UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF MEAT AND FISH IDENTITIES IN RAW AND PROCESSED FOOD SAMPLES USING PCR-RFLP TECHNIQUE

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Chairman : Professor Son Radu, PhD

Faculty : Food Science and Technology

The opening up of international food markets has resulted in the establishment of new regulations to ensure fair practices in the food trade. The identification of animal species is one of the areas of major concern for food hygiene laboratories, in forensic medicine and in the quality control of animal products. Food quality and safety will be strongly improved by the EC legislation (178/2002) on food traceability, which came into force in January 2005. The restriction fragment length polymorphism (RFLP) methodology has advanced genotyping of animal species, although further improvements are definitely needed. This study describes an investigation into the use of a PCR-RFLP technique as a routine analytical tool for species testing since accurate analytical methods are needed to ensure compliance with the new regulations. PCR-RFLP procedure was improved for the genotyping of beef, pork, buffalo meat, beef frankfurter (three brands), minced beef (two brands), pork frankfurter (two brands) and pork cocktail (one brand). Eight types of meat, 19 types of fish and 16 types of processed food samples were included as control samples. A

highly conserved segment within the cyt *b* gene was selected for PCR amplification by the universal primers cyt *b1* and cyt *b2* with the hope that it would amplify the cyt *b* gene from all the tested species. Apart from tuna fish and meats from quail, chicken, goat, beef, pork, buffalo, deer and rabbit samples, most of the fish samples were not identified using the cyt *b* primers. Genotyping of species by the present RFLP method was accomplished with amplifying a 359 bp region within the cyt *b* gene and digesting the amplified product using *Alu*I, *Hind*III, *BsaJ*I, *Rsa*I, *BstN*I, *Mse*I, *Nsi*I and *BstU*I enzymes. The specificity of the method was successfully assessed by RFLP analysis of meats from quail, chicken, goat, beef, pork, buffalo, deer, rabbit and tuna fish. PCR-RFLP technique showed high discriminatory power, but not all the species tested were identified. The concerted implementation of these conditional protocols for species identification was evaluated with beef frankfurter, minced beef, pork frankfurter and pork cocktail samples, and was found to be discriminatory for species identification. Commercial frauds through species substitution were not detected and the expected meat was present from the processed food samples tested. This PCR-RFLP based assay demonstrated to be an easy technique in routine analysis of raw and processed food for the detection of meat species.

Abstrak ini dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN SAMPLE DAGING DAN IKAN MENTAH DAN YANG DIPROSES DENGAN MENGGUNAKAN KAEDAH PCR-RFLP

Oleh

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Pengerusi : Profesor Son Radu, PhD

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Peraturan baru telah dilaksanakan dengan pembukaan pasaran antarabangsa yang berdasarkan bahan makanan. Identifikasi spesis haiwan adalah bidang yang diberi keutamaan dalam makmal kebersihan makanan, perubatan forensik dan dalam pengawalan kualiti produk makanan berasaskan haiwan. Keselamatan dan kualiti makanan telah diperbaharui melalui *EC legislation* (178/2002) yang telah berkuatkuasa pada Januari 2005. Penyelidikan ini melibatkan kajian berdasarkan PCR-RFLP sebagai kaedah analisis lazim dalam makmal untuk mengenalpasti spesis memandangkan kaedah analisis yang tepat diperlukan bagi memenuhi keperluan peraturan baru. Kaedah PCR-RFLP telah diperbaharui bagi menjalankan analisis genom daging lembu, khinzir, kerbau, sosej daging (tiga jenis), daging kisar (dua jenis), sosej panjang khinzir (dua jenis) dan sosej pendek khinzir (satu jenis). Lapan jenis daging haiwan, 19 jenis ikan dan 16 jenis produk makanan dijadikan sebagai sampel kawalan. Cyt *b* genom dipilih untuk PCR amplifikasi dengan menggunakan sepasang primer berstruktur umum, cyt *b*1 dan cyt *b*2. Primer ini digunakan untuk mengamplifikasi cyt *b* gen untuk semua spesis yang dikaji. Selain daripada daging

ikan tuna, burung puyuh, ayam, kambing, lembu, kerbau, rusa, arnab dan khinzir, kebanyakan daripada sampel ikan tidak dapat dikenalpasti dengan menggunakan primer cyt *b*. Kajian genom dapat dilaksanakan melalui keadah RFLP dengan mengamplifikasi gen cyt *b* yang bersaiz 359 bp dan pemotongan produk amplifikasi dengan menggunakan enzim *Alu*I, *Hind*III, *BsaJ*I, *Rsa*I, *BstN*I, *Mse*I, *Nsi*I and *BstU*I. Spesifikasi kaedah ini telah berjaya diperolehi dengan analisis RFLP daripada daging burung puyuh, ayam, kambing, lembu, kerbau, arnab, khinzir dan ikan tuna. Teknik PCR-RFLP menunjukkan kuasa pengenalpastian yang tinggi tetapi bukan semua spesis yang dikaji dapat diidentifikasi melalui teknik ini. Kebolehan teknik ini dalam mengidentifikasi spesis dibuktikan melalui sampel sosej daging, daging kisar, sosej panjang khinzir dan sosej pendek khinzir. Penipuan komersial dengan mencampur spesis tidak dapat dikesan dan daging yang betul didapati dalam produk makanan yang dikaji. Teknik yang berdasarkan PCR-RFLP yang digunakan dalam kajian ini didapati sangat sensitif dan cekap dalam analisis lazim bagi daging mentah dan produk makanan yang diproses bagi mengesan penipuan.

