SHORT-LENGTH DNA BIOMARKER FOR THE DETECTION AND QUANTIFICATION OF MALAYAN BOX TURTLE (*CUORA AMBOINENSIS*) MATERIALS IN FOOD CHAIN AND TRADITIONAL CHINESE MEDICINES

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INSTITUTE OF GRADUATE STUDIES
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Field of Study: **BIOLOGY AND BIOCHEMISTRY**

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ABSTRACT

Malayan box turtle (*Cuora amboinensis*) (MBT) is a protected species in Malaysia and prohibited (haram) animal species in Muslim foods and medicines. However, because of its purported health benefits, its clandestine trades in black markets, especially for use in tonic foods and traditional Chinese medicines (TCM) are quite rampant. The widespread availability of commercial food items and TCM across Malaysia may offer the opportunity of turtle product trafficking under the covert of halal brands, needing to develop a convenient and reliable method both for the qualitative and quantitative tracing of turtle materials in food chain and medicines. Several polymerase chain reaction (PCR) assays have been proposed for the detection of MBT species under various routes but they are based on long-length targets which break down under the state of decomposition, making them unsuitable for the forensic detection in food chain, medicines and other potential routes. To overcome this knowledge gap, for the first time, a short length DNA target was developed for the qualitative and quantitative detection of MBT tissues by conventional PCR, PCR-RFLP and SYBR green real-time PCR systems. It combined a 120 bp-site of the MBT mitochondrial cytochrome b gene and a 141bp-site of 18S rRNA gene as the universal marker for the eukaryotes. The assay specificity was checked against 20 different species and biomarker stability was tested under various food processing conditions, including boiling, autoclaving and micro oven heating under pure, admixed and commercial food matrices. The limit of detection (LOD) of the conventional PCR and PCR-RFLP assays was 0.0001 ng MBT DNA under pure state and 0.01% (w/w) MBT meat under admixed and commercial matrices. In contrast, the LOD of the SYBR green duplex PCR system was 0.00001 ng DNA and 0.001% (w/w) MBT meat under mixed matrices. PCR amplified target was further authenticated by sequencing and restriction digestion with *Bfa*1 endonuclease and distinctive fingerprints (72, 43 and 5 bp) were obtained. The MBT target was further quantified by a duplex SYBR green real time PCR

system consisting of MBT target and internal positive control, wherein the melting curve clearly reflected two distinctive peaks at 74.63 ± 0.22 °C and 81.40 ± 0.31 °C for the MBT and eukaryotic targets, respectively, under pure, admixed and commercial matrices. The quantification limit (ng) was 0.00001for pure meat, 0.0030 ±0.00001 for binary mixtures, 0.0021 ± 0.00008 for meatball, 0.0042 ±0.0037 burger and 0.0013 ±0.00006 frankfurter products. The analysis of 150 reference meat samples reflected 98.19 to 166.57 % target recovery, 92.23-98.15 % PCR efficiency and 0.001% LOD under various matrices. A total of 183 commercial meat products were screened but no turtle contamination was found. Finally, 153 and 120 TCM samples were surveyed by PCR-RFLP and SYBR Green PCR and 40% and 23% of them were found to be MBT-positive (0.00157 to 0.0612 ng/µL), respectively. Thus the methods were suitable for real-world application and they confirmed the widespread speculation that MBT materials are widely used in Chinese medicines and herbal desserts.

ABSTRAK

Kura-kura Kotak Malaya (*Cuora amboinensis*) merupakan spesies yang dilindungi di Malaysia dan merupakan haiwan yang tidak halal di dalam makanan Muslim dan ubat-ubatan. Walau bagaimanapun, disebabkan kelebihan kesihatannya, perdagangan haram dalam pasaran gelap berleluasa, terutama penggunaannya dalam barangan makanan tonik dan ubat-ubatan tradisional. Produk makanan komersial Cina dan ubat-ubatan tradisional yang meluas di seluruh Malaysia mampu memberikan peluang pemerdagangan produk kura-kura yang bertopengkan produk halal, menjadikannya keperluan untuk membangunkan kaedah yang mudah dan boleh dipercayai bagi mengesan tisu kura-kura dalam rantaian makanan dan ubat-ubatan. Beberapa tindakbalas rantai asai polimeras (PCR) telah dicadangkan untuk mengesan spesies MBT menggunakan pelbagai cara tetapi bersandarkan sasaran berjarak panjang yang terurai di bawah keadaan penguraian, menjadikan kaedah-kaedah ini tidak sesuai untuk pengesanan forensik dan di dalam pengesanan rantaian makanan, perubatan dan lain-lain kaedah-kaedah yang berpotensi. Untuk mengatasi jurang pengetahuan ini, buat pertama kali, kami membangunkan sasaran DNA berjarak pendek untuk pengesanan kualitatif dan kuantitatif tisu MBT oleh PCR konvensional, PCR-RFLP dan sistem SYBR Green PCR masa nyata. Ia menggabungkan 120bp-tapak MBT gen mitokondria cytochrome-b dan 141bp-tapak gen 18S rRNA sebagai penanda universal bagi eukariot. Kekhususan asai telah disemak dengan 20 spesies yang berbeza manakala kestabilan sasaran penanda bio telah diuji dalam keadaan pengendalian makanan yang berbeza, termasuk pendidihan ekstrim, pengautoklafan dan pemanasan ketuhar mikro di bawah matriks makanan tulen, campuran dan matriks makanan komersial. Had pengesanan (LOD) PCR konvensional dan asai PCR-RFLP adalah 0.0001 ng MBT DNA di bawah keadaan tulen dan 0.01% (w/w) daging MBT untuk campuran dan matriks komersial. Sebaliknya, LOD sistem PCR SYBR Green dupleks adalah 0.00001 ng DNA dan 0.001% (w/w) daging MBT dibawah matriks

campuran. Sasaran PCR yang teramplifikasi telah disahkan melalui penjujukan DNA dan pencernaan terbatas menggunakan Bfa1 endonuklase dan profil terbatas tersendiri (72, 43 dan 5 bp) telah diperolehi. Sasaran MBT telah dikuantifikasi melalui system PCR masa nyata SYBR Green yang terdiri daripada sasaran MBT dan kawalan internal positif, di mana keluk lebur jelas menunjukkan dua puncak tersendiri pada 74.63 ± 0.22 °C dan 81.40 ± 0.31 °C untuk MBT dan sasaran eukariot, masing-masing, di bawah matriks tulen, campuran dan komersial. Had kuantifikasi (LOD) (ng DNA) adalah 0.00001 untuk daging tulen, 0.0030 ±0.00001 untuk campuran binari, 0.0021 ± 0.00008 untuk bebola daging, 0.0042 ±0.0037 burger dan 0.0013 ±0.00006 produk frankfurter. Analisis 150 sampel daging rujukan menunjukkan 98.19 ke 166.57% dapatan semula sasaran, 92.23-98.15% keefisienan PCR dan 0.001% LOD dibawah pelbagai matriks. Sejumlah 183 produk daging komersial disaring namun didapati tiada sampel terkontaminasi dengan kura-kura. Akhirnya, 153 dan 120 sampel ubat-ubatan tradisional Cina diselidik meggunakan PCR-RFLP dan PCR SYBR Green dan 40% dan 23% daripadanya didapati positif MBT (0.00157 ke 0.0612 ng/L), masing-masing. Jadi, kaedah-kaedah ini sesuai diaplikasikan dalam dunia sebenar dan mengesahkan spekulasi bahawa bahan MBT digunakan secara meluas dalam perubatan Cina dan pencuci mulut herba.

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LIST OF SYMBOLS AND ABBREVIATIONS

$	:	Dollar
%	:	Percent
/	:	Prime
°C	:	Centigrade
µg	:	Microgram
µL	:	Microliter
µM	:	Micromolar
≥	:	Greater than or Equal to
≤	:	Less than or Equal to
±	:	Plus-Minus
®	:	Registered Sign
A	:	Adenine
$A_{260/280}$	:	Ratio of UV Absorbance at 260 nm to 280 nm
ABI	:	Applied Biosystems
ATP 6	:	ATPase Subunit 6
BLAST	:	Basic Local Alignment Search Tool
BBC	:	British Broadcasting Cooperation
bp	:	Base Pairs
C	:	Cytosine
CE	:	Capillary Electrophoresis
CITES	:	Convention on International Trade in Endangered Species of Wild Fauna and Flora
COI	:	Cytochrome c Oxidase Subunit I
Cq	:	Quantification Cycle

Ct	:	Threshold Cycle
Cyt b	:	Cytochrome b
DBKL	:	Dewan Bandaraya Kuala Lumpur
DR	:	Daily Requirement
dH_2O	:	Distilled Water
D-loop	:	Displacement Loop
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxyribonucleoside Triphosphate
ds-DNA	:	Double Stranded- Deoxyribonucleic Acid
E (%)	:	Amplification Efficiency
EC	:	European Commission
Eq	:	Equation
ELISA	:	Enzyme-Linked Immunosorbent Assay
E-nose	:	Electronic Nose
Euk	:	Eukaryotic
FAO	:	Food and Agricultural Organization
F	:	Forward
FTIR	:	Fourier Transformed Infrared
g	:	Gram
G	:	Guanine
GC-MS	:	Gas Chromatography-Mass Spectrometry
h	:	Hour
HPLC	:	High Performance Liquid Chromatography
IDT	:	Integrated DNA Technology
LINE	:	Long Interspersed Nuclear Element
LC/MS	:	Liquid Chromatography Mass Spectroscopy

LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
Ltd	:	Limited
MBT	:	Malayan Box Turtle
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
Min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
Mt	:	Mitochondrial
mDNA	:	Mitochondrial Deoxyribonucleic Acid
NCBI	:	National Center of Biological Information
NADH2	:	NADH Dehydrogenase Subunit 2
ND5	:	NADH Dehydrogenase Subunit 5
ND6	:	NADH Dehydrogenase Subunit 6
Ng	:	Nanogram
nt	:	Nucleotide
O.D	:	Optical Density
PAGE	:	Polyacrylamide Gel Electrophoresis
PDCAAS	:	Protein Digestibility-Corrected Amino Acid Scores
PCR	:	Polymerase Chain Reaction
PCR-RFLP	:	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
R	:	Reverse
pg	:	Picogram
psi	:	Pounds Per Square Inch

rpm	:	Rotations Per Minute
RNA	:	Ribonucleic Acid
R^2	:	Regression Coefficient
SA	:	Suitable Amount
s	:	Second
T	:	Thiamine
Taq	:	Thermus aquaticus
TCM	:	Traditional Chinese Medicine
Tm	:	Melting Temperature
UV	:	Ultraviolet
WHO		World Health Organization
W/W	:	Weight/Weight

CHAPTER 1: INTRODUCTION

1.1 General Background

Nowadays consumers like to know the ingredients of food and medicine which they are purchasing and consuming from the food court, road side restaurants, medicine shops and supermarkets. A list of factors including lifestyles (e.g. vegetarianism and organic food), diet (e.g. calories and nutritional value), hazardous health issues (e.g. toxins and allergens), and religious and social factors are bringing variation in food prices and increasing the rate of adulteration incidents in food medicine and personal care products (Fajardo at el., 2010). Unlisted or mis-description, false labeling and/or fraudulent ingredients in food products, medicines and cosmetics are getting serious issues to health, business, wildlife and religious practices. Due to scientific breakthrough and innovation in food processing and packaging technologies, the substituted materials look very similar to the original materials and thus it is becoming rather challenging to differentiate the false ingredients from the original ones (Ghovvati et al., 2009). Thus ensuring food safety from the farm-to-fork through rigorous market monitoring is becoming increasingly difficult (Shackell, 2008). Recently, the British Broadcasting Corporation (BBC) reported that approximately 20-70% meat products (sausage, ground meat, meat balls, deli meats, and dried meats, burgers) in Mexico, Turkey, and African countries, 8% in the UK and 19.4% in the USA are mislabeled. (Ayaz et al., 2006; Brown, 2013; Cawthorn et al., 2013; D'Amato et al., 2013; Özpınar et al., 2013). Similarly, research groups have found 92% adulteration/contamination in traditional Chinese medicines in Australia, 68% in North America (Newmaster et al., 2013) and 4.2% in China (Han et al., 2016).

Recently some unexpected alien species, such as, rat, cat and dog meat has entered into the food chain (Fang et al., 2016), raising concern about the validity of the traditional food authentication techniques and target analytes because many of them are not considered in a typical food detection test. These are really alarming to the security of public health, consumers' religious faith, fair-trade economy, endangered wildlife and biodiversity (Schoppe, 2008). The mesmerizing belief of certain health benefits such as the distinctive flavor, high protein and low fat and cholesterol, the absence of health-threatening anabolic steroids in bush meat are posing special threats to wildlife and encouraging their overhunting and exploitation to harness health benefits (Hoffman & Wiklund, 2006; La Neve, Civera, Mucci, & Bottero, 2008). The bones, shells, skins and eggs of certain endangered species, such as Malayan box turtles, are believed to possess active healing attributes and invigorating elements that prolong youth and sexual life (Graham-Rowe, 2011; Hempen & Fischer, 2009). In the recent decades, the uses of traditional Chinese medicine (TCMs) which are being taken in conjunction with, or as an alternative to conventional Western medicine has greatly increased because of their natural attributes that are believed to give natural cures to many complex diseases (Ernst, 2004; Houghton & Mukherjee, 2009; Wang et al., 2005). The increasing popularity of TCM products has witnessed a dramatic turnover of US$ 60 billion in the international markets, posing an unexpected threat to wildlife (WHO, 2002).

The market demand for wildlife for food, medicine and cosmetic applications has greatly surpassed their natural availability and placing a restriction on their legal trade has just prompted their clandestine turnover in the illegal markets. These actions are deteriorating the ecosystems and causing the extinction of vulnerable species from the world map. The perceived efficacy of TCM is largely based on long-standing beliefs (Still, 2003) as the therapeutic benefits of TCM products have been validated for only a few cases (Sahoo et al., 2010). The Chinese herbal/traditional medicines often contain

numerous different plant and animal-derived products that combined together, exert a synergistic outcome (Xie et al., 2006; Yang, 2010). Despite having a weak scientific basis, the uses of traditional medicines and its supplements have dramatically increased and about 80% of the world population rely on them for primary healthcare, especially in the developing countries and uneducated societies where traditional faiths prevails in everyday activities. Between 2000 and 2005, the market value of traditional medicines has increased more than 3 fold from US$ 385 million in 2000 to US$ 1.29 billion in 2005 (Jayaraj, 2010).

Due to the proprietary nature of TCM manufacturing coupled with a lack of strict industry regulation, the biological origins or contents of TCM are not appropriately labeled or determined, raising questions about its quality, efficacy and safety standards (Heubl, 2010; WHO, 2002). Under these circumstances, the widespread uses of these medicines are a great threat to the healthcare systems since its associated health risks are largely unknown. Undeclared or misidentified TCM ingredients and adulterants can pose serious health risks to consumers (Gilbert, 2011; Sakurai, 2011); these include but are not limited to allergens (Ernst, 2000), plant toxins (Still, 2003), heavy metals, such as mercury, lead, copper and arsenic (Ernst, 2002), and pharmaceutically active compounds of undetermined concentration that may lead to toxicity after prolonged intake (Ernst, 2004). An Australian study found that high rates of adulteration (92%), substitution and mislabeling are rampant in TCMs, wherein the undeclared animal materials and heavy metal ingredients were either illegal or potentially hazardous to the consumers (Coghlan et al., 2015; Ernst & Coon, 2001). In the early 1990s, the misidentification of the toxic herb, *Aristolochia fangchi,* in the anti-inflammatory agent for *Stephania tetrandra* led to kidney failure and subsequent development of cancer in the urinary system of more than 100 women in Belgium (Gilbert, 2011).

Turtle species are natural scavengers of waste materials and hosts of several microbes and heavy metals. The health risks associated with the consumption or contact of turtle meat, eggs and shells include infections caused by bacteria (such as *Salmonella spp*. and *Vibrio spp*.), parasites (such as Spirometra, Trichinella, Gnathostoma, and Pentastomids), and various type of biotoxins such as lyngbyatoxins, cyanotoxins, cytotoxins, haemotoxins, mycotoxins, and neurotoxins (Magnino et al., 2009). Moreover, it is a sensitive social and religious issue as the consumption of turtle-derived materials is prohibited in certain religions such as Islam (Ali et al., 2015). According to the halal definition, meat of the domestic animals having a split hoof like cattle, buffalo, sheep, goat and camel are allowed for consumption by Muslims but meat from a carnivorous animal like cat, dog, pork and some wild life such as wolf, hyena, lion, tiger and turtle/tortoise, and crocodile is prohibited (Khattak et al., 2011). Global halal food markets are rapidly expanding because of amongst others, its special health and religion compliant attributes (Ali et al., 2014), wherein, the current turnover has reached to 1.8 billion dollars (Anonymous, 2014). Ready-made foods such as burgers, meatballs, pizzas, hot dogs, sandwiches, soups, cookies, candies, creams and numerous others are becoming increasingly popular among the working class and teenagers in worldwide because of their convenient features of ready-made availability in road-side restaurants and groceries (Ali et al., 2012).

In addition to TCM, there is constantly a growing tendency of mislabeling or adulteration in commercial meat products as well as trafficking of endangered species meat and their organs through popular meat products presented under the halal logo to withstand the competitive markets. In January 2013, the Food Safety Authority of Ireland (FSAI) found the presence of horse and pig DNA in meat products that were labeled to contain only beef. Out of 27 beef burgers labeled as 100% beef, 10 (37%) were tested positive for horse DNA and 23 (85%) were positive for pig DNA. Testing of raw

ingredient was performed and traces of horse DNA were detected in several batches (O'Mahony, 2013). In South Africa, 139 processed meats including minced meats, burger patties, deli meat and sausages were tested for mislabeling with results determining that 95 samples (68%) were mislabeled, and contained animal species not listed on the package (Cawthorn et al., 2013). The samples were purchased from four provinces that represent the most highly populated provinces in South Africa.

Since adulterant meats are typically less costly than higher priced declared meat, it often returns higher profit and encourages the industry to do it. Another possible reason might be the unintentional contamination that might come from sharing the same equipment and its improper cleaning while multiple meat species are handled. Sausages, burger, patties, meatball and deli meats are among the most common mislabeled meat products because of the difficulty in discriminating mixed species in ground meats by visual means alone (Cawthorn et al., 2013; Sentandreu & Sentandreu, 2014). Thus there is a need of a reliable method that allows the correct identification of MBT in processed meat products and TCM.

