR-RFLP of mitochondrial Cytb with the restriction enzymes. This method can be applied also in meat mixture to identify the adulteration with minute amount of undesirable meat in consumer meat products.

Key words: PCR-RFLP, mitochondrial Cytb, animal species, meat.

INTRODUCTION

Meat is an important source of protein for all peoples. However, in recent years, illegal game meat harvesting has become highly commercialized and unsustainable (Hofer, *et al.,* 1996).

The need for accurate and reliable methods for animal species identification has been increased during past decades particularly with the recent food scares and the overall crisis of biodiversity primarily

resulting from the huge ongoing illegal traffic of endangered species. Species determination relying on DNA analysis is a very important task in Veterinary Forensic identification. Also, species identification is essential in food quality control procedures or for the detection and identification of animal material in food samples (Pfeiffer, _et al._, 2004).

Determining the species origin of meat is an integral part of food regulatory control with respect to economic fraudulence. For example, game meat products are often a target for fraudulent labeling, because of the different prices between game and other meat species (Wolf, _et al._, 1999 and Brodmann, _et al._, 2001). A part from the possible economic loss, correct species identification is important to the consumer for other reasons such as medical requirements of individuals who may have specific food allergies, or religious dietary restrictions (Fajardo, _et al._, 2007). Numerous analytical methods that rely on protein analysis have been developed for species identification, such as electrophoresis technique (Shelef, 1986 and Skarpeid, _et al._, 1998), liquid chromatography (Ashmoor, _et al._, 1998) and immunoassays (Hsieh, _et al._, 1998). However, immunological tests often are sensitive enough to distinguish closely related species (Malisa, _et al._, 2005). A relatively new biotechnological field, known as species molecular identification based on the amplification and analysis of DNA, offers promising solutions (Teletchea, _et al._, 2005).

Also, in comparison with protein-based technique, DNA-based ones have proved to be more reliable because DNA is more stable under conditions associated with the high temperature, pressure and chemical treatments used in preparation of some food products (Frezza, _et al._, 2003 and Arslan, _et al._, 2006).

Among PCR-based techniques employed in the food industry to monitor adulteration of products from animal origin, the most frequently used are PCR amplification of marker gene fragment (s) with universal primers, coupled with techniques like nucleotide-sequencing or restriction fragment length polymorphism (RFLP) (Colombo, _et al._, 2004, Girish, _et al._, 2004 and 2005).

Mitochondrial DNA has been widely employed in phylogenetic studies as well as in forensic investigation of animals because it evolved much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species. It had been shown in a variety of studies to be very useful DNA-region for species determination (Hebert, _et al._, 2004). Other authors (Matsunga, _et al._, 1999, Mackie, _et al._, 1999 and Partis, _et al._, 2000) have chosen

mitochondrial DNA gene targets for the design of species specific PCR assays because mt DNA is present as a high copy number molecule, it provides sufficient sequence variation for the design of species specific PCR assay. Krcmar and Rencova, (2001); Myers, *et al.,* (2001); Lahiff, *et al.,* (2001) and Tajima, *et al.,* (2002) mentioned that use of PCR has improved food analysis because of its simplicity, species specificity and sensitivity for detection feed components with species-specific primers and analysis of restriction fragment length polymorphism (Partis, *et al.,* 2000 and Bellagamba, *et al.,* 2001) and meat from different animal species can be detected and identified.

As species identification studies are forensic tool in game meat identification or in food quality control for discrimination of the adulteration in meat products, this work is aimed to differentiate between a number of meat samples obtained from different domestic animals and to detect some cases of meat adulteration. It was based on identifying nucleotide polymorphic sites in 359 bp region of the cytb gene of the mitochondria of different tested animals. This region is widely used as target in PCR-RFLP.

MATERIALS and METHODS

Materials:

Primer was synthesized by Bio Basic Inc. Restriction enzymes HaeIII and RsaI purchased from Fermentas , Canada.

