DETECTION OF MEAT CONTAMINANTS IN PROCESSED MEATS USING POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISMANALYSIS

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ABSTRACT. *A method based on Polymerase Chain Reaction (PCR) - Restriction Fragment Length Polymorphism (RFLP) analyses to determine the identities of meats species in meat products was assessed. Raw meat (control) and processed meat samples wereanalyzed bytargeting their cytochrome b gene. Universal primers, cytb1 and cytb2 amplified a fragment of the cytochrome b gene of approximately 360bp. The cytochrome b fragmentsdisplayed meat species-specific RFLP profiles when digested separately with restriction endonucleases RsaI, BsaJI, BstNI, AluI, TaqI, NsiI and BstUI. The identity of a meat was resolved by comparing the RFLP pattern of the processed meat to the RFLP profiles of the raw meats (control standards). The RFLP analysis showed that the processed beef products were contaminated with chicken while the processed chicken products were free from contaminant.*

KEYWORDS. Cytochrome *b* gene; PCR-RFLP, amplicon

INTRODUCTION

Meat is the major source of good quality protein that supplements essential amino acids for our daily requirement. Preparation of processed meat products by mixing meats of different origin sometimes takes place in a single factory or uncertified factories. The mode of preparation therefore presents a major concern for many consumers, particularly in relation with the accidental inclusion of meatswhich are sensitive towards certain religions such as kosher food which are religion-sensitive for Jews and Muslim (Montiel-Sosa *et al.,* 2000). Occasionally, adulteration of higher value meats with cheaper meats is also found. For example, pork is a potential source for adulteration of higher value meat such as beef and veal (Chen *et al.,* 1998) but it is a non-permissive meat for the Muslims. Additionally, meat products containing undeclared species may impose a potential health risk to people with allergies to certain protein. Hence, there is an urgency to establish a reliable meat testing protocol to authenticate animalspecies.

Currently, meat species identification is conducted using various detection methods. They are mainly based on the analysis of certain proteins in meat usingthe isoelectric focusing, immunochemical, and electrophoretic methods (Koolmees, 1999). However, each of these methods has major drawbacks, due to its dependence on the ability to characterize proteins. Many proteins are heat-labile, lose their biological activity and conformation soon after death and are subjected to modification in different cell types even within the same individual. For

these reasons, DNA-based analyses are preferred, and have become popular for the identification and differentiation of meats and meat-based products (Meyer *et al.,* 1995).

DNA in an organism carries more genetic informationthan the protein. This is due to the degeneracy of the genetic code as one goes from DNA to protein (Worf*et al.,* 2000). Moreover the information content of DNA is the same in all cell types of an animal, thus meat from any part of an animal (skeletal or organ) can be used as sample of identification. In comparison, with protein, DNA is a remarkably stable molecule allowing its extraction from many different types of samples (Worf*et al.,* 2000).

PCR methods (Chikuni*et al.,* 1994) have been used for identification of meats and meat products. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP) of mitochondrial DNA has offered the greatest advantage (Bellagamba*et al.,* 2001) when compared to other DNA-based methods. PCR easily amplifies target regions of template DNA in a much shorter time (Saiki *et al.,* 1985). RFLP of PCR products constitute a simpler alternative to sequencing for the identification of genetic variation between and within species (Borgo*et al.,* 1996). RFLP analysis is carried out to determine the species origin of meat samples. Amplicons (amplified DNA) are cleaved into smaller DNA fragments by a series of restriction endonucleases, followed by an agarose gel electrophoresis to determine the interspecies RFLP profiles. Hence, the objective of this project was to assess the PCR-RFLP method for meat species authentication.

MATERIALS AND METHODS

Meat samples

Ten different types of meat samples were obtained from animals such as pig, cow, buffalo, goat, deer, rabbit, ostrich, turkey, chicken and duck. Three independent samples of each meat type were purchased from 3 different locationsin Selangor and Sabah, Malaysia. Processed meat samples, chicken burger meat, chicken sausage, chicken nugget, beef burger meat and beef sausage of different commercial brands were obtained from Kota Kinabalu, Sabah. DNA from raw chicken meat and raw beef were utilized as positive control in this part of the experiment. All the samples were stored at -20^{o}C until used to prevent enzymatic degradation of the DNA.

