ASEAN Food Journal 14 (1): 51-59 (2007)

Meat Molecular Detection: Sensitivity of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism in Species Differentiation of Meat From Animal Origin

[1]Ong, S.B., [3]Zuraini, M.I., [1]Jurin, W.G., [2]Cheah, Y.K., [1]Tunung, R., [1]Chai, L.C., [1]Haryani, Y., [1]Ghazali, F.M. and [*1]Son, R.

[1]National Food Safety Research Centre, [1]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3]Faculty of Hotel and Tourism Management, Universiti Teknologi MARA, Kampus Dungun, Kompleks Dura, 23000 Dungun, Terengganu, Malaysia

Abstract: Three restriction enzymes were used in Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) using the mitochondrial cytochrome b region to establish a differential diagnosis which detect and discriminate between three meat species: pork, cow and chicken**.** DNA was extracted from samples containing meat of a single animal such as raw pork (*Sus scrofa domesticus*), chicken (*Gallus gallus*) and cow (*Bos taurus*) as well as mixed samples of two species of animals in different ratios. The amplified 359 base pairs (bp) portion of the mitochondrial *cyt* b gene from pure or mixed samples in different ratios was cut using three different restriction enzymes resulting in species specific restriction fragment length polymorphism (RFLP). This technique proved to be extremely reliable in detecting the presence of low levels of target DNA obtained from a 0.25 mg component in a particular mixed meat sample. This revealed the *cyt* b region as highly conserved and consequently a good molecular marker for diagnostic studies. Thus, this technique can be applied to food authentication for the identification of different species of animals in food products.

Keywords: PCR-RFLP, differentiation, meat, food, authentication

INTRODUCTION

Many meat products nowadays may contain several species in different proportions mixed together and undetectable by the naked eye or by eating. Meat adulteration has become a common practice in most countries. In the field of food analysis, species determination is mostly sufficient, but simultaneous detection of several species in a single food product is desirable. Identifying the species of a plant or animal can be uncertain whenever the usual species characteristics such as size, shape and appearance (the morphological characters) are removed on processing and only a portion of flesh is available (Mackie *et al*., 1999). This proves to be a serious issue in terms of ethics, religion, health, trade and commerce.

Food manufacturers as well as food processing factories may purposely add different types of meats to a particular meat product so as to add bulk or make up the volume of the product. For example, pork is a

*Corresponding author.
E-mail address: *son@putra.upm.edu.my (Son, R.)*

potential source for adulteration of higher value meat such as beef and veal (Chen *et al*., 1998). Meat products containing undeclared, exotic or less desirable species also may impose a potential health risk to people with allergies to certain proteins. There are consumers who are intolerant to mutton or sensitive to chicken meat and vegetarians who do not consume meat at all. Due to this, species differentiation of raw materials to be used for the industrial preparation of foods and the detection of animals' species in final food products is becoming more and more important as the society progresses in step with science.

Currently, species identification had been achieved through different methods, which may be chosen for test application depending on the purpose. They are mainly based on the analysis of certain biomolecules, such as protein-isoelectric focusing; immunochemical, immunoassay, electrophoretic methods (Zerifi *et al*., 1991), fatty acids (Verbeke and Brabander, 1980) or they are based on specific microscopic structure elements determination (Koolmees, 1999). However, each of these methods has major drawbacks, which are a consequence of their dependence on the ability to characterize proteins. Protein expression is tissue dependent and they may be denatured on processing and heating, leading to subsequent loss of analytical specificity (Hunt *et al*., 1997). Other techniques will require blotting, staining, long period for results production, preparation of antibodies and single-stranded DNA. As for immunological methods, a disadvantage of the majority of them is that they detect soluble plasma proteins and it has been argued that this is not 'meat' and may arise from adventitious contamination with blood from other species (Hunt *et al*., 1997). Therefore, such techniques have limited applications in food analysis.

For these reasons, nucleic acids based analyses are becoming more and more popular for the identification and differentiation of food and food products (Meyer *et al*., 1995). DNA especially, has been exploited for species identification due to its stability at high temperatures and its structure being conserved within all tissues of an individual. This has resulted in the development of species-specific DNA probes (Chikuni *et al*., 1990; Ebbehøj and Thomsen, 1991), polymerase chain reaction (PCR) assays (Chikuni *et al*., 1994; Meyer *et al*., 1994), random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al*., 1991) and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

In this study, the sensitivity of PCR-RFLP for the detection of pork, chicken and cow meat was evaluated. The approach involved mixing two different samples in different ratios followed by amplification of the cytochrome b gene of mitochondrial DNA and RFLP using appropriate restriction enzymes to allow detection and differentiation of the different species in the mixed samples.