Collection of Samples

1) Meat samples:

Five samples of muscle tissue were obtained from each of ten species of animals (cattle, buffalo, sheep, pig, dog, horse, donkey, rat, mice and camel). The fresh meat samples (cattle, buffalo, sheep, pig and camel meat) were obtained from the slaughter houses, dog, horse and donkey meat were collected from the Veterinary hospital, Faculty of Veterinary Medicine, Assiut University, while rat and mice were obtained from the experimental animal house, Faculty of Medicine, Assiut University. Fresh tissues were transported on ice, and finally frozen at -20C° in the laboratory for DNA analysis.

2) Meat mixture:

The samples of meat mixtures were minced and prepared by adding 0.5 and 1.0 % (w/w) pork, donkey and dog meat samples to cattle meat. The mixtures of meat were prepared in a total weight of 200 g. 2g of each sample was taken separately from 5 different areas of each mixture.

Methodology:

DNA extraction:

DNA was isolated with a QIAamp. DNA mini kit (Qiagen, Valencia, CA, USA) and we followed the manual provided by the manufacturer to isolate DNA (Wang, *et al.*, 2004).

PCR Primer:

The universal primers used were shown to be complementary to the conserved regions to the human and vertebral mitochondrial DNA cytb genes. The primer sequences used were as follows:

Cytochrome b1: 5'- CCATCCAACATCTCAGCATGATGAAA-3' (forward primer)

Cytochrome b2: 5'- GCCCCTCAGAATGATATTTGTCCTCA-3' (reverse primer) according to (Kocher, *et al.*, 1989).

Polymerase Chain Reaction (PCR):

Amplication was performed in 100 µl of a solution containing 67mM Tris (pH 8.8), 6.7 mM MgSo4, 16.6 mM (NH4) 2So4. 10 mM 2-mercaptoethanol, each dNTP at 1mM, each primer at 1 µM, genomic DNA (10 – 1000 ng), and 2 – 5 units of Thermus aquaticus polymerase (Perkin-Elmer/Cetus). Each cycle of the polymerase chain reaction consisted of denaturation for 1 min. at 93 C°, hybridization for 1 min at 50 C° , and extension for 2 – 5 min at 72 C°. This cycle was repeated 25 – 40 times depending on the initial concentration of template DNA in the sample (Kocher, *et al.*, 1989).

Generation of single-stranded DNA and sequences:

Electrophoresis of 10 µl of the amplified mixture was done in a 2% agarose gel (NuSieve, FMC) in 40 mM Tris acetate (pH 8.0) and the DNA was stained with ethidium bromide. The gel fragment containing the amplified product was excised from the gel and melted in 1 ml of distilled water, and 1 µl of this mixture was used as a template in a second chain reaction to generate single-stranded DNA for sequencing giving the PCR product (Kocher, *et al*, 1989).

Restriction endonuclease digestion and DNA analysis by agarose gel electrophoresis:

PCR products of meat samples were digested by restriction endonuclease HaeIII and RsaI. Meat mixtures digested by Hae III restriction enzyme only. Two units of each enzyme were added to 7 µl of PCR product in a final volume of 20 µl digestion mixture at 37 °C for 3 hr. The digested products were analyzed in 2% agarose gel in 1X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA). The sizes of products

were estimated by comparison with a Bio-100 DNA Ladder (Unit of Molecular Biology, Assiut University, Assiut)

RESULTS

I – Results of different meat species samples

For all investigated species, 359 bp of the cytochrome b gene were examined. Sequences were deposited in GeneBank under accession numbers (Table 1). DNA extracted from various meat species was successfully amplified using the cytb primer previously described. A single PCR amplicon, corresponding in size to the predicted 359 bp fragment was observed in the first gel for all investigated species (Figure 1).

The PCR products were digested with HaeIII and RsaI giving two different co-existing restriction patterns (Fig. 2 & 3 and Table 2 & 3). There is only one HaeIII restriction enzyme site at 73 bp in cattle and buffalo. We can differentiate between them using RsaI restriction enzyme, where it give one cleavage site in buffalo at 326 bp and no cleavage site in the other animal.