DNA extraction

DNA was extracted from 25 mg of meat and processed meat samples using the DNeasyprotocol for Animal Tissue provided with the DNeasy™ Tissue kit (Qiagen).

Oligonucleotide Primers

A pair of primer was employed in PCR reaction. The universal primers – cytb1 CCA TCC AAC ATC TCA GCA TGA TGA AA and cytb2 GCC CCT CAG AAT GAT ATT TGT CCT CA which weredescribed by Kocher *et al.* (1989) were used to amplify cytochrome b gene.

Polymerase Chain Reaction (PCR)

Amplification of the mitochondrial cytochrome b gene was carried out in a final volume of 100 µl containing 100 ng - 200 ng of extracted DNA, 1x PCR reaction buffer (50 mMKCl, 10 mMTris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mMdNTPs (Sigma), 10 pmol of each primer and 1.25 units if *Taq* DNA polymerase (Roche). PCR reaction was performed with a Perkin Elmer

(GeneAmp PCR system 2400) thermal cycler. The PCR temperature program was as follow: A pre-PCR treatment of 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 5 s, annealing at 55ºC for 30 s, and primer extension at 72ºC for 40 s. An incubation at 72ºC for 2 min followed the final cycle for complete synthesis of elongating DNA molecules. Negative control (no DNA added) was also included in parallel witheach set of amplification. Ten µl of PCR product was analysed in a 2% agarose gel (Sigma) in TAE buffer, pH 8.0. Electrophoresis was performed on a minigel apparatus (Bio-Rad), at 74 V. A molecular standard 1Kb plus DNA ladder (Life-Technologies) was analyzed in the gel. The electrophoretic bands were visualized over ultra-violet (322 nm) after staining with ethidium bromide (0.5 µg/ml).

Restriction enzymes Digestion

PCR product of mitochondrial cytochrome b gene was subjected to restriction enzyme digestion with 7 types of restriction enzymes, namely *Rsa*I, *Bsa*JI, *Bst*NI, *Alu*I, *Taq*I, *Nsi*I and *Bst*UI. Five units of each enzyme were applied to 10 µl of amplified DNA in a final volume of 20 µl digestion mixture containing 1x reaction buffer (10 mMTris-HCl, 50-100 mMNaCl, 10 mM MgCl2 and 1 mMdithiothreitol). The *Bst*NI and *Taq*Iwere supplemented with 0.5 µl of 100mg/ml BSA (bovine serum albumin). The digestion mixture was incubated overnight for optimal result and incubation temperature was set according to the types of restriction enzyme. Twenty µl of the digested samples were analyzed ona 2% agarose gel (Sigma) in TAE buffer at 74 V. The gels were stained with 0.5 µg/ml ethidium bromide and visualized using theGel Documentation and Analysis System (AlphaImager™ 1220).

RESULTS AND DISCUSSION

Total DNA was extracted from an internal portion of pig, cow, buffalo, goat, deer, rabbit, ostrich, turkey, chicken, and duck meats. Universal primers, cytb1 and cytb2 amplified the cytochrome b gene from all the DNA of animal samples. The sizes of the amplicons were approximately 360 bp (Fig.1). The 360bp fragment of the cytochrome b gene was reported to be highly polymorphic (Bellagamba*et al.* 2001), and could be used to differentiate meat species. When the amplicon was cleaved with restriction enzyme, the restriction map or RFLP profiles produced differed from animal species to another. This analysis termed as PCR-RFLP analysis was performed on 3 independent meat samples of each meat species to ensure that the restriction map of the cytochrome b gene for each animal species was consistent. The restriction enzymes used for the digestion were; *Rsa*I, *Bsa*JI, *Bst*NI, *Alu*I, *Taq*I, *Nsi*I and *Bst*UI. Theunique restriction map of the amplified cytochrome b gene of a meat species formed the basis for animal species identification.