1.2 Study Rationale

Turtle species are especially vulnerable and 3% of them are already extinct, 9% critically threatened, 18% threatened and 2% are at risk. In Asian regions, 1% of turtles are extinct, 20% are critically endangered, 31% are endangered and 25% are vulnerable (Fund, 2002). Malayan box turtle (MBT) belongs to the *Cuora* genus, which encompasses a total of 12 turtle species, all of which are critically endangered (Spinks et al., 2012). It is a widely distributed turtle species in Asia, including Bangladesh, India, Malaysia, Myanmar, Thailand, Vietnam Laos, Philippine, Singapore, Cambodia and Indonesia (Schoppe, 2008c), and it has been listed in the vulnerable category in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora

(CITIES) and in the IUCN due to its overexploitation in foods, pets and traditional medicines (Chen et al., 2009a). A large number of this species are hunted by local indigenous people such Orang Asli in Peninsular Malaysia, Bedayuh and Iban in Sarawak, and ethnic Chinese community for consumption whilst some are also exported illegally to Europe and North America as pets, and used in China as foods and traditional medicines (Schoppe, 2008c). Turtle shells are highly exploited in TCM markets in East Asian countries especially in China and Taiwan, as it is believed that turtle shells provide analgesic and antipyretic medicinal effects as well as being seen as rich nutritional value sources of protein, gelatin, calcium and potassium in Chinese Materia Medica. Each year hundreds metric tonnes of turtle shells including *Cuora* species shells are exported to main land China from Southeast Asian countries either legally or illegally (Lo et al., 2006). Additionally, various food items such as soup, gel, pills and capsules are prepared with tortoise shells and sold widely in the East Asian countries (Hsieh et al., 2008).

To protect them from illegal exploitation, the Malaysia government banned export of all Malaysian native turtle including Malayan box turtle from Peninsula Malaysia and eastern Malaysia (Sabah and Sarawak state) since 2005 (Schoppe, 2008c) but importing countries (China and Singapore) reported the import of 33,969 individuals and 390 kg of plastron from Malaysia. In 2006, more than 21,884 Malayan box turtle were illegally exported to China, Hong Kong and Singapore from Malaysia (Schoppe, 2008b) and another report mentioned that more than 20,000 turtle species were exported to the East Asian countries from Malaysia in 2008 (Nijman, 2010). The Malaysian Customs Department seized 4.3 tonnes of illegally trafficked reptiles, including fresh water turtles, lizards, snake and tortoises; the illegal shipment was seized at the Malay–Thai border in December 2010 (Traffic, 2010), which reflects the fact that the illegal trade of reptiles is still rampant (Felbab-Brown, 2011). These actions are definitely deteriorating the ecosystems and causing the extinction of vulnerable species from the world map.

1.3 Current Detection Methods and Challenges

The current detection methods for food and medicinal products' authentication are numerous and they are mainly based on DNA and protein biomarkers (Nicolai et al., 2009; Coghlan et al., 2015). Morphological diagnostic traits do not work when products are degraded or highly treated making the microscopic tools obsolete for food forensic studies (Nejad et al., 2014). Protein and lipid-based species detection schemes are hopeless as these biomarkers are easily modified during processing treatments, providing misleading information about the source materials (Murugaiah et al., 2009). Initially, many tests were directed towards the identification of protein fractions in foods using isoelectric focusing and ELISAs (Bottero & Dalmasso, 2011). However, these techniques are gradually proving less effective due to low specificity under complex matrices and vigorous processing, such as chilling, salting, seasoning and heating which induces marked structural modification of proteins (Dooley et al., 2004). Protein-based approaches are particularly ineffective for TCM authentication because TCM preparations involve decoction and some excipients after a series of processing such as cooking, drying and stewing that significantly denature or degrade protein molecules, rendering them unidentifiable.

On the other hand, DNA-based species identification schemes are reliable and recently several DNA based methods, such as PCR–DNA sequencing (Lo et al., 2006), PCR-restriction fragment length polymorphism (PCR-RFLP) (Moore et al., 2003), and randomly amplified polymorphic DNA (RAPD) (Saez et al., 2004), have been documented for the detection of MBT (*Cuora amboinensis*) and other turtle species. However, all these reported methods involve very long lengths of target amplicons that break down during food processing treatments or under the state of decomposition such as found during the natural decomposition of the carcasses. Moreover, there was no found

document on quantitative detection of Malayan box turtle materials in foods and medicinal products. Additionally, remarkable research gaps were also found on assay sensitivity, stability and biomarker validation under various processed samples as well as validated assay with commercial food products screening. To overcome these limitations, for the first time this paper develops a very short-amplicon-length PCR assay (120 bp) for the quantitative detection of Malayan box turtle meat in raw, processed and mixed matrices, and experimental evidence is produced that such an assay is not only more stable and reliable but also more sensitive than those previously published. Since the mitochondrial genes are present in multiple copies (Rojas et al., 2011), if the target biomarkers are developed from a site of mitochondrial genes, assay reliability would be greatly increased as it is highly unlikely that all copies of the gene would be degraded together, even under the compromised state. Inclusion of an endogenous positive control would effectively eliminate the chances of any false negative detection (Sobhy & Colson, 2012). Authenticity of the PCR targets could be verified by restriction fragment-length polymorphism (RFLP) analysis and handling errors could be eliminated or reduced using an automated system.

1.4 Objectives of the Research

1.4.1 General objective

The objective of the present study is to develop a short-length DNA biomarker for the detection and quantification of Malayan box turtle (*Cuora amboinensis)* materials in the food chain and traditional Chinese medicines using conventional and Real-Time PCR techniques.

1.4.2 Specific objectives

(a). To identify and characterize short-length DNA biomarker targeting multicopy mitochondrial DNA of Malayan box turtle.

(b). To optimize and validate a conventional PCR and Lab on a Chip based PCR–RFLP system for the qualitative detection of Malayan box turtle material in foods and traditional Chinese Medicine.

(c). To develop and validate a SYBR green duplex qPCR assay for the quantitative detection of Malayan box turtle in the food chain and traditional Chinese medicines.

1.5 Thesis organization

This thesis consisted of five (5) chapters namely (1) introduction, (2) literature review, (3) methodology, (4) results and discussion and (5) conclusion and recommendation

Chapter 1: This chapter consists of current authentication issues that are related to food and medicinal products, especially on adulteration, mislabeling in the food chain and traditional medicinal preparations and the negative impact in health and biological conservation. It also briefly presents currently available meat authentication techniques, significance of using the mt- gene as a target, research gaps and study objectives.

Chapter 2: This chapter critically presents the current state of knowledge about the abundance and the authentication of animal materials in the food chain and medicinal products. This literature review chapter specifically emphasizes the turtle population, their smuggling routes, risks in the food chain and medicines and biodiversity protection.

Chapter 3: This chapter describes the methodologies and materials that are adapted to fulfill the scopes of the work and research targets. The materials, procedures, equipment, instruments, bioinformatics tools used for designing a biomarker, in-silico analysis of restriction sites and experimental works are systematically presented. The details of primer design, species selection, specificity, sensitivity, stability, meat product

formulation under various matrices, commercial meat product and traditional medicine screening procedure are stated as well.

Chapter 4: This chapter consists of research findings and discussions along with the significance of the studies that were performed for the identification of Malayan box turtle species in foods and medicines.

Chapter 5: This final chapter summarizes the overall findings of the research and recommendations for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Animal Materials in Foods Chain

The animal contributions into the human food chains are huge and probably one of the main catalysts for a mutual set of interactions between animals and humans for millions of years (Shipman, 2010). According to Henry Bunn, an anthropologist of the Wisconsin University, early human started to eat meat more than two million years ago (Wrangham, 2013). This historical evidence was reported after the successful analysis of carcasses of antelopes, gazelles and wildebeest left behind by *Homo habilis* at a site in Tanzania (Yirka, 2002). Over the period, *H. habilis* used to get meat mostly by scavenging and a smaller part by hunting. However, the large scale of wild meat was predominantly hunted by *H. erectus* to obtain protein; it was probably a major adaptive shift in human civilization (Leonard et al., 2007). A study on human evolution reflects when early humans started to eat meat and eventually hunt; their women started to give birth to more children during their reproductive life, contributing to the growth and spreading population all over the world (Psouni et al., 2012). A positive correlation was also observed between regular meat eating and body size. *H. erectus/ergaster* males had an average body mass of 66 kg compared to *H. habilis* which weighed 37 kg, while the body mass of females increased by 53%, from 32 kg for *H. habilis* to 56 kg for *H. erectus/ergaste* for females (McHenry & Coffing, 2000). Thus, meat has a very crucial link to our evolutionary heritage (Smil, 2002; Craig et al., 2001; Craig et al.,1999). The age-old interlacing of the collection, consumption, and societal integration of meat with hominin development has greatly influenced our biological and cultural modes of operation (Ehrlich, 2000). With the improvement of stone tools, sustained running ability, hominin accessed more animal-derived foods during the Pliocene period (Bramble & Lieberman, 2004; Domínguez et al., 2005; Schoeninger, 2012) and they preferred meat

from large animals as meat makes up 40% or more of the diet; a much-larger proportion than in other primates (Wrangham, 2013). Subsequently, the meat consumption opportunity expanded around 250,000 years ago when the earliest *Homo* invented fire (Goudsblom, 1992) and they made meat food more delicious through searing and roasting, and smoking and preserved it for later consumption.–Thus, many years ago, animals were considered as valuable source of meat food, containing high biological value proteins and valuable minerals such as iron, vitamin as well as zinc, selenium and phosphorus.