One HaeIII restriction enzyme site also recorded in dog, rat and mice at 232, 141 and 292 bp respectively, while RsaI enzyme gives two bands in dog at 282 and 326 bp and one band in rat at 305 bp, there is no cleavage site in mice.

Two HaeIII restriction enzyme sites where obtained in sheep and donkey at 73, 232 and 19, 153 bp respectively. RsaI restriction enzyme has no cleavage sits in sheep and donkey.

Three HaeIII restriction enzyme sites where obtained in pig and horse at 73, 227, 232 and 73, 232, 338 bp, respectively. In case of RsaI enzyme, no cleavage sites were observed in both species. In camel both restriction enzymes give no cleavage sites. The summary of these results present in Table 4.

II- Results of meat mixture samples

Test mixtures of meat at 0.5% and 0.1 % levels were identified after digestion by HaeIII restriction enzyme. The same cleavage sites which were specific to each individual were observed. In the first mixtures including cattle and pork at 0.5 or 1.0 % levels, the restriction patterns show three cleavage sites at 73, 227 and 232 bp which are specific to pig. At 73 bp two bands overlapping each other appeared indicate the specific cleavage site of cattle (Figure 4).
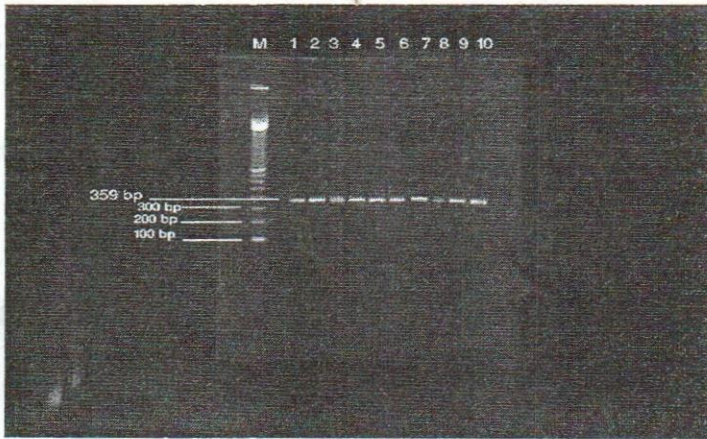
The second mixture of cattle and donkey with the same percentage, the restriction patterns showed two cleavage sites specific for donkey at 19 and 153 bp as well as the specific site for cattle at 73 bp (Figure 4). In The third mixture (cattle and dog at 0.5 or 1.0% mixture), the restriction patterns showed one cleavage site specific for dogs at 232 bp in addition to cleavage site specific to cattle at 73 bp (Figure 4).

Table 1 : The mitochondrial cytochrome b (cytb) gene sequences for each investigated animal species are available under the corresponding accession number from the gene bank database http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html