MATERIALS AND METHODS

Meat Samples

Three different types of meat samples from animal origin (pig, chicken and cow) were chosen and obtained from the market in Batu Pahat, Johor, Malaysia. The meat samples were stored at -20^{0}C before the extraction of the DNA in order to prevent the enzymatic degradation of DNA.

DNA Extraction

Twenty five milligrams of each meat sample was sliced using sterile dissecting scissors and forceps for preparation of pure animal meat samples containing only pork, chicken or cow meat (Table 1). For the preparation of mixed samples, meat from two different kinds of animals were sliced according to the specified weight to reach a total of 25 mg and placed in 1.5 ml microcentrifuge tubes. Different combinations of animal species and weight were achieved and a total of 12 tubes of mixed samples were labeled as A1, A2, A3, A4, B1,

B2, B3, B4, C1, C2, C3 and C4 (Table 1). DNA was extracted from the meat samples using the DNeasy Protocol for Animal tissue provided with the DNeasy™ Tissue Kit (Qiagen). For the analysis of DNA quality, readings were taken using a spectrophotometer (Pharmacia Biotech) at wavelengths ratio of 260 / 280 nm.

Amplification of the CYT b Gene by Polymerase Chain Reaction (PCR)

A pair of universal primers (CYT b1, 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3' and CYT b2, 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3') was employed in the PCR reaction for amplification of the cytochrome b gene region (Kocher *et al*., 1989) in a final volume of 20'µl containing 20 ng extracted DNA, 1x PCR complete buffer (BIORON), 10 mM dNTP's (Finnzymes), 5 pmol of each primer and 5 U/µl of *Taq* DNA polymerase (BIORON). The PCR step-cycle program was as follows: pre-denaturation of 94°C for 5 minutes to completely denature the DNA template followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds. Final extension at 72°C for 10 minutes followed the final cycle for complete synthesis of elongated DNA molecules.

Restriction Endonuclease Digestion of the CYT b Amplicon

PCR products of mitochondrial cytochrome b were immediately subjected to restriction enzyme digestion using *Alu* I (5'…AG / CT…3'; 10,000 U/ml), *Bsa* JI (5'…C / CNNGG…3'; 2500 U/ml) and'*Rsa* I (5'…GT / AC…3'; 10,000 U/ml) (New England Biolabs®). Five units of each enzyme were applied to 10 µl of amplified DNA in a final volume of 20 µl digestion mixture, which contained 1X reaction buffer. The digestion mixture was incubated in a water bath (Thermomix® BU) for 16 hours at the specific incubation temperature according to the restriction enzymes used.

The digested samples were electrophoresed through 2% agarose gel in 1X TAE buffer at 80V until the markers migrated to about two-thirds of the gel. The sizes of the bands produced were compared with the 50 bp ladder. The gels were stained with 0.5 µg/ml ethidium bromide for 5 minutes and destained for 10 minutes. The destained gels were viewed with Gel Doc 2000, Bio-Rad and printed using a Sony digital graphic printer (UP-D890).

RESULTS AND DISCUSSION

DNA was successfully extracted from the meat of chicken, cow and pork using the DNeasy Tissue Kit (Qiagen). The quality of the extracted DNA was examined using a spectrophotometer and gel electrophoresis (Table 2). The absorbance readings showed the purity of the extracted DNA. The bands of high intensity in the gel electrophoresis proved that the extracted DNA is sufficient and of good quality to be used for PCR amplification of the mitochondrial cytochrome b region (Figure 1).

The locus of cytochrome b has been well characterized among different vertebrate groups (Hatefi, 1985; Irwin *et al*., 1991). These studies revealed that the level of cytochrome b gene sequence variation is suitable for addressing general questions on inter-specific diversity. Universal primers CYT b1 and CYT b2 (Korcher *et al*., 1989) consistently amplified a fragment of 359 bp of the cytochrome b gene. An advantage in employing universal primers is that it obviates the requirement for an internal control, which is otherwise used to monitor the success of DNA amplification. Besides that, since each cell may contain up to 1000 copies of the cytochrome b locus, PCR assays based on its amplification should offer the advantage of increased sensitivity in comparison to single or low copy nuclear DNA targets (Partis *et al*., 2000). For all investigated samples, the 359 bp PCR product from the cytochrome b gene was amplified by the universal primers CYT b1 and CYT b2 and no unspecific PCR amplicons were detected. The

successful amplification of amplicons proved that the PCR is a highly sensitive method.