2.1.1 Meat

Meat has played a crucial role in human evolution and is an important component of healthy, well balanced diet because of its high nutritional values. Increasing populations and rapid income growth at the global scale has led not only to an increased demand for staple foods but also for preferred foods such as meat products. Thus, various forms of animal meat products have unique entry points into our commercial food chains; these include minced meat, sausages, burger patty and meatballs which are the most common meat products that are being widely consumed around the world regardless of the brands, geographical and ethnical preferences. This is because only meat can offer such a wide range of nutritional requirements; it is the best source of proteins, fats, vitamins, minerals and micronutrients which are essential for human growth and development. Moreover, the qualities of proteins from animal sources are superior to those from plant sources as all the eight essential amino acids for human growth and development are found in meat (Soares et al., 2014). The current rate of the global meat consumption is 41.2 kg per capita per year (BBC, 2013) and has been on an increasing trend for both domesticated and farmed wild animals (Klein, 2004) (Figure 2.1). According to the Food and Agricultural Organization (FAO), global annual meat

production is estimated to will have increased from 218 million tonnes in 1997-1999 to 376 million tonnes by 2030 (FAO, 2002).

The top sources of meat in our diet are domesticated animals such as cattle, pigs and poultry and to a lesser extent buffaloes, sheep, camels and goats. To a limited extent and on a regional basis, meat is also derived from wild animals such as turtle, deer, elk, rabbit, crocodiles, snakes and lizards (FAO, 2014; Klein, 2004). To keep up with this trend, food companies are vigorously competing to produce and supply more meat including raw meat itself and various meat products. However, consumers nowadays are becoming more anxious about their choice for healthy food and are showing trends to avoid high fat content meat. Another cause of anxiety is the increasing findings of fraud labelling and adulteration in meat products (Nicole, 2010). Therefore, those who are concerned about healthy diet regimes, are trying to reduce the high fat content meat, such as red meat on their dining table. As a response to consumer demands for healthy food, game/wild animals are being integrated into common foods chain using either legal or illegal procedures. As wild meat provides distinctive texture and flavor, low fat and cholesterol contents and is free from anabolic steroids or other drugs, they have become attractive components in new and exotic delicacies (Hoffman & Wiklund, 2006; La Neve et al., 2008) and as a result have created huge appeal to consumers. According to the North American Elk Breeders Association's report, a tremendous growth in the Elk farming industry was observed in the USA from 1997 to 2003, where the total market value was $ 150 million in that period. Similarly, the National Deer Farmer's Association, USA reported in 2003, about $1 billion worth of deer meat were marketed by 11,000 U.S. farms (Klein, 2004). Meanwhile in Southeast Asia, Malaysia is one of the most intensely hunting countries where approximately 108 million bush meat animals are killed for consumption each year (Bennett et al, 2002).

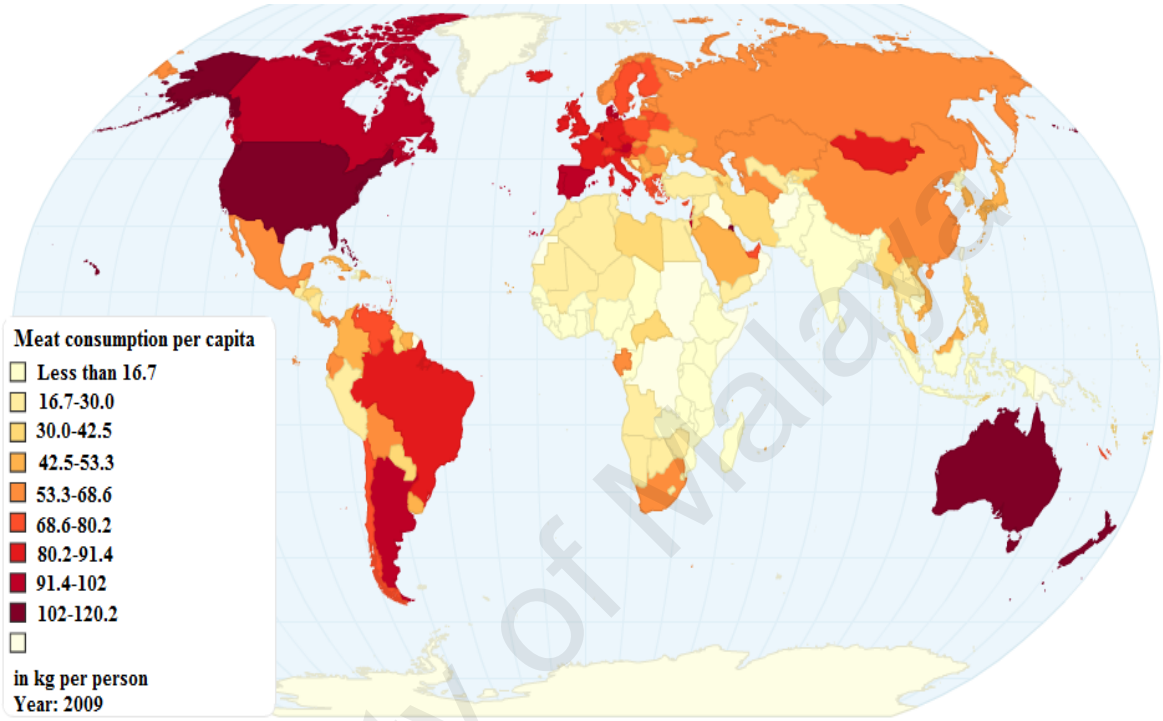


Figure 2.1: Global meat consumption per capita (Source: http://www.fao.org/docrep /005/y4252e /y4252e05b.htm)

2.1.2 Animal proteins

Protein is a core macronutrient and comes from a variety of sources, including meat, milk, fish, soy, and eggs, as well as beans, legumes, and nut butters. When proteins are digested, they leave behind amino acids, which the human body needs. In general, proteins derived from animal sources (i.e. milk, eggs & meat) are complete, but the human body's ability to use the protein varies. Unlike carbohydrates and fats, protein is not stored in the body; so, it is needed to be add in the diet regularly for the body to stay healthy. Similar to carbohydrates and fat, protein is a "macronutrient," which is needed in relatively large amounts. However, meat protein content can vary substantially. According to the Portuguese nutritional table data, the average protein content of meat is

22%, however, it can be as high as 34.5% (chicken breast) or as low as 12.3% (duck meat) (INSRJ,-2006).

Meat proteins are further categorized by their essential amino acids content. There are more than three hundred amino acids in nature but only twenty are indispensable to synthesize proteins (Wu, 2009). Within this twenty, eight cannot be produced by the human body which makes them essential and thus they have to be supplied by the diet. Both essential and non-essential amino acids cannot be produced by the human body unless all necessary raw materials are supplied from the diet. Thus, dietary proteins are needed for growth, maintenance and repair of the body, and can also provide energy. The amount of essential amino acids in the crude protein of beef is approximately as follows: 8.4% leucine, 8.4% lysine, 5.7% valine, 5.1% isoleucine, 4.0% phenylalanine, 4.0% threonine, 2.9% histidine, 2.3% methionine and 1.1% tryptophan. Nonessential amino acid contents in order of importance are glutamic acid, aspartic acid, glycine, arginine, alanine, proline, serine, tyrosine and cysteine (Giroux & Lacroix, 1998).

In most developed countries, average protein intakes for all age groups are in excess of the minimum requirements for good health resulting in the excess protein being used to provide energy. Meat and meat products (including poultry) are the main dietary source of protein in many countries. An overview of the developed countries reflects that the average available amount of red meat (pork, beef and veal, sheep) for daily consumption is approximately 110 g per person (FAO, 2009). Reviewing dietary guidance of developed countries reveals that the recommendations for meat and meat alternatives are 142 g/day on average or from 65 to 250 g/day for adults depending on gender, indicating that red meat consumption is within recommended levels in most developed countries. For example, in a recent analysis of U.S. National Health and Nutrition Examination Survey (NHANES), data for adults (19–50 years) reflects that the

total beef consumption equated to 49.3 g of the 142–198 g/day recommended by the USDA "MyPyramid" food plan (Zanovec et al., 2010). These data indicate that beef is moderately consumed despite its popularity with consumers.

2.1.3 Animal fats

Animal fats are principally composed of triglycerides and accumulate in the fat cells and become the richest dietary source of energy that supplies essential nutrients and essential fatty acids. Usually fat is made up of different types of fatty acids: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). The content of fat may differ significantly among beef, poultry and other meat products such as offal and specialties like sausages, ham, etc. According to the Portuguese nutritional composition table (INSRJ, 2006), there are 14% (calf) to 19% (adult) fats in beef retail cuts whereas it is 8% to 28% in pork. However, the USDA reports estimated values that range from 5.4% to 7.9% fats in beef retail cuts while pork fat range from 8% to 10.7% (USDA, 2011). Skin is probably the main source of fat in poultry meat, hence chicken and turkey skinless breasts have similar fat contents (INSRJ, 2006).

Animal fats are widely added in meat preparation to make the products softer and also to add taste and flavors. In particular, products such as meat loaves, frankfurter type sausages or liver pate, have meats and fats that are finely comminuted and the fat particles are enclosed in protein structures and thus become difficult to detect via visual methods. Fat contents of up to 40% may be hidden this way, which may be harmful to the consumers' health because most often relatively low grade and cheap raw materials have been used in processed foods. Fat abundancy also plays a role in food processing. For example-fat derivatives are used as biosurfactants in food industries as emulsifying, antimicrobial, antifungal and as well as antiadhesive agents. Commercially available

emulsifiers used in the food and drink sectors are typically derived from soy and eggs (Shepherd et al., 1995).

