DETECTION OF SPECIES-SPECIFIC GENETIC MARKERS IN FARM ANIMALS BY RFLP ANALYSIS OF CYTOCHROME B GENE

M. Ahmed, Aliaa El-mezawy

Abstract RFLP analysis was used on several meat sources to identify the species. It might be important to evaluate a method to identify different meat species. We tested Buffalo, Cattle, Goat and Sheep to enforce labeling regulation in the authentication of each of them. In this study, the presence of restriction sites on the polymerase chain reaction (PCR) products has been used for the specific identification of the four different meat species. The cytochrome b encoding gene (359 bp) as a part of the mt-DNA was amplified. The digestion of the PCR products using AulI and HaelIII gave specific restriction profiles that allowed a direct identification of farm animal species in raw and heat-treated meat samples. The results showed that RFLP analysis provided with a rapid and effective method to detect meat species and could be easily used for meat identification and authentication.

Key words: Farm animals, meat identification, RFLP, mt-DNA, cytochrome b gene.

Introduction and literature review

Mitochondrial DNA accumulates about 10 times as many mutations per unit as nuclear DNA and has thousands of copies per cell. Thus, amplification of a mitochondrial DNA segment is a relatively sensitive procedure, and the identification of the species can be based on mutations in the amplification products. A simple and convenient way of testing for a mutation is RFLP (Restriction Fragment Length Polymorphism) analysis, which uses an enzyme with a recognition

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sequence created or abolished by the mutation. Species identification using PCR-RFLP of a mitochondrial cytochrome \( b \) segment has been well documented (\textit{Partis et al.}, 2000). The technique is equally applicable to the identification of species origin in cheese products (\textit{Branciari, et al.}, 2000) as it is in meat products.

An alternative DNA detection system is based on the polymerase chain reaction (PCR) and the amplification of a segment of the mitochondrial cytochrome \( b \) gene (\textit{Murray et al.}, 1995). Subsequent cleavage by a restriction enzyme gives rise to a species-specific pattern. This method does not require the development of species-specific probes and, because it is PCR-based, is most suitable for critical samples in which DNA is largely degraded.

Several other methods have been described for the determination of species origin in meat products, the most straightforward of which is sequencing of the mitochondrial PCR product (\textit{Palumbi & Cipriiano}, 1998). This method is suitable for the identification of unknown species, but cannot be used to test samples of dual species origin. An alternative is the MIR-specific PCR, which generates species-specific fingerprints by amplification of DNA segments positioned between MIR (mammalian interspersed repeat) elements (\textit{Buntjer & Lenstra}, 1998). A third method uses one generic primer and several species-specific primers to differentiate between the most common meat species (\textit{Matsumaga et al.}, 1999). Other PCR-based methods have been designed specifically for the detection of porcine (\textit{Montiel-Sosa et al.}, 2000), bovine (\textit{Tartaglia et al.}, 1998), ostrich & emu (\textit{Colombo et al.}, 2000).

Consumers nowadays very seldom can identify the species in the products they purchase: cuts either fresh or frozen, more or less processed (souse-vide, marinated, dried, smoked, salted, etc.) and prepared ready-to-eat products are increasingly available. This opens the possibility of fraudulent adulteration and substitution of the expected species with others of less value (\textit{Martinez & Yman}, 1998). 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.

The conventional methodology used for the determination of species origin in meat and meat products have been predominantly based on the immunochemical and electrophoretic analysis of proteins. More modern techniques now allow the identification of species-specific
markers as mt-DNA RFLP, RAPD-PCR and SSR techniques which has two major advantages over protein analysis: samples heated to as high as 120° C can still be analyzed and discrimination between related species, with farm animals such as (Buffalo, Cattle, goat & sheep), or chicken & turkey…e.g., is possible (Lenstra et al., 2001). An identification of animal species could be performed by mitochondrial DNA (mt-DNA) analysis. According to Abdulmawjood & Buelte (2002), mt-DNA analysis requires the isolation of the mt-DNA molecule and a digestion of the mt-DNA with a variety of restriction endonucleases. The resulting fragment patterns are then examined for polymorphisms within and among populations studied. A combination of PCR amplification of mt-DNA and restriction fragment length polymorphism (RFLP) analysis has been used in species and stock identification studies (Lenstra et al., 2001, Abdulmawjood & Buelte 2001, Abdulmawjood & Buelte 2002).

RFLP technique relies on the amplification of variable regions of the target genome with the amplicon, then being digested with one or more specific restriction enzymes. Although many enzymes still need to be tested in the initial phase to be able to identify the polymorphism, it is today an easy, reliable and relatively cheap marker to use (Dodgson et al., 1997). PCR-RFLP is, therefore, probably the simplest PCR procedure for comparisons of sequence polymorphism. Potential disadvantages of the RFLP technique include the dimorphic nature, since a RFLP only indicates the presence or absence of a cleavage site and, therefore, does not provide a great deal of genotypic information and RFLP (Erlich, 1991).