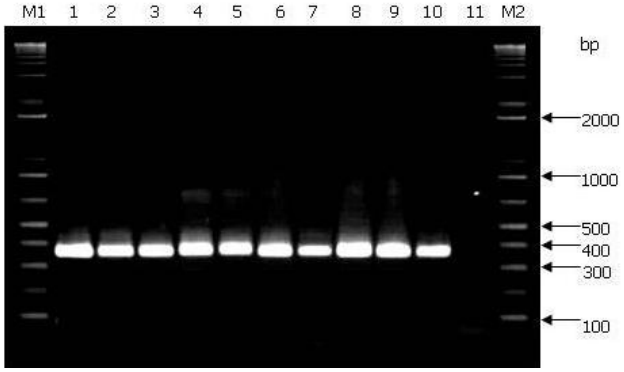


Figure 1.Amplified cytochrome b gene. Lanes: M1, 1 Kb Plus DNA ladder (LifeTechnologies); 1, pig; 2, cow; 3, buffalo; 4, goat; 5, deer; 6, rabbit; 7, ostrich; 8, turkey; 9, chicken; 10, duck; 11, negative control (a PCR mixture without any DNA template).

In general, it was observed that the restriction enzymes could not digest some of

the cytochrome b amplicons completely for all the meat species (Figs. 2 - 4). Hence, part of the 360 bp cytochrome b amplicons remained as the top band on the agarose gel. Nevertheless, the digested portion of the cytochrome b amplicons gave banding patterns (RFLP profile) that allowed the meat species to be differentiated. Digestion with restriction enzyme *Rsa*Iproduced characteristic DNA fragments in ostrich, turkey, and chicken amplicons. A single restriction site for restriction enzyme*Rsa*I was found in the DNA sequences of ostrich, turkey, and chicken cytochrome b amplicons, yielding 2 DNA fragments (Fig. 2a) of approximately 150bp and 210 bp for ostrich (lane 7) and chicken (lane 9), and 110bp and 150bp for turkey (lane 8). In this study, a single restriction site was also found in cow, buffalo, and goat, yielding 2 DNA fragments. Two faint DNA fragments in sizes of approximately 160 and 180bpwhich could not be separated effectively from goat were shown in Fig.2a (lanes 4). This was due to the low resolution of agarose gel and a result of incomplete digestion. Restriction enzyme*Rsa*I digested cytochrome B DNA bands for cow and buffalo did not produce patterns that were easily distinguishable from other meat sources. In comparison, the RFLP profile of cytochrome b amplicons between chicken and ostrich were very similar. Hence, additional enzyme would be required to differentiate between these two meat species. Restriction enzyme*Rsa I* did not cleave the cytochrome b amplicons of pig, deer, rabbit and duck.

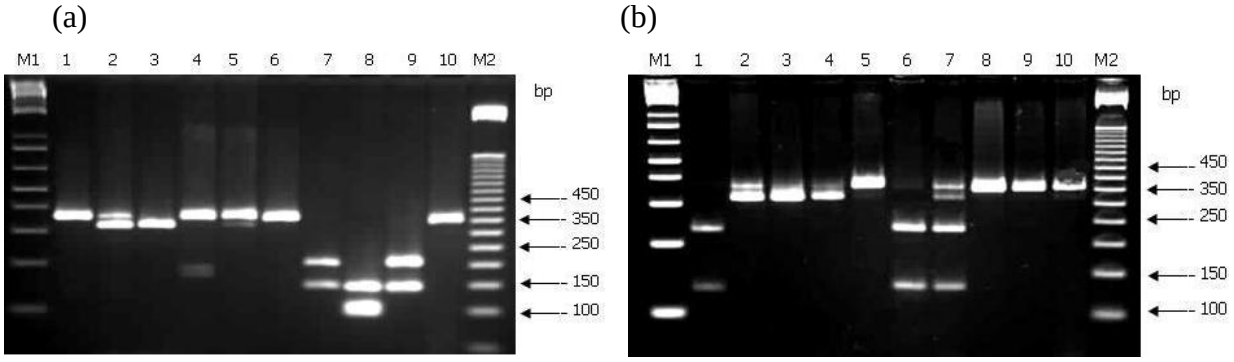


Figure 2 (a)*Rsa*I and(b)*Bsa*JIrestriction profiles of the cytochrome b amplicon. Lanes: M1, 1 Kb Plus DNA ladder (Life Technologies); M2, 50 bp DNA ladder; 1, pig; 2, cow; 3, buffalo; 4, goat; 5, deer; 6, rabbit; 7, ostrich; 8, turkey; 9, chicken; 10, duck.