It is now well recognized that different fatty acids have different consequences on blood cholesterol levels (including beneficial as well as harmful effects), hence it is important to consider the fatty acid profile of a food. Palmitic acid and stearic acid are the main SFAs present in red meat (Fink-Gremmels, 1993; MAFF, 1998) while oleic acid is the principal MUFA in meat and typically ranges between 30 and 40% of the fat in meat (Fink-Gremmels, 1993; MAFF, 1998). The predominant PUFAs in meat are linoleic (n-6) and α-linolenic acid (n-3), which are known as essential fatty acids. In spite of having low PUFAs content, meat and meat products (including poultry) contribute substantially to intakes in the UK, providing 18% of n-6 PUFAs and 17% of n-3 PUFAs, while 23% of overall fat intake (Henderson, Gregory, Irving, & Swan, 2004).

2.1.4 Vitamin and minerals

As with other animal foods, animal meat is an excellent source of bioavailable vitamins and minerals. Red meat provides up to 25% RDI (recommended daily intake) of multivitamins such as riboflavin, niacin, vitamin B6 and pantothenic acid per 100 g intake. Up to two thirds of the daily requirement of vitamin B12 is derived from red meat foods (Williams, 2007). Although, liver is known as a reservoir of heavy metals, hormones and xenobiotics, it is also the best source of vitamin A and folate than lean muscle meat tissue (Kerry, 2009). Chicken breast is an excellent source of niacin (100 g supplies 56% of DR) and vitamin B6 (27% of DR) whereas turkey breast supply as low as 31% of niacin DR and 29% of vitamin B6 DR from 100 g intake (USDA, 2011). For all these vitamins, older animals tend to have higher concentrations; so the levels in beef are generally higher than those in veal, mutton and lamb. Likewise, red meat is also the greatest source for trace elements such as zinc, selenium phosphorus and iron. A 100 g of

red meat provides around 37% of selenium DR, 26% of zinc DR and 20% of potassium DR (USDA, 2011). According to Henderson et al (2003), in the UK 17% of the total dietary iron comes from meat and its product whereas the amount is 22% in New Zealand (Russell et al., 1999). Absorption of zinc from a diet high in animal protein is greater than from plant foods, causing about 50% higher requirement for vegetarians than meat eaters (NHMRC, 2006). Meat and meat products in Ireland provide around 41% of total zinc intake (Cosgrove et al., 2005) and 31–34% intake in Denmark, UK and New Zealand (Credoc, 2003; DIFVR, 2005; Henderson et al., 2003; Russell et al., 1999).

Other elements such as sodium and potassium are important for muscle contraction, nerve transmission, digestive and muscular function and maintaining pH balance. A certain amount of both these elements in diets are derived from red meat where potassium is present 5 times higher than sodium. Copper is a mineral that is found throughout the body. It helps to make red blood cells and keeps nerve cells and the immune system in the right gear. Raw lean meat cuts provide 0.055 to 0.190 mg/100 g of copper for beef and 0.190 to 0.240 mg/100 g for mutton (Chan, 1995).

2.2 Animal Materials in Pharmaceuticals

Chemicals from nature have played a vital role in health care and prevention of diseases for thousands of years (Ji et al., 2009). The ancient Chinese, Indian and North African civilizations provide written evidence for the use of medicinal chemicals from plants, animals or micro-organisms for curing various diseases (Phillipson, 2001). A major part of these chemicals comes from animals and its metabolic byproducts such as secretions and excrements. Indeed, animals are therapeutic arsenals that have played significant roles in the healing processes, magical rituals, and religious practices of the peoples from all five continents (De et al., 2000).

Globalization of technologies and new innovation ideas lead to the expansion of animal based medicines around the world. It has been recorded that the annual global trade in animal-based medicinal products accounts for billions of dollars per year (Kunin & Lawton). An assessment study on 252 essential chemicals by the World Health Organization showed that 11% were from plants and 8.7% from animals (Marques, 1997). Out of 150 recommended drugs in the U.S.A, 27 have animal origins (WRI, 2000), reflecting the wide spread consumption and uses of animal materials in these medicines. Similarly, Tatham & Patel (2014) stated that some drugs prescribed by primary care doctors elsewhere in the world contain ingredients derived from animals. For example, several vaccines for both children and adults containing animal by-products are manufactured with gelatin, chicken embryo, pork serum and embryo cells. These usually go unnoticed by the consumers, who have no knowledge that they are taking medicine with animals by-products. Numerous animals are being used to develop medicines across the world (Figure 2.2).

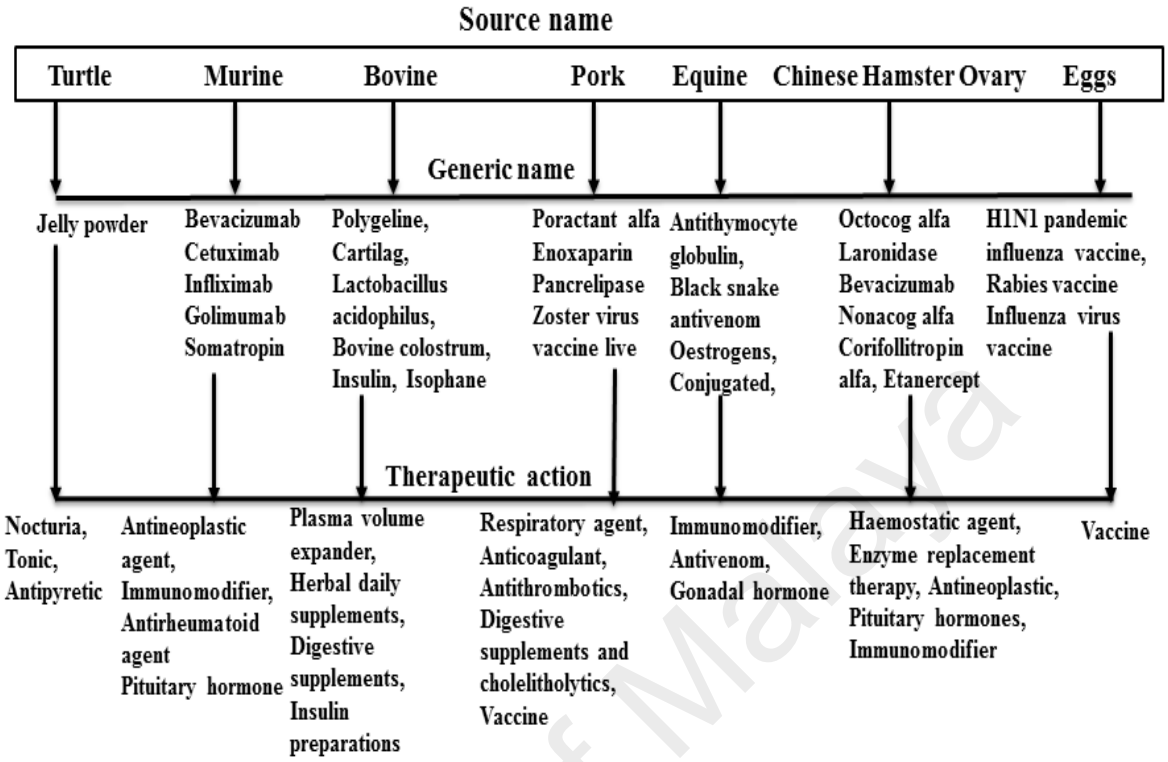


Figure 2.2: Schematic diagram of animal sources of therapeutic agents (Source: https://www.health.qld.gov.au/__data/assets/pdf_file/0024/147507/qh-gdl-954.pdf).

Gelatin is an animal protein has been used as a carrier of Zoster vaccine (Grabenstein, 2013). Zoster vaccine is a live vaccine used to prevent or reduce post herpetic neuralgia caused by the varicella zoster virus. The introduction of insulin was a breakthrough in the treatment of diabetes, as insulin is effective in restoring normoglycaemia, suppressing ketogenesis and in delaying or arresting diabetic complications (Garg & Misra, 2002). This life saving drug is derived from pig pancreas as well as bovine serum. Heparin is a sulfated polysaccharide of animal origin used as an effective anticoagulant to prevent the formation of blood clots in the veins, arteries, or lungs (Triplett, 1979). It is obtained from porcine (intestines), bovine (lungs), ovine and caprine (Huang et al., 2012), but porcine intestinal heparin has restricted applications because of the emergence of transmissible spongiform encephalopathy from other ruminant animals (Concannon et al., 2011) and also due to religious prohibition.

2.3 The Need for Animal Material and Turtle Authentication

The identification of animal species in food and medicinal products has enormous importance in public health as they are potential careers of various zoonotic threats and food-borne diseases as well as certain chronic disorders such as cancer and cardiovascular diseases (CVD). Studies demonstrate approximately 75% of infectious diseases in humans are caused and originated from domesticated and wild animals (Blancou et al., 2005). There are approximately 1,500 pathogens, which are known to infect humans and 61% of these cause zoonotic infections via direct or indirect contact (Taylor et al., 2001). The potential pathways include but are not limited to (i) transmission by animal bites and scratches (Zambori et al., 2013); (ii) meat food or materials from infected animals as well as improper food handling and cooking (Gerba et al., 1996; Roe & Pillai, 2003; Tauxe, 1997); (iii) through farmers and animal health workers who spend a significant time with animals and thus are exposed to certain pathogens or catch health risk bacteria and thus they could also become carriers and spread the infection to other humans (Levy et al., 1976); (iv) wildlife animals who can serve as hosts for a variety of known and unknown emerging zoonotic pathogens (Karesh et al., 2005).