Species	sequence	Accession Number	Reference Sequence
Cattle	ccatcaaacatttcatcatgatgaaatttcggttccctcctgggaatctgoctaatoctacaaatoctcacaggoctattcctagcatacnctaca catccgacacaacaacagcattctcctctgttacccatatctgccgagacgtgaactacggctgaatcatccgatacatacacgcaaacgga gcttcaatggtttttatctgcttatatatgcacgtaggacgagccttatattacgggtcttacactttctagaaacatgaaatattggagtaatcct tctgcacagtaataagccacagcatttatagaatacgtcctaccatgaggacaaatatcattctgaggagc	AF490528	11907 – 12266
Buffalo	ccatcaaacatctcatcatgatgaaactttggctctctcctaggcatctgoctaattctgcaaatoctcaccggcctattcctagcaataca ctacacatccgacacaacaacagcattctcctccgtcgcccacatctgccgagacgtgaactatcgatacatacacg caaacggagcttcaatattttccaatctgcttatatatacacgtaggacgagccatatactacgggatcatataccttctagaaacatgaaac atcggagtaattctattattcgcagtaatagccacagcatttataggatacgtactgccafgaggacaaatatcattctgaggggc	D82892	69 – 428
Sheep	ccatcaaatatttcatcatgatgaaactttggctctctcctaggcatttgcttactttacagatttctaacaggcctattcctagcaatacactatacacctg acacaacaacagcattctcctctgtaacccacatttgccgagacgtaaactatggctgaattatccgatatatacacgcaaacggggcatcaatattt ttatctgcctatttatgcatgtaggacgaggcctatactatggatcatatacctttcctagaaacatgaaacatcggagtaatcctcctatttgcgacaata gccacagcattcataggctatgtttttaccatgaggacaaatatcattctgaggagc	AF010406	14227 – 14586
Pig	ccctcaaacatctcatcatgatgaaacttccctcttaggcatctgoctaatcttgcaaatoctaacaggcctgttcttagcaatacattacacatcc agacacaacaacagcttctcatcagttacacacatctgtcgagatgtaaattacggatgagttattcgctacctacatgcaaacggagcatccatgtt ctttatttgcctattcatccacgtaggccgaggcctatactacggatcctatatattcctagaaacatgaaacattggagtagtcctactatttaccgttata gcaacagccttcatagggctacgtctcctgccctgaggacaaatatcattctgaggagc	AB015079	69 – 428
Dog	ccgtctaacatctctgcttgatggaacttcggatccttactaggagtatgcttgattctacagattctaacaggttattcttagctatgcactatacatcg gacacagccacagctttttcatcagtcacccacatctgccgagacgttaactacggctgaattatccgctatatgcacgcaaatgcgcttccatatt ctttatctgcctattcctacatgtaggacgaggcctatattacggatcctatgtattcatagaaacatgaaacatggaattgtactattattcgcaaccat agccacagcattcatgggctatgtactaccatgaggacaaatatcattttgaggagc	NC_002008	14251 – 14610
Horse	ccctcaaacatttcatcatgatgaaacttcggctccctcctaggaatctgcctaatcctccaaatcttaacaggcctattcctagccatacactacaca tcagacacgacaactgccttctcatccgtcactcatctgccgagacgttaactacggctgaattatccgatatatacatgcaaacggggctccacatgat tatttttatctgcctcttcattcacgtaggacgaggcctcctactacggctcttacacattcctagagacatgaaacattggaatcatcctacttttcacagt tatagctacagcattcatcgggctatgtcctaccatgaggccaaatatccttttgaggagc	D82932	69 – 428
Donkey	cacataggcttggtcctggcctttttattagttattaataaaattacacatgccagtatccgcaccccagtgagaatgcctctaaatcgcactctacga tcaaangggagcaggtatcaagcacactacaaagtagctcataacaccttgctaggccacaccccacgggacacagccagtgataaaaattaagct atgaacgaaagttcgactaagttatattaaactagggttggtaaaattcgtgccagccaccgcggtcatacgattaacccanattaataaaccctccg gcgtaaagcgtgtcaaagaccccatactaaaatanagttaaaaocccagttaagctgtaaaaagctac	NC_001788	69 – 428
Rat	tgacaaacattcgaaaatctcatcctctactcaaaattattaatcactccttttattgaccttccgctccatccaacatctcatcatgatgaaactttggct ctctatttaggagtatgcctaataattcaaatcatttacaggcctgttcctagcaatacactacacatccgacacttctaacagcatttcatcagttacccac atctgccgnngacgtaaactacggctgactaattgatatttacatgccaacggagcttcaatatttctttatctgcctattcctccacgtaggacgaggaat gtactatggatcttcaccttctagaaacatggaaacatcgaaatcatgtctacta	EF186523	41 – 399
Mice	tcgcgcgtgcgagcgggcgcgcgagctggccccgggaggcgcgatggcggaccgccaggcgtctgcgtggccgaggagcgcagcggc cactgcgcagtggtggacggacactcctctacgtgtggggggcgctacgtgtctattgaagacaatgaggtatattgccaaatgatgaaatgtggac ctacgatattgatagtgggctgtgtggaaaatgcacctaatggaaggggagctccccccctccalgtcgggaagctgcggggcttgcatccacggaag gctctatgtctttgaggctatgacgacaaapgatacagcaaccggctttacttttgtcaatttacgaacaagag	EF605403	41 – 399
Camel	cctccaatatttcatcatgatgaaacttcggctctctactaggagtttgcttaattatacaaatcctgacaggactattcctggcaatacattatacatccga cacaacaacagccttctcttcagtcgcacacatctgccgagatgttaactacggctgaatcattcgatatttacatgctaacggagcttccatattcttcat ttgcttatatattcacgtgggtcgcgggctttattacggctcgtatacctttcagaaacctgaaacgttggaattgtttttattgttcacagtaatagcaacag cttttcatgggctacgtccttccatgaggacagatatattcattctgaggagcaa	U06426	69 – 428