A *Bsa*JI restriction enzyme's site was present in the cytochrome b amplicons of pig, rabbit and ostrich, yielding two fragments of approximately 130bp and 230 bp, whilst the cytochrome b amplicons of deer, turkey, chicken and duck were not cleaved by *Bsa*JI (Fig. 2b). *Bsa*JI cleaved the cytochrome b amplicons of cow, buffalo and goat but did not produce patterns that were easily distinguishable from other meat sources. The sizes of *Bsa*JIrestricted DNA fragments of pig, rabbit and ostrich appeared indistinguishable by electrophoretic analysis. Therefore, the digestion by an additional restriction enzyme would be required to differentiate between these species.

Restriction enzyme, *Bst*NI cleaved the cytochrome b ampliconof duck to produce two fragments of approximately 50bp and 300bp (Fig 3a, lane 10) but did not cleave the PCR amplicons of other meat species. Restriction enzyme,*Alu*Icleaved the cytochrome b amplicons of pig, cow and buffalo yielding two fragments with sizes of approximately 120bp and 240bpfor pig, 120 bp and 140 bpfor cow,and 110 bpand140bp for buffalo (Fig. 3b). Restriction

enzyme,*Alu*I did not cleave the cytochrome b amplicons of goat, deer, rabbit, ostrich, turkey, chicken and duck. This indicated that pig, cow and buffalo meats could be distinguished from other species using RFLP profile generated by this restriction enzyme.

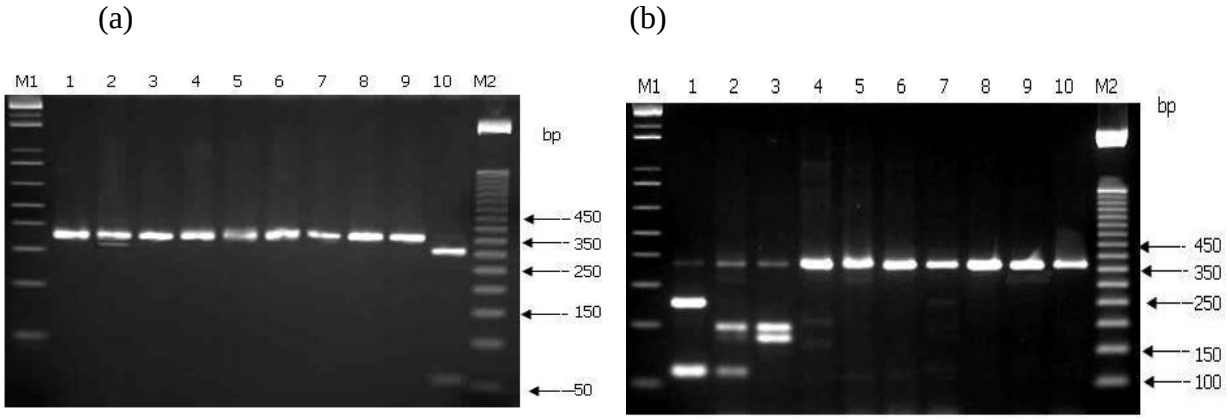
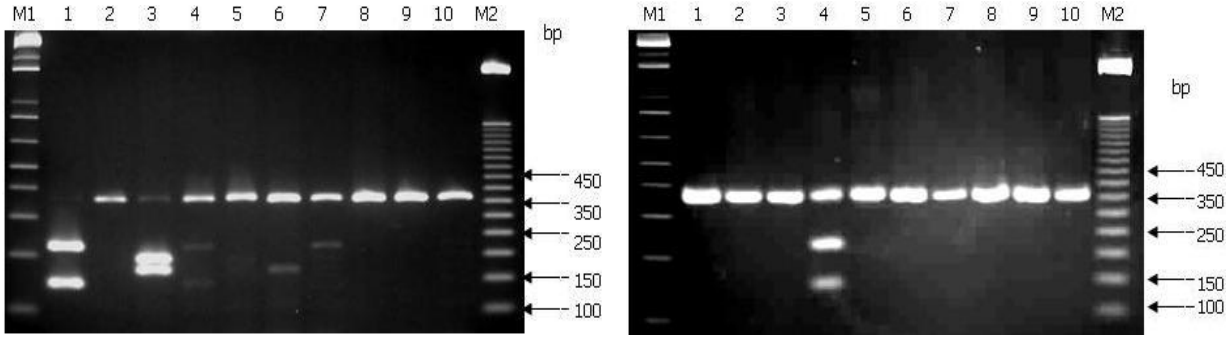