Some of the animals are especially vulnerable and have been identified as a reservoir of various pathogens. Turtles and tortoises are among these groups, where several potential zoonotic agents such as parasites and enteric bacteria have been detected and which are the causative agents for about 70,000 cases of human *salmonellosis* that happen in each year in the United States alone (Wong et al., 2003). Moreover, turtle and tortoise species are known as wild scavengers since they consume waste materials in nature. Thus, the consumption of turtle meats may have adverse human health effects due to the presence of pathogens and environmental contaminates. In 2013, a survey conducted by the Department of Health, Philippines on a foodborne illness

(Chelonitoxism) event in Rawis, a small village located in the coastal area of Arteche, Philippines, where four consumers ranging in age from 23 to 80 years died after consuming turtle meat. Three of these were from the same family and consumed turtle meat and the intensity of food poison was so acute that all of them died from the consumption of turtle soup and internal organs; whereas 75% and 25% consumers had died after the consumption of turtle eggs and heart parts, respectively (Ventura et al., 2015). This incident supports the alarming risk that comes with the consumption of turtle products. However, turtle and some others wildlife are integral parts of traditional medicines as some recent medicinal textbooks of traditional Chinese Medicines (TCM) still recommend the use of various animal tissues such as tiger bones, antelope, buffalo or rhino horns, deer antlers, dog testicles, bear or snake bile in TCM formulas. Although there are not enough scientific studies on the physiological effects of animal materials in traditional medicines, it has been continued to be included because of the traditional belief and rituals that have propagated from generation to generation. Several studies have suggested that there are strong possibilities of the transmission of diseases from animal preparations. For example, monkeys are identified as harboring the herpes B virus which is also known as the Ebola virus in Human and is transmissible to man with potentially grave consequences. Several researchers have considered the propagation worldwide of the sweeping HIV virus may have originated from monkeys. In some regions, wild animals are hunted (poached) for their meat rather than for their uses in medicines.

Even though animal meat is consumed to get basic nutrition, indiscriminate consumption might not only affect the food chain but also risk their health as well. A number of epidemiological studies have reported that high consumption of red meat or processed meat is associated with increased risk of several cancers and cardiovascular disease (CDV) (Cross et al., 2007; Giovannucci et al., 1994; Kelemen et al., 2005; Kontogianni et al., 2008). Although the risks are small, they could be important for public

health as many people worldwide eat meat, and its consumption is increasing day by day even in the low and middle-income countries. According to the most recent export by the Global Burden of Disease project, more than 34,000 cancer related deaths are caused by diets high in processed meat. Similarly, the same organization has stated that red meat was responsible for 50,000 cancer deaths a year worldwide (WHO, 2015).

Processed meats mostly contain pork or beef, but they may also contain other red meats, poultry, offal and meat by-products such as blood. Constituents of these red meat products that have been proposed to be responsible for these associations include the fat content, fatty acid composition and the possible formation of carcinogenic compounds, such as heterocyclic amines (HCAs) while cooking the meat at high temperatures (Bingham, Hughes, & Cross, 2002). Besides, oxidation of red meat derived fat leads to the formation of oxyesterols and aldehydes that may alter transforming growth factor beta (TGF-β)-mediated signal and cell proliferation (Biasi, Mascia, & Poli, 2008). Moreover, the high saturated fat content of red meat contributes to obesity and general inflammation, insulin resistance, and intestinal dysbiosis (Calle & Kaaks, 2004; Schulz et al., 2014). Recent epidemiological studies show consistent associations between saturated fat intake and the risk of prostate and breast carcinomas (Pelser et al., 2013; Xia et al., 2015). Moreover, red meat is a potential source of contaminating inorganic toxins, such as arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), pesticides among many others (Domingo & Nadal, 2016). These toxins can be derived from the cooking processes or from industrial sources during meat processing.

2.4 Religious Prohibition and Social Factors

Religions have played an important role in the selection of the menu throughout the human civilization (Rehman et al., 2010). The impact of religion on food consumption depends on the individuals who are following the teachings of the religion along with

their understanding and interpretations of the religious tenet. Religious requirements and its adherence influence the feelings and attitudes of people towards food consumption (Jamal, 2003). Most of the religions have strict guidelines that determine the food consumption to show a respect to God as well as its health attributes (Meyer-Rochow, 2009). For Muslims, the Islamic Shariah law is one of the most important foundations in their social and cultural life. Thus, Muslim consumers strictly follow dietary laws enshrined in the holy Quran, Hadiths of prophet Mohammad (SM) and in certain cases based on the opinions of a group of Islamic Scholars. According to the Islamic dietary laws, Muslims are prohibited from eating or using any product derived from pigs as well as prohibited body parts and ingredients such as blood and plasma even from halal animals, or permissible body parts such as the flesh if the animal is slaughtered in a non-halal way such as shooting or electrical shock (Nakyinsige et al., 2012). Likewise, Jewish dietary laws consider animal's ingredients must comply with the kosher laws. Both the Muslim Halal and Jewish Kosher law require that animals which may be consumed must chew their cud and have split hooves; pigs don't chew their cud and thereby pork and pig derivatives are clearly prohibited as food materials in Islam and Judaism (Regenstein, Chaudry, & Regenstein, 2003). Although cow meat and its products are lawful as food for the Jewish, Christian and Muslim consumers, they are with the exception of milk, unacceptable to the followers of Hinduism; this is because cow is considered as sacred animals by Hindus (Meyer-Rochow, 2009).

On the other hand, vegetarianism is strongly linked to a number of religions that originated from ancient India. While Judaism, Christianity and Islam have not strongly promoted vegetarian diets; religions that originated from ancient India such as Hinduism, Jainism and Buddhism, strongly practice vegetarianism in their everyday menu. While vegetarianism is mandatory for everyone in Jainism (Burt, 2016), it is advocated by some influential scriptures and religious authorities of Hinduism and Buddhism (Davidson,

2003). Most of these religious tenets are focused on promoting a healthy lifestyle and preventing illness caused by food consumption. For example, halal is an all-encompassing concept which encourages a Muslim to seek and use products, ventures and services that promote cleanliness in all aspects of a person's life. Thus, halal food means that a product or service is safe for consumption, produced in a clean environment and healthy. Therefore, religion is one of the main factors determining food avoidance, taboos and special regulation particularly with respect to meat consumption (Ali et al., 2014). Culture and social lifestyle also play an important role in food selection. Lifestyle, such as practicing vegetarians consume only plant originated materials whilst some Buddhists believe that killing animals is a great sin(Phelps, 2004; Stewart, 2015). Considering all of these religious views, food and drug manufacturers should have a loyalty and responsibility to provide reasonable information related to all aspects of food and drug production. Consumers also need to be assured that the information they are being provided by a company is true and accurate (Holm & Kildevang, 1996)

Malaysia, is a multiracial country with various ethnic groups and religious tenets, although Islam is the official religion where 50% of the population practice Islamic doctrines (Fischer, 2008). Malaysia has shown great interest in the Halal industry development including food, pharmaceutical and personal care products as well as Halal finance systems (Riaz & Chaudry, 2003). Malaysia also imports food, cosmetic and medicinal products from several non-Muslim countries such as Australia, New Zealand, India and Thailand where the bulk market is not Halal compliant (Elasrag, 2016). Muslims around the world are facing similar problems when they purchase consumer items from non-Muslim countries. These food and consumer products could contain haram substances because the manufacturers in the foreign countries and importers/exporters may not well understand the concept of Halal which is a fundamental aspect in Islamic life. Fortunately, since 1982 Malaysia along with other Muslim

countries have strong legislation and surveillance laboratories under the Halal regulatory board, JAKIM, to monitor the Halal markets and create trust to the Halal-consuming populations (Othman, Ahmad, & Zailani, 2009). Thus, exporter and importers are required to meet the Malaysian standard for Halal Food Production, Preparation and Storage-General Guideline (MS 1500:2004) (Malaysian Standard). Despite strict monitoring of Halal status, recently non Halal beef was sold in Malaysia; this has placed most Muslim consumers on red alert in determining the presence of prohibited animal ingredients in marketed foods as well as medicinal products (Anonymus, 2015).

2.5 Regulatory Laws

The regulation of health products and food is an important activity that not only supports our health but also gives us the right tools for authentication. Nowadays, both in developed and developing countries, food and drug assurance systems are generally getting more stringent to ensure both the real and perceived food safety problems are addressed. Regulations are the rules issued by the Governor of a Council to carry out the intent of statutes (Acts or legislation) enacted by the Government. They are the instruments of legislative power and have the force of law. Regulations contain more specific information and requirements than Acts. These can include definitions, licensing requirements, performance specifications, exemptions, forms and other details. The complexity in regulations for the health and food product sectors reflect consumers' demand for safe food and drugs, as well as firms' reputation for providing safe food and drugs and maintaining global market shares. The regulations also differ significantly across health products and food types, such as raw and processed food and drugs, low and highly perishable food products, or for food and drugs containing low or high incidences

of risks for human health. The following Malaysian regulatory system, statutes and rules provide a framework for health to regulate health products and food in country

2.5.1 Malaysia food act 1983

Generally, "food law" is used to apply to legislation which regulates the production, trade and handling of food and hence covers the regulation of food control, food safety and relevant aspects of food trade. To protect the public from unhealthy food or health hazards and fraud in the preparation, sale and use of food, the Malaysian Government enacted the Food Act law on 9th March 1983. Moreover, Malaysia is on the edge of making itself as the Halal food hub of the world. Malaysia is a Muslim-majority country and hence there is a demand for Halal foods in the local markets and Malaysia also wants to earn revenues from the export of Halal foods. Also, Halal foods has gained attention among non-Muslims communities due to its quality attributes, hygiene and safety standard (Wahab et al., 2016). The global Halal food market value is estimated to be US$ 3.7 trillion by 2019 (WAM, 2016). This big market may contribute as a potential engine for economic growth and societal development and Malaysia aspires to be a leader in the Halal food benchmarking. In fact, the United Nations has cited Malaysia as the world's best example of benchmarking of Halal food in Geneva in 1997 (Bohari et al., 2013). Therefore, food material including processed foods is stringently regulated by the Malaysia Food Act 1983 and Malaysia Halal Standard (Act, 1983; Wahab et al., 2016). This act protects the public from health risks and fraud in the food preparation, sale, use of food, and for matters incidental or connected in addition to that, throughout Malaysia. Additionally, Malaysia is the first to announce a global Halal center and create a restricted agency for Halal monitoring at the national level (Iberahim et al., 2012).