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I wish especially to thank my family, especially my mother, for their endurance, patience and encouragement.

I certify that an Examination Committee has met on 22 August 2006 to conduct the final examination of Chandrika Murugaiah on her Master of Science thesis entitled "Determination of Meat and Fish Identities in Raw and Processed Food Samples Using PCR-RFLP Technique" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act of 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 12 October 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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CHANDRIKA MURUGAIAH

Date: 12 September 2006

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LIST OF ABBREVIATIONS

Alu	*Arthrobacter luteus*
ATP	adenosine triphosphate
Bp	base pair
BsaJ	*Bacillus stearothermophilus*
BstN	*Bacillus atearothermophilus*
BstU	*Bacillus stearothermophilus*
CAPS	cleavable amplifiable polymorphic sequences
cm	centimeter
cyt *b*	cytochrome b
dH2O	distilled water
DNA	deoxiribonucleic acid
dNTPs	deoxyribonucleotides
ds DNA	double-straded DNA
EDTA	ethylenediamine teraacetic acid
g	gram
g	*gravity*
Hind	*Haemophilus influenzae*
IEF	isoelectric focusing
kb	kilobase
kDa	kilodalton
M	molar
mg	miligram
min	minute

ml	milliliter
Mse	*Micrococcus species*
mt	mitochondrial
NaCl	sodium chloride
nDNA	nuclear DNA
ng	nanogram
Nsi	*Neisseria sicca*
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RAPD	random amplified polymorphic
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
Rsa	*Rhodopseudomonas sphaeroides*
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSCP	single strand conformation polymorphism
ssDNA	single-stranded DNA
Taq	*Thermus aquaticus*
µg	microgram
µl	microliter
V	volt
w/v	weight/volume

CHAPTER 1

INTRODUCTION

There is a clear trend in the food international market towards labeling products with information about their composition and quality. Due to the increase in international trade in seafood and seafood products, authentication has become a necessity (Bossier, 1999; Etienne *et al.*, 2001). There is a need for suitable methods of identification to ensure compliance with the labeling regulations and thereby to prevent the substitution of fish species (Etienne *et al.*, 2001).

Consumers require clear and accurate information to make informed choices about their diet and the foods they buy. Their choice might also reflect lifestyle or religious concerns (preference for organic products for vegetarian, absence of pork for Jews and Muslims), or health concerns (e.g. absence of peanuts, lactose or gluten for individuals with particular allergies). Therefore, the description and/or labeling of food must be honest and accurate, particularly if the food has been processed and unable to distinguish one ingredient from another. The information that must be given is enshrined in the laws of most developed countries so that the food supplied must be exactly as in its label. In other words, the food must be authentic and not misdescribed.

According to the Ministry of Agriculture Food and Fisheries (MAFF), UK, (MAFF, 1993) food can be misdescribed by several ways, include: (i) abstraction or omission of valuable constituents; (ii) extending or adulteration of food with a base ingredient;

(iii) the non-declaration of processes and (iv) over-declaring a quantitative ingredient or substitution by undeclared components.

In 1962, the Codex Alimentarius Commission was established for implementation of the joint FAO/WHO standards programme. The aims of Codex Alimentarius include protecting the health of the consumer, ensuring fair practices in the food trade, coordination of all food standard work, publishing regional and world standards, recommending international standards for individual foods and making provision with respect with food hygiene, contaminants, additives, labelling and so on. The Codex recommendations are often used by bodies like European Union (EU) to formulate their standards (CODEX, 2006).

According to United States Department of Agriculture (USDA), food is considered adulterated when the food article: consists of any filthy, putrid, decomposed or diseased animal or vegetable material; is insect infested or unfit to human consumption; is prepared, packed or stored under insanitary conditions, contains any poisonous ingredients; has been substituted by any inferior or cheaper substance; has had any constituent abstracted; is packed in a container of any poisonous or deleterious substance; has any unpermitted additive present in an amount exceeding the prescribed limit; consist of a quality falling below the prescribed standard; or is not as purported or claimed (USDA, 2004).