Following digestion using the three kinds of restriction enzymes, pork DNA was found to be successfully digested by *Alu* I and *Bsa* JI. A 50 bp ladder (New England BioLabs®) was used as molecular size markers. Single restriction sites occur in pork DNA for both the *Alu* I and *Bsa* JI. For *Alu* I, 2 bands of 115 bp and 244 bp were produced and for *Bsa* JI, 2 bands of 131 bp and 228 bp were produced (Table 1). Results of experimental RFLP indicate that the 359 bp amplicon from pork DNA can be restricted using *Alu* I and *Bsa* JI enzyme. The 359 bp DNA fragment from chicken meat was cut by the restriction enzyme *Rsa* I but not by *Alu* I and *Bsa* JI. The single restriction sites of *Rsa* I in the amplicon from the chicken DNA produced two bands of 149 bp and 210 bp. The 359 bp amplification products from 25 mg of cow meat produced three and two fragments when digested with the restriction enzymes *Alu* I and *Bsa* JI, respectively. However, *Rsa* I do not cut the amplicons from cow DNA (Table 1). Based on the calculation of corresponding RFLP-patterns of the three investigated species, the three restriction enzymes used were found to give reasonable restriction patterns allowing differentiation of the three investigated species (Table 1). It is important to note that in many cases only two or three restriction enzymes are sufficient to be used for the identification of a single species. This is due to the possible complicated identification of a single species within a mixture of several species as some mixtures would result in very complex restriction patterns. Thus, the results obtained suggested that the three restriction enzymes used were sufficient for the identification and differentiation of the three investigated species.

Fraudulent substitutions of alternative meat species in meat products have led to the need for reliable and specific methods of meat species determination (Hunt *et al*., 1997). A variety of analytical approaches based on protein analysis or immunological assay have been reported previously, but these techniques have limited applications. Therefore, researchers have resorted to nucleic acids analyses, which are more stable, robust, efficient and versatile. PCR-RFLP is one of these techniques employed in differentiating the contents in food products. For the determination of the sensitivity of the PCR-RFLP in detecting minute amounts of DNA in the mixed samples, various combinations of the pork-cow and pork-chicken meat were investigated (Table 1). Samples A1 to A4 and B1 to B4 consist of mixed DNA from pork and cow in different percentages or weights. The 359 bp cyt b fragment was amplified and subjected to digestion using the three restriction endonucleases and the presence of both pork and cow in the mixed sample was confirmed by the resulting fragments that represented the cutting sites of the enzymes in the 359 bp amplicons from pork and cow meat, respectively (Table 1). Samples C1 to C4 consists of mixed DNA from pig and chicken in different percentages or weights and the restriction endonuclease digestion of the 359 bp *cyt* b fragments produced the expected fragments (Figure 2) that represent the pork and chicken meat, respectively (Table 1). The results obtained showed that PCR-RFLP using *Alu* I, *Bsa* JI and *Rsa* I is sensitive enough to detect and differentiate pork, cow and chicken DNA from a mixed sample containing as little as 0.25 mg of the meat. Moreover, the absence of variability in the results highlighted a precise reproducibility of the method. In conclusion, this work describes the establishment of a molecular diagnostic test of PCR-RFLP to discriminate between the three species of meat, allowing detection of falsely declared meat or meat products made up of a single species or mixed samples.

Effective molecular meat diagnosis requires a system that ensures the declared meat species is correct. The optimized procedure in this study represents a valid PCR-based method to test meat for fast and accurate results. This will contribute to an analytical approach that is advanced and capable of

Table 1: Percentage, weight of meat from different species present in the samples and the calculated restriction fragment length (bp) polymorphism of the mt *cyt b* gene of pork,chicken and cow