2.5.2 The criteria and justification of Malaysia food act 1983

The regulatory and enforcement bodies will take necessary legal action if they find the following offenses and evidences (Act, 2006):

1. Strictly about clean and sanitary condition of premises or equipment

- **Procedure:**
  - a. Director may order premises or appliances be put into clean and sanitary condition 2. If any offence and evidence with harmful materials at food premise or foods contain harmful ingredients to health or food unfit for human consumption or any portion of an animal unfit for food, or any diseases, filthy, decomposed or putrid animal or vegetable substance
- **Procedures:**
- Report letter as a warning (specified period)
- Penalty: imprisonment for a term not exceeding (5) five years or to a fine or to both.

3. If any person who prepares, packages, labels or sells any food in a manner that is false, misleading or deceptive as regards its character, nature, value, substance, quality, composition,

- **Procedures:**
- Writing warning report: not exceeding 14 days of any premises providing or selling food
- Closure selling food
- Imprisonment for a term not exceeding three (3) years or a fine or both

4. Regulatory body will take the necessary steps if they find adulterated food or if any substance or ingredient has been extracted, wholly or in part, or omitted, from the food and by reason of such extraction or omission.

- **Procedure:**
- Removal of food from food premises
- Writing warning notice letter
- Order to remove or withdraw from sale at specific time
- Prohibition against the sale
- Penalty: imprisonment for a term not exceeding (5) five years or to fine or to both
- Order license to be cancelled and food to be disposed of

2.5.3 Malaysian standard

The Malaysian Standard is under the Department of Standards Malaysia which is the recognized body for the standardization and accreditation industry in Malaysia. The application of the Malaysian Standard is a voluntary use except where it is made mandatory by regulatory authorities involved by means of regulations, local by-laws or any other similar ways. Article 4.2.1. in MS 1500:2009 has clearly defined Halal food, its production, preparation, handling and storage procedures step by step (Amendment 2009). This category of Malaysian Standard reviews of the food industry start from the preparation, handling and serving process of Halal food and products including trade and business. It is a fundamental requirement guideline which contains knowledge of nutrient matter. The premises criteria discussed in this guideline are divided into 4 (four) elements: The criteria and justification of premise in MS 1500:2009.

2.5.3.1 Layout design

- Plan should provide proper flow of the process, employee, good hygiene and safety application as applicable to any pest infection and cross-contamination between and during operations
- Clean facilities and proper food hygiene supervision designed
- Provided and maintaining the sanitary facilities
- Appropriately designed for loading and unloading bay

2.5.3.2 Flow of product processing

- Started with raw materials receiving until finish product without cross contamination
- Entirely separated and well insulated from a pig farm or its related activities .

2.5.3.3 Conditions

- Maintain the good repair and condition to prevent contamination from any pest and breeding sites potential access
- To prevent pets and other animals from entering the premises compound

2.5.3.4 Slaughtering

- Only for Halal slaughtering and processing
- All carcasses process like deboning, cutting, packing and storing should be done completely in approval premises; the competent authority require this standard

2.5.4 U.S. legislations

There is a long established system of food safety control and regulation which occurs at the federal (interstate commerce and import) and regional (intrastate commerce) level. Two main agencies are involved at the federal level, namely, Food and Drug Administration (FDA) and the Food Safety Inspection Service (FSIS) of the United States

Department of Agriculture (USDA). The Food, Drug and Cosmetic Act 1938 (FDCA) sets out the authority of the FDA whilst the Meat and Poultry Inspection Acts and the Egg Inspection Act set out the authority of FSIS. But In some situations, FSIS and FDA follow similar practices while in other situations, the practices of these two agencies are quite distinct. State agencies also have an active role in overseeing food processing businesses within their respective states, but their role is in collaboration with the federal agencies. U.S federal law has categorized foods into interstate commerce "; foods not in interstate commerce" and which are regulated by state law. Most foods fit the definition of being in interstate commerce, however, because they are moved across a state line, or an ingredient has been moved across a state line. Federal agencies have defined the adulterated and misbranded food products which are not allowed to be sold in the USA.

2.5.4.1 Adulterated foods

- If any food contains any substance, food additive, or pesticide chemical residue which may render it injurious to health or unsafe
- If any food consists of any filthy, putrid, or decomposed substance
- If any food is unfit for food
- If any food has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated
- If any food is the product of a diseased animal or an animal that died other than by slaughtering or professional killing
- If food container is composed of any substance which may render the contents injurious to health
- If any food has been intentionally irradiated.

2.5.4.2 Misbranded food

It means any food that

- Its labeling or advertising is false or misleading
- It is offered for sale under the name of another food
- It is an imitation of another food but the label does not bear the word "imitation"
- Its container is made or filled to be misleading
- Its label does not provide

i) the name and place of the manufacturer, and

ii) an accurate statement of the quantity of the contents in terms of weight, measure, or numerical count

2.5.5 European Commission legislations

The food processing sector in the European countries is tightly regulated by state and European commission legislation, although there is no specific definition about food adulteration and misbranded food but it has traceability guidelines that have five major steps to prevent food fraud under the article 18, 19 and 20 of Regulation (EC) 178 (2002) sections. Thus the articles 18, 19 and 20 of the Regulation (EC) 178 (2002) cover the responsibilities of food and feed business operators to inform the public on an appropriate label about the content of the prepared food (Law, 2002), as follows:

- The traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed shall be established at all stages of production, processing and distribution.
- Food and feed business operators shall be able to identify any person from whom they have been supplied with a food, a feed, a food-producing animal, or any substance intended to be, or expected to be, incorporated into a food or feed. To this end, such operators shall have in place systems and procedures, which allow for this information to be made available to the competent authorities on demand.

- Food and feed business operators shall have in place systems and procedures to identify the other businesses to which their products have been supplied. This information shall be made available to the competent authorities on demand.
- Food or feed which is placed on the market or is likely to be placed on the market in the Community shall be adequately labelled or identified to facilitate its traceability, through relevant documentation or information in accordance with the relevant requirements of more specific provisions.
- Provisions for the purpose of applying the requirements of this Article in respect of specific sectors may be adopted in accordance with the procedure laid down in Article 58, paragraph 2, referring to Committee and Mediation Procedures.
- If an operator considers, or has reason to believe that a food/feed which they have imported, produced, processed, manufactured or distributed is not in compliance with the food/feed safety requirement, they will immediately initiate procedures to withdraw the food/feed in question from the market where the food/feed has left the immediate control of that initial food/feed business operator and inform the competent authorities thereof.

2.6 Turtle Species in Foods and Pharmaceuticals

Turtle species have been exploited for meat, eggs, and traditional medicine since the 16th century (Alves & Santana, 2008; Klemens, 2000). In many East Asian cultures, turtle meat and eggs are considered a culinary delicacy with perceived health and medicinal benefits. The annual trade volume of live turtles for consumption in Asian countries have exceeded 13,000 metric tons, a high proportion of this is believed to be collected from the wild (Dijk et al., 2000). The largest consumers are predominantly in East Asia, i.e., China, Hong Kong, Japan, Korea, Singapore and Taiwan, as well as Chinese ethnic communities around the world. But, among them China is the leading consumer country in the world and its international trade has been cited as the greatest

threat to Asian turtles (Figure 2.3) (Cheung & Dudgeon, 2006; Gong et al., 2009). Turtle plastron is the essential part of traditional medicine preparations which comprise about 32% protein, 7% collagen and 50% calcium carbonate. In 1992-1998, Taiwan imported more than120 metric tons turtle shell from main land China and Southeast Asia (Figure 2.4) (Lo et al., 2006) however, food trade involving the largest quantities of turtles takes place in Asia.

The consumption of turtle meat and eggs are greatly encouraged by the traditional belief of health benefits such as natural source of high quality proteins for certain communities. In Malaysia, indigenous people such as the Orang Asli of Peninsular Malaysia and the Bedayuh and Iban of Sarawak are greatly involved o consume turtle meat as their protein source. These ethnic groups traditionally appreciate turtles and other wildlife as their food (Sharma et al., 2000; Sim et al., 2002) and Sharma et al., (2000) noted that most indigenous groups consume the flesh of the species purely for food and not for medicinal purposes (Sharma et al., 2000).

In contrast, the Chinese communities around the world consider it as tonic food as well as being medicinal materials (Cheng, 2014). In Hong Kong and Singapore, turtle soup is widely accepted as an exotic dish and largely consumed during the winter season because of the belief that turtle meat warms its consumer's blood (Lau, 2000)(Figure 2.4). Conversely, Malaysian Chinese, eat turtle meat for its assumed medicinal values and they feed flesh to the children to cure for nocturnal urination in bed (Sharma et al., 2000). Recently, turtle jelly has become more popular as desert food or as a medicinal product and is found in traditional medicine shops around the Asia. The dark brown turtle jelly powder is obtained from the plastron of *Cuora trifasciata, Cuora amboinensis, Mauremvs mutica* and *Notochelvs platvnota species, Callagur borneoensis* and *Pelodiscus sinensis* and prepared with various medicinal herbs (Figure 2.5). This plastron jelly is also

considered as anticancer and detoxifying drugs and applied as traditional medication (Lau, 2000).