Fig. 1: Amplification of a 359 bp fragment of the cytochrome b gene from different meat species, M: 100 bp molecular size marker, 1: cattle, 2: buffalo, 3: sheep, 4: pig, 5: dog, 6: horse,7: donkey,8: rat,9: mice, and 10: camel.
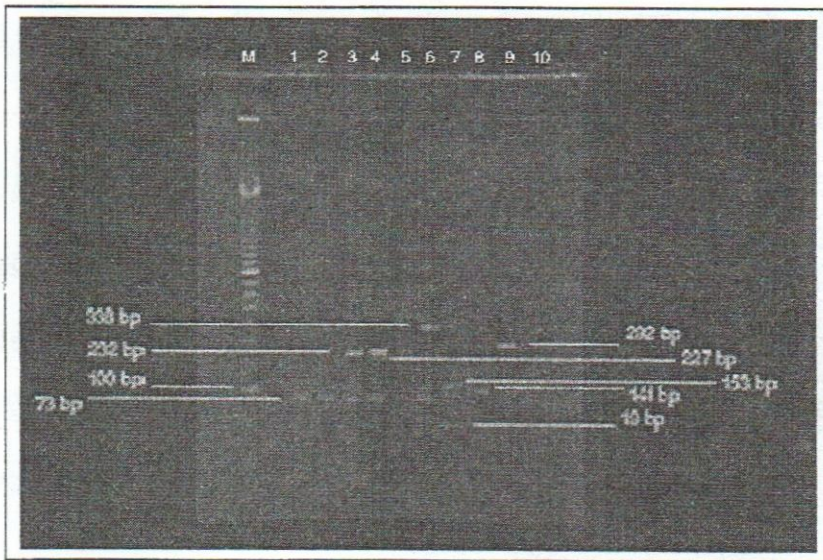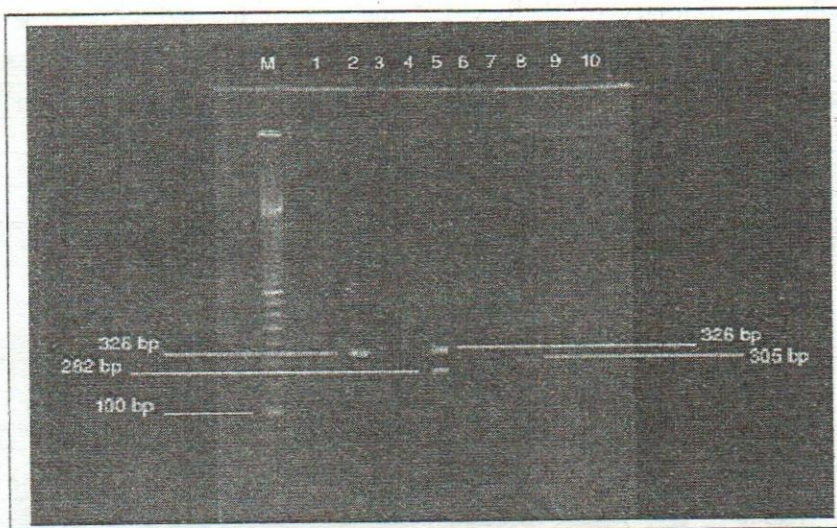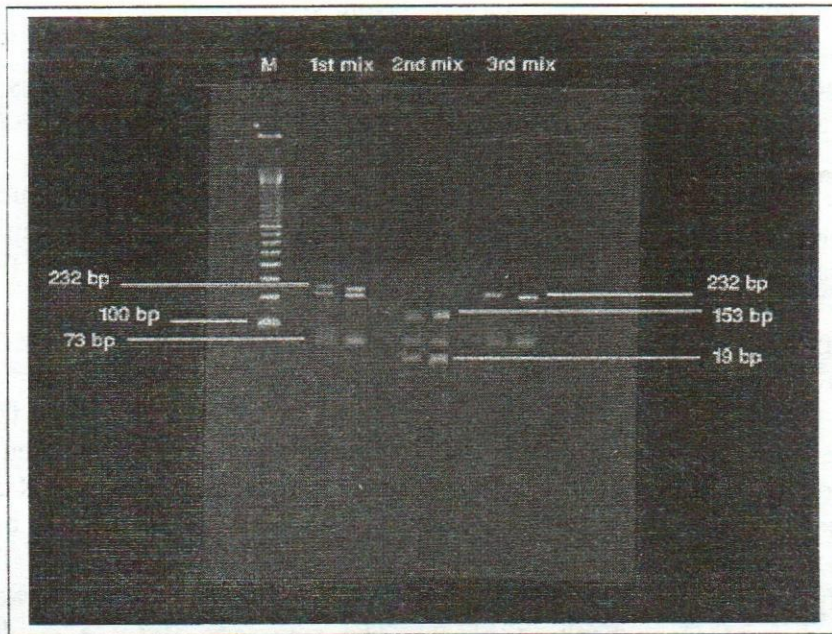