Sample Type	Percentage and weight of meat samples used			Calculated restriction fragment length (bp) of the *cyt* b amplicon		
	Pork	Chicken	Cow	*Alu* I	*Bsa* JI	*Rsa* I
Pork	100% (25.00 mg)	na	na	115 244	131 228	359
Chicken	na[a]	100% (25.00 mg)	na	359	359	149 210
Cow	na	na	100% (25.00 mg)	19 160 180	39 320	359
A1	1% (0.25 mg)	na	99% (24.75 mg)	19 115 160 180 244	39 131 228 320	359
A2	3% (0.75 mg)	na	97% (24.25 mg)	19 115 160 180 244	39 131 228 320	359
A3	5% (1.25 mg)	na	95% (23.75 mg)	19 115 160 180 244	39 131 228 320	359
A4	10% (2.50 mg)	na	90% (22.50 mg)	19 115 160 180 244	39 131 228 320	359
B1	99% (24.75 mg)	na	1% (0.25 mg)	19 115 160 180 244	39 131 228 320	359
B2	97% (24.25 mg)	na	3% (0.75 mg)	19 115 160 180 244	39 131 228 320	359

Table 1: Continued

Sample Type	Percentage and weight of meat samples used			Calculated restriction fragment length (bp) of the *cyt* b amplicon		
	Pork	Chicken	Cow	*Alu* I	*Bsa* JI	*Rsa* I
B3	95% (23.75 mg)	na	5% (1.25 mg)	19 115 160 180 244	39 131 228 320	359
B4	95% (22.50 mg)	na	10% (2.50 mg)	19 115 160 180 244	39 131 228 320	359
C1	1% (0.25 mg)	99% (24.75 mg)	na	115 244 359	131 228 359	149 210 359
C2	3% (0.75 mg)	97% (24.25 mg)	na	115 244 359	131 228 359	149 210 359
C3	5% (1.25 mg)	95% (23.75 mg)	na	115 244 359	131 228 359	149 210 359
C4	10% (2.50 mg)	90% (22.50 mg)	na	115 244 359	131 228 359	149 210 359

1 - na, not applicable

delivering the desired performance for identification of small amounts of DNA to support an efficient surveillance system for species substitution that is lacking nowadays.

ACKNOWLEDGEMENTS

This research was supported by the funds from a short-term Universiti Teknologi MARA and Universiti Putra Malaysia IRPA grant.

Table 2: Amount of DNA extracted from the various samples examined

Samples	A_{260}/A_{280}	DNA (μg/ml)
A1	1.67	50
A2	1.13	25
A3	2.13	125
A4	1.25	25
B1	1.77	95
B2	1.67	25
B3	1.33	30
B4	1.00	5
C1	1.48	75
C2	1.67	40
C3	1.36	40
C4	1.64	90
Pork	1.50	30
Chicken	1.63	35
Cow	2.29	40

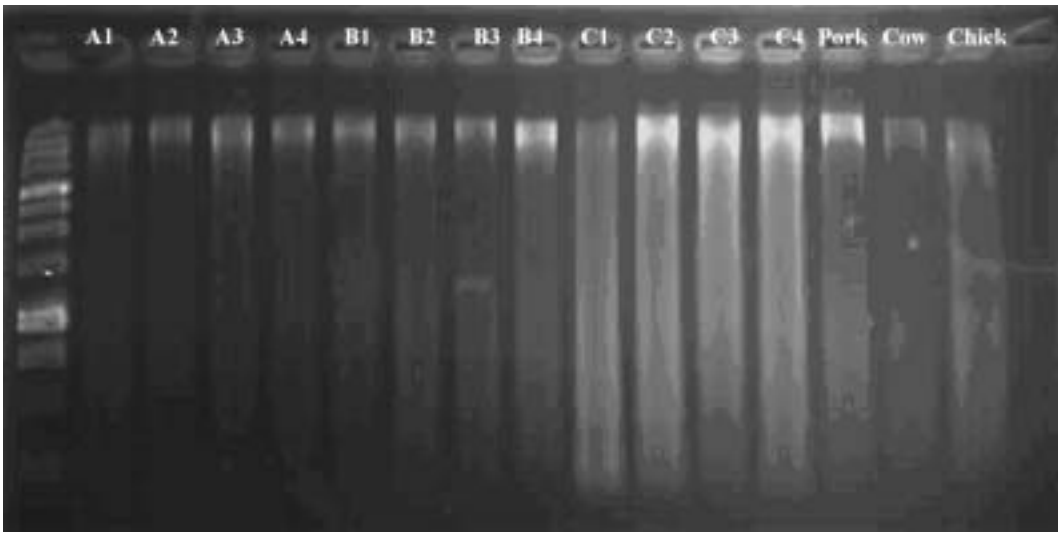

Figure 1: Agarose gel electrophoresis showing the extracted genomic DNA from all the samples. The unmarked lane on the far left contains the 50 bp ladder molecular weight markers