It has become a challenging task to identify the species origin of meat and fish, especially in processed meat products. Furthermore, the identification of animal species is one of the areas of major concern for food hygiene laboratories. It is also

of considerable importance in forensic medicine and in the quality control of animal products. According to the Minister of International Trade and Industry, the Malaysian Government is committed to make Malaysia the Hub of Halal Food (MITI, 2004). Food quality and safety has been strongly improved by the EC legislation (178/2002) on food traceability, which came into force in January 2005 (EC, 2002).

Methods of food analysis have taken advantage of the rapid development of DNA fingerprinting techniques. DNA based techniques have the advantage that one does not need a standard for each tissue because all the cells in an individual have the same DNA. DNA based techniques like FINS (forensically informative nucleotide sequencing), RFLP (restriction fragment length polymorphism), SSCP (single-stranded conformational polymorphism), RAPD and LP-RAPD (long-primer random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) all can contribute to establishment of methods for authentication (Bossier *et al.*, 1999). Single nucleotide polymorphisms (SNPs) and restriction fragment length polymorphism (RFLP) are two approaches using polymerase chain reaction (PCR), which have proven to be very useful. Meyer *et al*. (1994) described the use of the RFLP technique for the detection of pork in cooked meat products. In this instance the RFLP detected was in the gene encoding cyt *b*.

Today, the use of the cyt *b* gene is nearly universal for determining the species of animals, birds and fish in raw and processed food products. The cyt *b* gene is located on mitochondrial DNA (mtDNA) and thus has two advantages (Woolfe and Primrose, 2004). The mtDNA is present in multiple copies compared to nucleus

DNA (nDNA) in every cell thus making its detection easier and the mitochondria are likely to remain intact during processing, thereby, minimizing DNA degradation.

In this study, restriction site analysis of PCR products of mtDNA was applied to identify different types of fish, meats and processed food. The 359 bp portion of cyt *b* gene on the mtDNA was amplified by cyt *b1* and cyt *b2* universal primers. Species differentiation was determined by digestion the 359 bp amplicon with different types of restriction enzymes, which generated species-specific electrophoresis banding patterns. The use of PCR-RFLP analysis of the conserved region of the cyt *b* gene provides a simpler, quicker and cheaper alternative to sequencing for direct identification of species origin.

This study aimed to establish method for the identification of muscles from fish and meat in processed and unprocessed food samples using PCR-RFLP analysis of a conserved region in the mitochondrial cyt *b* gene.

The specific objectives of this study were:

1) To establish method for the identification of adulteration in processed food samples.
2) To establish the standard RFLP profile obtained from different meat and fish samples

CHAPTER 2

LITERATURE REVIEW

2.1 Species Identification of Raw and Processed Fish and Meat

Consumers have become more demanding in the choice of foodstuff to avoid commercial frauds or for health issues as for example allergies towards specific components or ingredients (Comi *et al.*, 2005). Problems of authentication call for the availability of reliable and rapid methods to assess the hygienic quality of food and to identify food components in meat or fish-based foods. Species identification is important for the implementation of the labeling regulations as set by many countries (Mermelstein, 1993; FAS, 2006). Food labeling regulations require that the species of meat in meat products to be accurately declared to the consumer (Hird *et al.*, 2003). It is vital for preventing possible commercial frauds and guaranteeing the quality and the safety of meat (Sasazaki *et al.*, 2004). It is very important to assess that species of high commercial value are not sold, partially or entirely substituted with other species of lower commercial value (Comi *et al.*, 2005).

Identification of species in food is becoming a very important issue concerning the assessment of food composition, which is necessary to provide consumers accurate information about the products they purchase (Rodriguez *et al.*, 2003). There is a need for a new analytical technique, which is sensitive and inexpensive to discriminate the

origin of species in minced pork and beef (Skrokki and Hormi, 1994). Beef has been always adulterated with low-cost meat such as pork.

Identification of processed food is necessary as the customer has the right to be informed about products being bought and consumed (Pancorbo *et al.*, 2004). Law requires that products should be labeled with official names, thus creating a foundation for discouraging fraud. Regulation by the EC legislation (178/2002) on food traceability (EC, 2002) requires all stakeholders within the food supply chain must be able to identify the source of all raw materials. There is, therefore, a need for rapid methods for determining the species origin of a biological sample.

Determination of genetic relationships among closely related species is important in animal breeding program (Rao *et al.*, 1996). Cattle (*Bos indicus*), buffalo (*Bubalus bubalis),* goat *(Capra hircus)* and sheep *(Ovis aries)* belong to a single family Eovidae, order Artiodactyla. They are thought to have originated from a single ancestral species and are closely related. However, information on the extent of genetic relationships and diversities at the molecular level in these species is not yet available.

The quantitative detection of meat and fish species in mixed samples has been approached using high-performance liquid chromatography (HPLC) (Walker *et al.*, 2003; Armstrong *et al.*, 1992). The HPLC method has proven to be useful for the identification of many different animal species, but the detection limits are restrictive (Walker *et al.*, 2003). The detection of nuclear DNA (nDNA) sequences has also been