Fig. 2: Shows the restriction profiles of the different meat samples DNA after digestion with restriction enzyme HaeIII, yielding species-specific restriction fragments for each sample. M: 100 bp molecular size marker, 1: cattle, 2: buffalo, 3: sheep, 4: pig, 5: dog, 6: horse, 7: donkey, 8: rat, 9: mice, and 10: camel.

Table 2: The result of the specific restriction enzyme (HaeIII) profile of cytb PCR amplicans (359bp) in different animal species.

Species	PCR-RFLP products (bp)								
	19	73	141	153	227	232	292	338	359
Cattle		■							
Buffalo		■							
Sheep		■				■			
Pig		■				■			
Dog		■				■			
Horse		■				■		■	
Donkey	■			■					
Rat			■						
Mice							■		
Camel									

Fig. 3: Shows the restriction profiles of the different meat samples DNA after digestion with restriction enzyme RsaI, yielding species-specific restriction fragments for each sample. M: 100 bp molecular size marker, 1: cattle, 2: buffalo, 3: sheep, 4: pig, 5: dog, 6: horse, 7: donkey, 8: rat, 9: mice, and 10: camel.

Table 3: The result of the specific restriction enzyme (RsaI) profile of cytb PCR amplicans (359bp) in different animal species.

Species	PCR-RFLP products (bp)								
	36	73	142	236	282	305	314	326	359
Cattle									
Buffalo								▓	
Sheep									
Pig									
Dog					▓			▓	
Horse									
Donkey									
Rat						▓			
Mice									
Camel									

Table 4: Summary of PCR-RFLP analysis of the cytochrome b gene with HaeIII and RsaI restriction enzymes.

Species	GC↓CC cut in bp	GT↓AC cut in bp
	HaeIII	RsaI
Cattle	One band at 73 bp	No bands
Buffalo	One band at 73 bp	One band at 326 bp
Sheep	Two bands at 73 bp & 232 bp	No bands
Pig	Three bands at 73 bp, 227 bp & 232 bp	No bands
Dog	One band at 232 bp	Two bands at 282 bp & 326 bp
Horse	Three bands at 73 bp, 232 bp & 338 bp	No bands
Donkey	Two bands at 19 bp & 153 bp	No bands
Rat	One band at 141 bp	One band at 305 bp
Mice	One band at 292 bp	No bands
Camel	No bands	No bands

Fig. 4: Shows the restriction profiles of the different meat mixtures DNA after digestion with restriction enzyme Hae III, yielding species-specific restriction fragments for each sample. M: 100 bp molecular size marker, 1: 0.5 % pork meat in cattle meat, 2: 1.0 % pork meat in cattle meat, 3: 0.5 % donkey meat in cattle meat, 4: 1.0 % donkey meat in cattle meat, 5: 0.5 % dog meat in cattle meat 6: 1.0 % dog meat in cattle meat.