Figure 3 (a)*Bst*NI and(b)*Alu*Irestriction profiles of the cytochrome b amplicon. Lanes: M1, 1 Kb Plus DNA ladder (Life Technologies); M2, 50 bp DNA ladder; 1, pig; 2, cow; 3, buffalo; 4, goat; 5, deer; 6, rabbit; 7, ostrich; 8, turkey; 9, chicken; 10, duck.

Restriction enzyme, *Taq*I digested the cytochrome b amplicons producing DNA fragments of approximately 150bp and 200bp for pig (Fig. 4a, lane 1) and goat (Fig. 4a, lane 4), and DNA fragments of approximately 170bp and 200bp for buffalo (Fig. 4a, lane 3). Restriction enzyme, *Taq*Igenerated RFLPthat could notbe used discriminate between goat and pig because the sizes of digestion fragments were very similar in their sizes (Fig. 4a, lanes 1 and 4). Restriction enzyme, *Taq*Idigested the cytochrome b ampliconsto produce DNA fragments of approximately 180 bp for rabbit and 220 bp for turkey.RFLP generated byrestriction enzyme *Taq*I for buffalo, rabbit and ostrich could distinguish the 3 meat species from those of pig and goat species.*Taq*Idid not cleave the cytochrome b amplicons of buffalo, deer, turkey, chicken and duck.

A single cleave site for restriction enzyme, *Nsi*I was found in the cytochrome b amplicon of goat, yielding 2 DNA fragments of approximately150bp to 220 bp (Fig 4b, lane 4). *Nsi*I did not cleave the cytochrome b amplicons of other species of meats. Hence, goat meat could be easily distinguished from others species of meat samples by digesting its cytochrome b ampliconwith restriction enzyme *Nsi*I. Restriction enzyme, *Bst*UIdigested the cytochrome b amplicons of turkey and rabbit to produce two DNA fragments each with sizes of approximately 150 bp and 250 bp(Fig. 4c, lanes6 and 8).The size of *Bst*UI restriction fragments between rabbit and turkey appeared indistinguishable on the agarose gel. Cytochrome b amplicons of other species of meat samples were not cleaved by *Bst*UI.

(a) (b)



(c)

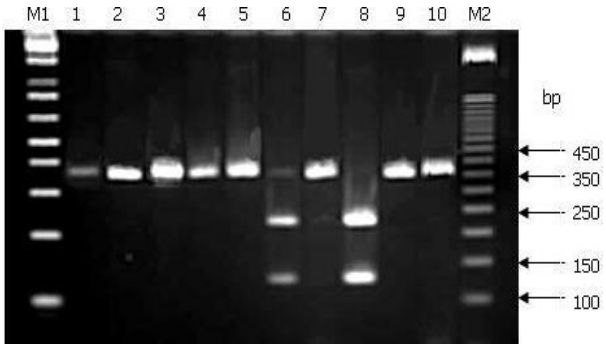


Figure 4 (a)*Taq*I, (b)*Nsi*I and (c)*Bst*UI restriction profiles of the cytochrome b amplicon. Lanes: M1, 1 Kb Plus DNA ladder (Life Technologies); M2, 50 bp DNA ladder; 1, pig; 2, cow; 3, buffalo; 4, goat; 5, deer; 6, rabbit; 7, ostrich; 8, turkey; 9, chicken; 10, duck.