The development of molecular biological techniques has created new possibilities for the selection and genetic improvement of livestock. The discovery of the PCR had a major impact on the research of eukaryotic genomes and contributed to the development and application of various DNA markers. DNA markers have already found wide application in parentage verification, individual identification of meat and control of genetic disorders. The ultimate use of DNA markers would be to identify quantitative trait loci (QTL) in order to practice genotypic selection (Marle-Koster & Nel, 2003).

The present study was designed to investigate the application of the molecular methods in the identification of farm animal's meat (Buffalo, Cattle, Goat and Sheep). This was based on PCR-RFLP of a conserved mitochondrial DNA region of the cytochrome b gene of different animal species.
Materials and Methods

Meat samples
Meat samples of four species, Buffalo, Cattle, Goat and Sheep from muscle purchase were collected from New Borg El-Arabi City, Alexandria, Egypt.

DNA Extraction
DNA was extracted from muscle sample following the method described by Bardakci & Skibinski (1994) with some modifications. Approximately 0.5 g of the tissue was cut into small pieces and suspended in 1000 μl STE (0.1 M Na Cl, 0.05 M Tris and 0.01 M EDTA, pH 8). After adding 30 μl SDS (10 %), 30 μl proteinase K (10 mg/ ml) were added. The mixture was incubated at 50 °C for 30 min. DNA was purified by successive extraction with phenol, phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) respectively. DNA was precipitated with ice-cold absolute ethanol and washed with 70 % ethanol. The pellet was dried and resuspended in 200μl mill Q water.

PCR Primers
Two primers were used to amplify cytochrome b gene (Table 1).

Table 1. The sequences of the primers (mt-DNA cytochrome b gene) used and their annealing temperatures

<table>
<thead>
<tr>
<th>Primer/ Forward/ Reverse/ Primer</th>
<th>Sequence/Niz 5’- 3’</th>
<th>Annealing Tm/Sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA TCC AAC ATC TCA GCA TGA TGA AA</td>
<td>GCC CCT CAG AAT GAT ATT TGT CCT CA</td>
<td>54/30</td>
</tr>
</tbody>
</table>

PCR Amplification of mt-DNA cytochrome b gene
PCR amplifications were performed following the procedure of Abdulmajwood & Buelte, (2002) with some modification. The reaction were carried out in 25 μl consisted of 1.0 U Taq DNA polymerase (Finnzymes), 25 pmol dNTPs, 25 pmol of each primer, 2.5 μl of 10X Taq DNA polymerase buffer and 50 ng of genomic DNA. The final reaction mixture was placed in a DNA thermal cycle (Eppendorf AG 22331,
Hamburg, Germany). The PCR program included an initial denaturation step at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min. The samples were cooled at 4°C.

**Agarose Gel Electrophoresis**

The amplified DNA fragments were separated on 3% agarose gel and stained with ethidium bromide. 100 bp DNA marker was used in this study. The amplified pattern was visualized on an UV transilluminator and photographed by Gel Documentation system (Alpha ImagerTM1220, Documentation & Analysis system, Canada).

**RFLP for PCR products of mt-DNA cytochrome b gene**

The restriction enzymes *AluI* & *Hae*III were used to yield specific restriction profiles that allowed a direct identification of Buffalo, Cattle, Goat & Sheep raw meat. PCR products resulted from cytochrome b gene amplification was digested with 10 U restriction endonuclease/12 µl of DNA products, 4 hour incubation. The DNA fragments were separated on 3 % agarose gel and stained with ethidium bromide. ØX174 DNA marker was used in this study. The fragments pattern was visualized on an UV transilluminator and photographed by Gel Documentation system (Alpha Imager TM 1220, Documentation and Analysis system, Canada).

*Results*

The PCR products have been generated by the universal oligonucleotide primers (Table 1). The amplification of the gene encoding cytochrome b yielded a 359-bp amplicon as shown in Figure 1A. The restriction enzymes *AluI* & *Hae*III were used in the RFLP analysis of cytochrome b gene. The RFLP patterns of the digested PCR products of the farm animal’s cytochrome b gene as depicted in Figure 1B showed differences among the species under investigation (Buffalo, Cattle, Goat & Sheep). Using the restriction enzyme *AluI*, a single restriction site was found in Buffalo and cattle cytochrome b gene. Two fragments with a size of 190 and 169 bp were detected. Using the restriction enzyme *Hae*III, cytochrome b gene of Goat was digested to 3 fragments with a size of 239, 74 and 55 bp. The restriction enzyme *Hae*III recognized 2 restriction sites in sheep cytochrome b gene. Three fragments with a size of 153, 74 and 32 bp were detected. The fragment sizes after digestion of cytochrome b gene of four different farm animals' species using two restriction enzymes are shown in Table 2.
Table 2. Predicted fragment sizes after digestion of cytochrome b gene of different farm animals species via two restriction enzymes  