DISCUSSION

Recently the need of further information about the composition of food products, in particular of meat products, is increased, so identifying the species of origin of meat products represents a considerable target for food inspection, underlined by the enforcement of community laws and new analytical methodologies are necessary. Actually stringent control measures require methods that ensure an efficient species identification in meat products.

Application of PCR-RFLP to food analysis has shown to be useful because this technique is accurate, simple and with moderate cost (Meyer, *et al.,* 1995). DNA polymorphism is useful for identifying the source of meat, because we can easily isolated DNA and conduct PCR

with milk, blood, bone and meat. (Desjardins and Morais, 1990). Our primary goal was to differentiate the source of meat among cattle, buffalo, sheep, pig, dog, horse, donkey, rat, mice and camel. To distinguish these ten kinds of meat, we amplified cytb gene that is a highly conserved region (Desjardins and Morais, 1990 and Irwin, *et al.*, 1991). PCR has been successfully applied for the differentiation of species in meat products, including cooked products, which characterized by high specificity and sensitivity as well as to its rapid processing time (Sun and Lin, 2002). We can also count the bands on the gel to differentiate between meat from different animal species, by combination of the HaeIII and RsaI restriction enzymes, we can easily distinguish between the different kinds of meat.

The mt 359 primer specifically amplified DNA from all of the samples used in this study by giving a consistent 359 bp fragments (Figure 1). Digestion of mt 359 PCR products with Hae III and RsaI enzymes generated fragments of different sizes ranging from 19, 338 bp, which were sufficiently diagnostic (Figure 2 & 3 and Table 2 & 3). To identify the species, DNA sequence information to different animals was obtained from Gene bank (Table 1). The summary of these results present in Table 4.

When the PCR products were digested with HaeIII and RsaI. There is only one HaeIII restriction enzyme site at 73 bp for cattle and buffalo. We can differentiate between them using RsaI restriction enzyme, where it gives one site in buffalo at 326 and no cleavage site in the other animal.

One HaeIII restriction enzyme site also recorded in dog, rat and mice at 232, 141 and 292 bp respectively. Two HaeIII restriction enzyme sites where obtained in sheep and donkey at 73, 232 and 19, 153 bp respectively. Three HaeIII restriction enzyme sites where obtained in pig and horse at 73, 227, 232 and 73, 232, 338 bp, respectively. In case of Rsa I enzyme, no cleavage sites were observed in both species. In camel both restriction enzymes give no cleavage sites. These findings support the argument by kohn and Wayne, (1997) that the moderate interspecies variation of mt DNA cytb gene is well studied for use in species identification. Mayer, *et al.*, (1995) amplified the 359 bp fragments of the cytb gene using PCR followed by restriction digestion with Alu1, Rsa 1, Taq 1 and Hinf 1 to identify pig, cattle, buffalo, sheep, goat, horse, chicken and turkey meat. In another report, Paris *et al.*, (2000) generated DNA finger print for 22 animal species by amplifying 359 bp regions within the cytb gene and digesting the amplified products with

Hae III and Hinf 1. All species could be discriminated except the kangaroo and buffalo. The complete sequencing of Cytb gene is time-consuming and laborious due to the size of this locus being about 1140 bp (Hsieh, *et al.*, 2001). In this study we report on the use of a partial Cytb sequence for identification of the meat obtained from different animal species. The test uses primer that amplifies a 359 bp fragments with known sequence from Gene bank data base. The sequence can be used to identify all the animal species tested. The cytb gene of mitochondrial DNA (mt DNA) has been used in species identification (Kocher, *et al.*, 1989; Irwin, *et al.*, 1991; Lau, *et al.*, 1998; Cook, *et al.*, 1999 and Su, *et al.*, 1999), in taxonomic and phylogenetic studies (Kuwayama and Ozawa, 2000). The high copy number of mt DNA compared to nuclear DNA makes this locus ideal for analysis from highly degraded DNA (Pfeiffer, *et al.*, 2004). These results indicate that the meat samples from different species can be identified through this method.