Strategies for meat species identification based on the RFLP patterns

The present study demonstrated that it was possible to authenticate animal or meat species that were normally consumed in Malaysia using the PCR-RFLP method. Pig, cow and buffalo were easily differentiated from other meat species by digesting their cytochrome b amplicons with *Alu*I that generated unique RFLP profiles. Buffalo, rabbit and ostrich were distinguished from other meat species by digesting their cytochrome b amplicons with *Taq*I. Amplicons of turkey, duck and goat species were digested by *Rsa*I,*Bst*NIand *Nsi*Irespectively to generate unique RFLP profiles that segregated them from other meat species tested.Chicken was differentiated from ostrich by digesting their cytochrome b amplicons with *Rsa*I and *Taq*I. Cytochrome b amplicon of deer meat sample were not digested by any of the restriction enzymes used in this study. Hence, other types of enzymes were required to obtain RFLP profile for deer. Matsunaga*et al.* (1998)reported that the RFLP profiles of cytochrome b generated by restriction enzymes,*Eco*RI,*Bam*HI and *Sca*Icould possiblybe used to differentiate deer meat from other meat species.

Authentication of processed meat

To test the feasibility of the PCR-RFLP method in meat authentication, anexperiment was carried out to determine the identity of processed meat. DNA was isolated from processed chicken sausage, chicken burger meat, chicken nugget, beef burger meat and beef sausages. Smeary genomic DNA bands were observed in all the lanes in Fig. 5a indicating that the DNA from the processed meat samples was partially degraded. Degradation might have taken place during the processing of the food products in the factory or during storage. However, the DNA

obtained was sufficient and the quality was good enough for PCR amplification (Fig. 5b). The degraded DNA did not affect the PCR amplification process where single dense DNA bands of the cytochrome b gene with the sizes of approximately 360 base pairs were amplified for all the processed meat (Fig. 5b). This indicated that the PCR-based method was versatile and was able to amplify theminute amount of intact DNA from processed meat.

Digestion of the cytochrome b ampliconsof raw and processed beef samples with *Alu*I produced two DNA fragments with the sizes of approximately 170 and 190bp (Fig. 6a, Lanes 4, 5 and 7). The expected DNA bands of approximately 170 and 190 bp appeared as a single band while the 50 bp DNA band ran off the gel. Nevertheless, the resultswere able todifferentiatethe raw and processed chicken samples from raw and processed beef products as *Alu*I did not cleave thecytochrome b amplicon from chicken. Digestion with *Rsa*I cleaved raw chicken and all the processed chicken products producing two distinct DNA bands of approximately 150bp and 210bp. The banding patterns of the digestion of raw chicken samples when digested with restriction enzyme,*Rsa*I (Fig. 6b, Lane 6) shared similar RFLP profile with the chicken burger meat, chicken sausage, and chicken nugget (Fig. 6b, Lanes 1 – 3 respectively) indicating that all the processed chicken products were not contaminated by other meats.

Surprisingly it was found that both the beef burger meat and beef sausages were contaminated with chicken meat. Restriction enzyme, *Rsa*Idid not cleave the raw beef (Fig. 6b Lane 7, control for beef) but cleaved the cytochrome b amplicons of the two beef products (Fig. 6b ,Lanes 4 and 5). Apart from the 326 bpDNA fragment of beef-specific profile, two distinct DNA bands of approximately 150 and 210bp were observed in the beef burger meat and beef sausage samples. The RFLP profiles of the two processed beef matched the RFLP profile of the chicken. These tests were repeated three times using fresh DNA isolated from the two beef products and the results obtained were consistent. This observation was further confirmed by sequencing several amplified cytochrome b fragments from the beef products. The sequences confirmed the presence of chicken DNA in the beef burger meat and beef sausage (data not shown).This showed that the beef burger meat and beef sausages were most probably contaminated with chicken meat. The contamination could be due to the same set of machineries being used to process the two types of meat products in the factory. In cases like these, the PCR-RFLP technique wasshown to be useful, simpleand rapid to detect the presence of other meat contaminants.