<table>
<thead>
<tr>
<th>Species/Vrsta</th>
<th>Size of DNA fragment (bp) after digestion/ Veličina DNK fragmenta (bp) nakon digestije</th>
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<tbody>
<tr>
<td></td>
<td>&lt;sup&gt;Alb&lt;/sup&gt; &lt;sup&gt;HaeIII&lt;/sup&gt;</td>
</tr>
<tr>
<td>Buffalo/ Bivo</td>
<td>190, 169</td>
</tr>
<tr>
<td>Cattle/ Goveda</td>
<td>190, 169</td>
</tr>
<tr>
<td>Goat/ Koza</td>
<td>-</td>
</tr>
<tr>
<td>Sheep/ Ovca</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>239, 74, 55</td>
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<td></td>
<td>159, 126, 74</td>
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Discussion

A restriction site of the polymerase chain reaction (PCR) product has been used for the specific identification of farm animal meat. In the present study the part of the gene encoding cytochrome b was amplified using the universal oligonucleotide primers (Table 1) and the digestion of the PCR products using specific restriction enzymes was carried out. Lenstra et al. (2001) used similar oligonucleotide primers with different animal species; the sequences were analyzed to find conserved and variable regions for the selection of restriction enzyme. PCR technology allows an amplification of specific regions of DNA, facilitating the detection of genetic differences among species or populations. With this PCR approach, it is important to establish genes showing variations for the species under study (Brown, 1983). It should be stressed that, it is probable, the hypervariable sequences of the mt-DNA molecule will prove more useful in studies of intraspecific variation (Hansen & Loechcke, 1996 and Lenstra et al., 2001) relatively conserved sequences may be ideally suited for interspecific and interfemal comparisons (Lockwood et al., 1993). A comparison of the sequence with other farm animal species showed differences in their restriction sites and allowed an identification of each of them. <sup>Alb</sup>I, was used to yield specific restriction profiles that allowed a direct identification of Buffalo and Cattle raw meat. <sup>HaeIII</sup> was used to yield specific restriction profiles that allowed a direct identification of Goat and Sheep raw meat. Restriction enzymes (BamHI, EcoRI, SacI and TaqI) have often been shown to be useful in obtaining RFLP patterns for haplotype individual
identification (Spike et al., 1996). The enzymes AluI and HaeIII were previously used by Lenstra et al. (2001) and Abdulmawjoood & Buelte (2002) to differentiate several meat species and also for the authentication of meat origin. The RFLP patterns of the digested PCR products of the farm animal’s cytochrome b gene as depicted in Figure 1B showed differences among the species under investigation (Buffalo, Cattle, Goat and Sheep). However, in this study we provide a simple, accurate and universal method for identifying meat. Further studies must be done to design farm animal species-specific primers and/or probe for the direct diagnosis and further analysis of the other mt-DNA regions to provide more sequence data for the differentiation of these animals.

Direct sequence analysis of the PCR products of the cytochrome b gene were performed with the oligonucleotide primers designed by Kocher, et al., (1989). This slightly modified primer pair had already been used to amplify the cytochrome b gene in more than 100 species, including mammals, birds, amphibians, fish and some invertebrates. In addition, these primers had been used to assess inter- and intraspecific differentiation of Atlantic cod (Gadus morhua) populations (Pepin & Carr, 1993), salmon species (Lockwood et al., 1993), tuna species (Bartlett & Davidson 1991), snail species (Abdulmawjoood & Buelte 2001), farm animals (Lenstra et al., 2001) and poultry (Abdulmawjoood & Buelte, 2002). These authors showed interspecific variations, which might be useful for identifying the various species. However, the high cost of this technique and the need of individual sequences for detailed comparison make it inappropriate for the analysis of large numbers of samples.

Conclusion

As an alternative to the high cost direct sequence analysis, the present study describes an approach based on PCR-RFLP analysis of cytochrome b gene conserved region. The application of PCR- RFLP technique to a conserved region of the cytochrome b gene provides an accurate, quick, and cheap alternative method to sequencing could be enhance the direct identification and authentication of farm animals species.
DETEKCIJA GENETSKIH MARKERA SPECIFIČNIH ZA VRSTU KOD DOMAČIH ŽIVOTINJA KORIŠĆENJEM RFLP ANALIZE CITOHROMA B GENA

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Rezime

References


Figure 1. A. PCR amplification products generated by cytochrome b primers. B. RFLP patterns of cytochrome b gene digested with restriction enzymes AluI & HaeIII. Lane M: DNA marker, Lane B: Buffalo, Lane C: Cattle, Lane G: Goat and Lane S: Sheep

Slika 1. A. Proizvodi PCR amplifikacije stvoren od strane prajmera citohroma b. B. RFLP obrasci citohroma b gena digestirani pomoću restriktivnih enzima AluI i HaeIII. M: DNK marker, B: bivo, C: goveda, G: koza, S: ovca