In addition to species determination using PCR-RFLP analysis, we tested for different DNA ratios of mixed samples to detect the possibility of meat adulteration (Figure 4). The result of the three mixtures samples after their digestion by the same restriction enzyme (Hae III), showed the pattern of bands which specific to each species in the mixture. In the first mixture including cattle and pork at 0.5 or 1.0 % levels, the restriction patterns show three cleavage sites at 73, 227 and 232 bp which are specific to pig. At 73 bp two bands overlapping each other appeared indicate the specific cleavage site of cattle. The second mixture of cattle and donkey with the same percentage, the restriction patterns showed two cleavage sites specific for donkey at 19 and 153 bp as well as the specific site for cattle at 73 bp. In The third mixture (cattle and dog at 0.5 or 1.0% mixture). The restriction patterns showed one cleavage site specific for dogs at 232 bp in addition to cleavage site specific to cattle at 73 bp.

Ilhak and Arslan, (2007) suggested that the number of PCR cycles of amplification play an essential role in identification of meat in mixes. Therefore, in a case where a very low level of meat is suspected of being mixed into the main meat batch, the meat batch should be homogenized before sampling, multiple samples should be taken and the number of PCR cycles should be increased (i.e 35 cycle). Results of the present study was supported by the findings published by Mayer *et al.*, (1994 and 1995), Hopwood, *et al.*, (1999) and Partis *et al.*, (2000) who reported that PCR could be used for identification of meat mixes till 1 %

and 0.5 % levels and PCR was the best method of choice for identifying meat species in muscle foods. The problem of substitution or adulteration of costly meat with a cheaper one, whether by accident or intention, is not a new one (Kon, *et al.,* 1998). The use of the mitochondrial genome as marker for the development of techniques such as PCR-RFLP is widely extended choice (Verkaar, *et al.,* 2002, Pfeiffer, *et al.,* 2004 and Fajardo, *et al.,* 2006).

In meat plants processing more than species of meat, it may be inevitable that one species of meat may be contaminated with other during meat operation, such as cutting and griding via knives, grinders, choppers and cutting boards. PCR analysis of such samples may result in positive results for a violation due to its high sensitivity (Meyer, *et al.,* 1994 and Sawyer, *et al.,* 2003) even through contamination was unintentional and at a very low level. Therefore, precaution should be exercised when interpreting the results of species identification by PCR and analysis of multiple samples should be taken from each lot for an objective evaluation. These results might be useful for effective control of adulterated consumer meat products and violations of labeling requirements for meat products. PCR species determination can also be used to monitor ruminant feeds for any beef tissue, which has been banned in many countries in an effort to control the spread of bovine spongioform encephalomyelitis (Ilhak and Arslan, 2007).

The applicability of PCR-RFLP methodology may be restricted in the analysis of admixed meats (sausage, pates, minced meat products) including two or more species in their composition, because the results obtained after digestion of PCR products might show a combination of miscellaneous restriction patterns representing all the possible species included in the adulterated sample (Girish, *et al.,* 2005). Considering these aspects PCR using species-specific primers directed to short DNA fragments offers an excellent alternation for meat identification, since this technique would allow species identification of the target species from a pool of different DNAs, without the need for further sequencing or digestion of the PCR products with restriction enzymes (Fajardo 2007).

In conclusion, the data clearly illustrate that it is possible to identify the species from meat or meat mixture using PCR-RFLP with restriction enzyme provided that comparison to know species pattern. The PCR-RFLP enables the observation of fragment contaminations in meat products. By combining diagnostic restriction sites of two mitochondrial genes, the mitochondrial PCR-RFLP assay gives a

positive identification of all investigated species via the presence of a restriction site, except the camel which need another restriction enzyme to digest its PCR product.

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