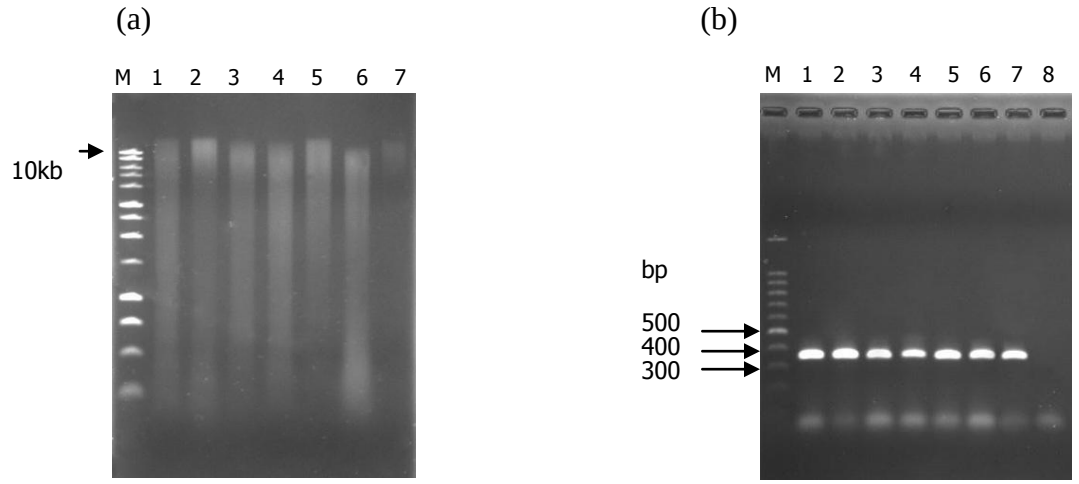


Figure 5(a) DNA extracted from processed meat on a 1% agarose gel. M- 1kb DNA ladder, Lane 1- Chicken sausage, Lane 2- Chicken burger meat, Lane 3- Chicken nugget, Lane 4- Beef burger meat, Lane 5- Beef sausage, Lane 6- Raw Chicken, Lane 7- Raw Beef. (b) PCR products from thecytochrome b gene of processed meats on a 1.5% agarose gel. M- 100bp ladder, Lane 1- Chicken sausage, Lane 2- Chicken burger meat, Lane 3- Chicken nugget, Lane 4- Beef burger meat, Lane 5- Beef sausage, Lane 6- Raw chicken, Lane 7- Raw beef, Lane 8- negative control.

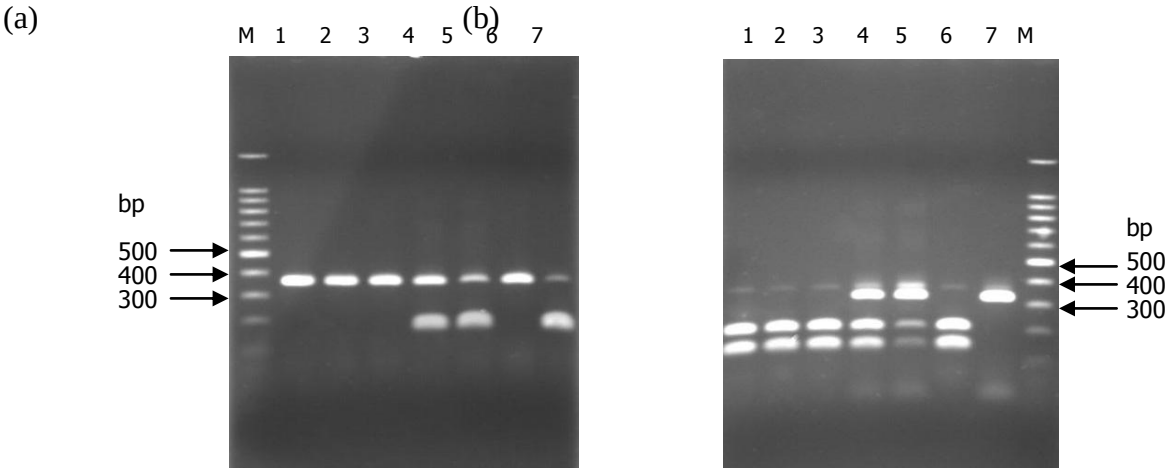


Figure 6 (a) *Alu*I and (b) *Rsa*I restriction profile of cyt*b* PCR products amplified from processed meat samples. M- 100bp ladder, Lane 1- Chicken sausage, Lane 2- Chicken burger meat, Lane 3- Chicken nugget, Lane 4- Beef burger meat, Lane 5- Beef sausage, Lane 6- Raw chicken, Lane 7- Raw beef.