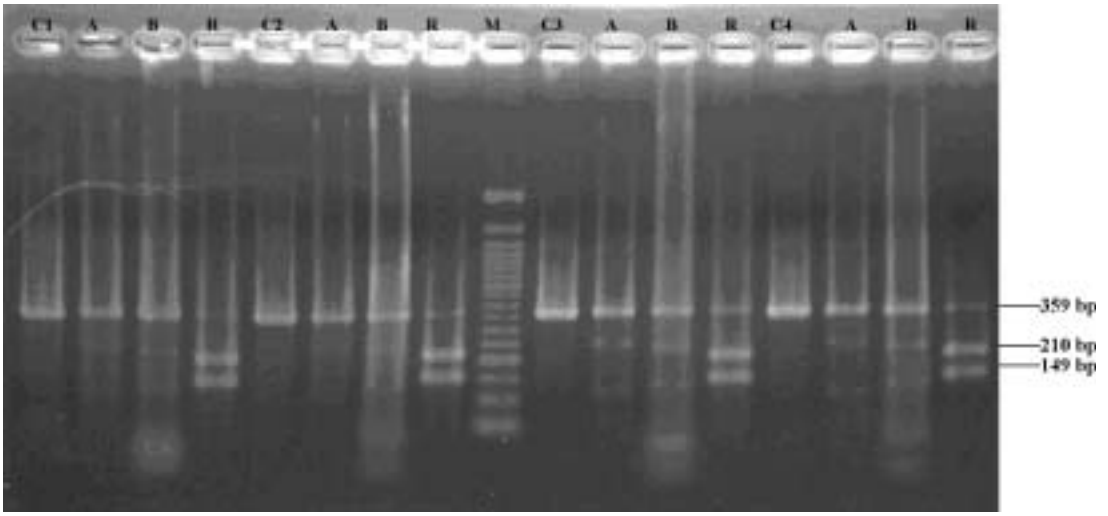

Figure 2: Agarose gel electrophoresis showing PCR-RFLP products of samples C1-C4 (mixed samples of chicken and pork meat as defined in Table 1) and restricted fragments using three restriction endonucleases: A = *Alu* I, B = *Bsa* JI, R = *Rsa* I. M, 50 bp ladder molecular weight markers

REFERENCES

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

Chikuni, K., Ozutsumi, K., Koishikawa, T. and Kato, S. 1990. Species identification of cooked meats by DNA hybridization assay. Meat Science, 27: 119-128.

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

Ebbehøj, K. F. and Thomsen, P. D. 1991. Species differentiation of heated meat products by DNA hybridization. Meat Science, 30: 221-234.

Hatefi, Y. 1985. The mitochondrial electron transport and oxidative phosphorylation system. Annual Review of Biochemistry, 54: 1015-1069.

Hunt, D. J., Parkes, H. C. and Lumley, I. D. 1997. Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. Food Chemistry, 60: 437-442.

Irwin, D. M., Kocher, T. D. and Wilson, A. C. 1991. Evolution of the cytochrome b gene of mammals. Journal of Molecular Evolution, 32: 128-144.

Korcher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pabo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. Procedure National Academy of Science USA, 86: 6196-6200.

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

Mackie, I. M., Pryde, S. E., Gonzales-Sotelo, C., Medina, I.., Perez-Martin, R., Quinteiro, J., Rey-Mendez, M. and Rehbein, H. 1999. Challenges in the identification of species of canned fish.

Trends in Food Science and Technology, 10: 9-14.

Meyer, R., Candrian, U. and Luthy, J. 1994. Detection of pork in heated meat products by the polymerase chain reaction. Journal of AOAC International, 77: 617-622.

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

Partis, L., Croan, D., Guo, Z., Clark, R., Coldham, T. and Murby, J. 2000. Evaluation of a DNA fingerprinting method for determining the species origin of meats. Meat Science, 54: 369-376.

Verbeke, R. and Brabander, H. F. 1980. Identification of animal fat species. Proceedings of European Meeting of Meat Research Workers, 26: 150-153.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using arbitrary primers. Nucleic Acids Research, 18: 7213-7218.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingley, S. V. 1991. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Research, 18: 6531-6535.

Zerifi, C., Lanie, C. and Benard, G. 1991. SDS-PAGE technique for species identification of cooked meat. Fleischwirtschaft, 71: 1060-1062.