CONCLUSION

PCR-RFLP based DNA typing method was employed to differentiate and determine the RFLP profiles of the ten meat species that were commonly sold in the market in Malaysia, namely, pig, cow, buffalo, goat, deer, rabbit, ostrich, turkey, chicken and duck. A pair of universal primers that flanked a portion of the mitochondrial cytochrome b gene was used to generate an

ampliconof approximately360bp for the ten meat species. RFLP analysis on the 360bpamplicons by restriction enzymes – *Rsa*I, *Bsa*JI, *Bst*NI, *Alu*I, *Taq*I, *Nsi*I and *Bst*UIgenerated interspecies polymorphisms except for the deer. Hence, these intraspecies homologies provided an excellent standard for interspecific differentiation. In this study,there was no restriction site for all the restriction enzymes used in the cytochrome b amplicon of deer. Therefore, additional restriction enzymes would be needed to determine the enzymes that wouldcleave the cytochrome b amplicon of deer.

The cytb1 and cytb2 primers employed in this study were considered to be universal and were shown to amplify the cytochrome b gene from all the meat species tested which encompassed mammals, ruminants and birds. The cytochrome b PCR-RFLP species identification assay yielded excellent results for identification of fresh, frozen and cooked meats. The results of the analyses of the processed chicken burger meat, chicken sausage, chicken nugget beef burger meat and beef sausage samples showed that the PCR-RFLP analyses technique couldbe used to detect contaminants in meat products sold in the market. Analyzed processed meat could be authenticated and certified to gain the confidence of the consumers. This technique could also be used to detect the presence of non-permissive meat such as pork in processed meats for halal certification.

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REFERENCES

Bellagamba, F., Moretti, V. M., Comincini, S. &Valfrè, F. (2001). Identification of species in animal feedstuffs by polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial DNA. J. Agri.& Food Chem., 49: 3775-3781.

Borgo, R., Souly-Crosset, C., Bouchon, D. &Gomot, L. (1996).PCR-RFLP analysis of mitochondrial DNA for identification of snail meat species. J. Food Sci. 61:1-4

Chen, F.C., Peggy Hsieh, Y-H.& Bridgman, R.C. (1998) Monoclonal antibodies to porcine thermal-stable muscle protein for detection of pork in raw & cooked meats. J. Food Sci.63: 201-205

Chikuni, K., Tabata, T., Kosugiyama, M. &Monma, M. (1994).Polymerase chain reaction assay for detection of sheep & goat meats. Meat Sci., 37: 337-345.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pabo, S., Villablanca, F. X. & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in mammals: amplification & sequencing with conserved primers. Proc. Natl. Acad. Sci. 86: 6196- 6200.

Koolmees, P. A. (1999). Histology as an additional technique for species identification in meat products.*InBergwerff, A. A. (Ed).* SpeciesIdentification in Meat Product, p. 35-41. Utrecht, The Netherlands: ECCEAMST.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Nakai, H., Shibata, K., Yamada, J.&Shinmura, Y. (1998).Determination of mitochondrial cytochrome b gene sequence for red deer (*Cervuselaphus*) & the differentiation of closely related deer meats. Meat Sci. 49: 379-385

Meyer, R., Hofelein, C., Luthy, J.&Candrian, U. (1995). Polymerase chain reaction- restriction fragment length polymorphism analysis: a simple method for species identification in food. J. AOAC. 78: 1542-1551.

Montiel-Sosa, J.F., Ruiz-Pesini, E., Montoya, J., Roncalés, P., López-pérez M.J. & Pérez-Martos, A. (2000) Direct & highly species-specific detection of pork meat & fat in meat product by PCR amplification of mitochondrial DNA. *J.* Agric. Food Chem. 48: 2829-2832

Saiki, R.K., Schart, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. &Arnheim, N. (1985) Enzymetic amplification ß-globin genomic sequences & restriction site analysis for diagnosis of sickle cell anemia. Science. 230: 1350-1354.

Wolf, C., Burgener, M., Hübner& P.,Lüthy, J. (2000). Identification of flatfish by PCR-RFLP. J. Sci. Food & Agric. 83: 752-759.