Figure 2.3: Wild turtle meat and egg soup are openly sold at restaurant in Singapore (Source: http://www.sbestfood.com/wp/tanserseng.htm).

Turtle and tortoise shells possess small portions of keratin, magnesium, vitamin D and trace minerals such as zinc (Damiens et al., 2012; Mitchell, 2014) but collagen and calcium are the two main chemical constituents that are found in the turtle or tortoise shells (Damiens et al., 2012). While the collagen usually makes up the 7% of the turtle plastron, calcium makes up half of the tortoise plastron and carapace. Calcium has been known for building strong teeth and bones but it is also associated with nervous and neuromuscular disorders. Calcium deficiency usually lead to nervousness, hyperactivity, irritability, insomnia, muscle cramps, limb numbness, etc. (Fang, Zhang, & Liu, 1989). A

popular medicine "*Longmu Zhanggu Tang"* made from turtle and tortoise shells with others materials such as oyster shell, astragalus, atractylodes, codonopsis, hoelen, dioscorea, schizandra, jujube, licorice, and *gallus* and is applied for the treatment of rickets disease because this medicine improves serum calcium and phosphorus levels, and bone mass (Lili, 1987). Plastron is sometimes described as an exoskeleton, partially or completely solidified by calcium carbonates and phosphates. Ingestible collagen can inhibit arthritis (perhaps by deflecting immune attacks against the joints to the ingested collagen) and as well as angiogenesis (blood vessel formation) to inhibit tumor growth (Dunussi-Joannopoulos et al., 2005). Animal gelatin is recommended for arthritis treatment as well as soreness and back and spinal pain, joints enlargement, leg pains, and heel pains in published traditional Chinese medical books and medicines are composed with tortoise and turtle shell, donkey skin gelatin, and deer antler gelatin (Cai et al., 1995). Gelatin polypeptides contribute to stop vaginal bleeding and uterine fibroids in women older than 40 years. Administered medicines to these diseases are derived from tortoise shell as well as other wild animals by-products (Sionneau, 1997). There may be substances in the shell that stimulate the body to produce hormones and control and prevent thyroid disorders. There are also small amounts of fats, magnesium, trace minerals, such as zinc, and vitamins, including vitamin D, in the tortoise shells (http://www.itmonline.org/arts/tortois2.htm). It is still difficult to explain some of the traditional indications for tortoise shell based on the limited knowledge of its constituents and their effects.

Figure 2.4 Turtle plastron are sold in traditional Chinese medicine shops in China (Source: http://www.thefullwiki.org/Traditional_Chinese_medicine).

2.7 Malayan Box Turtle and Availability

The Malayan box turtle (*Cuora amboinensis)* is one of 18 freshwater turtle and tortoise species which are the native species of Malaysia (Sharma, 1999). These species belong to the *Cuora* genus, which encompasses a total of 12 turtle species (Table 2.1), all of which their habitats are found in different geographical locations across the Asian peninsulas (Spinks et al., 2012). A wide distribution of these species is found across South and Southeast Asia countries such as Malaysia, Thailand, Myanmar, Cambodia, Vietnam, Brunei, Indonesia, Laos, Philippines (Ernst & Altenburg, 2000; Trust, 2004), Bangladesh (Khan, 1982), and India (Moll & Vijaya, 1986). However, it is a very common hard shell fresh water turtle found in Malaysia including the Borneo islands (East Malaysia) and Singapore (Lim & Das, 1999). Life expectancy of this species is about 25–30 years and a maximum age of 38.2 years was recorded (Bowler, 1977). According to Schoppe (2008), it takes 14-16 months to reach sub-adulthood having a MeCL (median carapace length) size > 115 mm and to reach maturity, female species take 5-6 years and males 8-9 years. However, suitable habitats, environment, and sufficient feeding might enhance them to

mature at an age of 3-5 years (Praedicow, 1985). An adult female turtle can lay 3-5 eggs during the rainy season but its population numbers are based on fertile and infertile eggs (Nutaphand, 1979). It is estimated that 61% of the female of the total population is found in Peninsular Malaysia and 49% in Sarawak, east Malaysia and the ratio of male to females is 1:1.6 in Peninsular Malaysia and 1:1.2 in Sarawak. An overview of the Asian box turtles are given in table 2.1.

Table 2.1: Asian box turtles – An overview

Species	Countries of Origin	Population Status	Use and Trade
Cuora amboinensis Malaysian box turtle	Malaysia, Bangladesh, Cambodia, India, Indonesia, Lao PDR, Myanmar, Philippines, Singapore, Thailand, Vietnam	Endangered	Food markets and TCM. High level of exploitation for Chinese food markets in East Asia
Cuora aurocapitata Golden-headed box turtle	China	Critically endangered	Food markets and TCM. One of the most requested and most expensive species in the Chinese food markets
Cuora flavomarginata Chinese box turtle	China, Japan	Endangered	Food markets and TCM. High level of exploitation in China and Hong Kong,
Cuora galbinifrons Flowerback box turtle	Cambodia, China, Lao PRD, Vietnam	Critically endangered	Food markets and TCM. High level of exploitation for food markets in Southeast and East Asia
Cuora mccordi, Mc Cord´s box turtle	China	Critically endangered	Food markets and TCM
Cuora pani, Pan´s box turtle	China	Critically endangered	Food markets and TCM
Cuora trifasciata, Three-striped box turtle	Cambodia, China, Lao PDR Vietnam	Critically endangered	Food markets and TCM. Most expensive and requested turtle in the Chinese markets
Cuora yunnanensis, Yunnan box turtle	China	Probably extinct in the wild	Not observed in trade, maybe already extinct
Cuora zhoui, Zhou´s box turtle	China	Critically endangered	Food markets and TCM. One of the most requested and expensive species in the China food markets
Cuora cyclornata, Vietnamese three-striped box turtle	China, Vietnam, Lao PRD	Critically endangered	Food markets and TCM. One of the most requested and expensive species in the China food markets
Cuora bourreti, Vietnamese flowerback box turtle	Vietnam, Lao PRD	Not recorded	Food markets and TCM. One of the most requested and expensive species in the China food markets
Cuora serrata	China	Critically endangered	Food markets and TCM:

Source: https://www.cites.org/eng/cop/11/prop/36.pdf

2.8 Existential Threats

The Malayan box turtle population in Malaysia occupies about 2.6 million acres of peat swamp forests area (Schoppe 2008b). Nevertheless, the existence of the species is now in jeopardy due to over-exploitation. A large number of this species is caught for domestic consumption, but it has the highest consumption by East Asian consumers and for use in traditional Chinese medicine (TCM) (Schoppe, 2008b) (Figures 2.3 and 2.4). All specimens for domestic and international use are wild-caught and linked with an economic motivation. Moreover, its size and reputation of having higher meat quality make the Malayan box turtle highly preferred for consumption in Asian food markets. Likewise, Malayan box turtle shell is reported to be analgesic and antipyretic and a rich source of proteins and calcium in Chinese Materia Medica (Schoppe et al., 2012). Being wildlife protected and a commercially high valued species, the possibility of illegal trafficking and trade of Malayan box turtle are rampant in Southeast Asia including Malaysia. A conservative estimate is that the illegal trade amounts to 10 times the volume of legal trade (Schoppe, 2009), and this covers live specimens and the shell trade. Thus, a higher portion of this species is harvested through forgery ways. According to the Vanda Felbab-Brown's (International and internal expert on security and illicit economies), illegal trade of wildlife is $8-10 billion per year in Southeast Asia, resulting in deterioration of ecosystems and extinction of vulnerable species (Felbab-Brown, 2011).

Besides, the rapid and extensive destruction of tropical forests has become a serious threat to their native biota (Zanovec et al., 2010). The current deforestation rate is particularly severe in Malaysia, where natural habitats are being destroyed at relatively higher rates than those of other tropical regions (Fink-Gremmels, 1993). If this continues, Malayan box turtle will be extinct from this region in the near future. Similarly, drainage

and irrigation works, unregulated chemical and pesticide use in paddy land also have a significant impact upon the environment and this may lead to the destruction of wetlands and cause a population decline (Gregory & Sharma, 1997; Lim & Das, 1999).

Moreover, Malayan box turtles have a slow reproductive cycle characterized by late maturity and a limited number of eggs. It is therefore feared that the continuous high volume exploitation in combination with its slow productivity might lead to serious population reductions and finally to a local extinction (Schoppe, 2009). Turtle species are known as natural scavengers and they eat vegetable, fallen fruits and soft invertebrates such as worms and slugs and waste materials in wetlands (Anonymus, 2016a). Therefore, its temporary or permanent removal from its natural habitats is not only a threat to their existence but also may lead to an imbalance of the ecosystem structure, the significance and consequences of which cannot currently be determined.

2.9 Routes of Trafficking

To protect them from overexploitation, Malayan box turtle was first assessed by the International Union for Conservation of Nature and Natural Resources (IUCN). It was Red Listed as the Threatened Species in 1996, "Low Risk or Near Threatened" before it was upgraded to vulnerable in 2000 (Schoppe, 2008b). In the same year, all species under the genus *Cuora* were listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), bringing requirements of legal provenance and sustainable management for any international trade in the species (Henderson et al., 2004). Prior to the export of Appendix II species from a country of origin, a so-called non-detriment finding (NDF) needs to be conducted to determine the number of individuals that can be harvested without a negative impact on the survival of wild populations (Henderson et al., 2004). Despite strong legislations internationally, thousands are harvested annually in Malaysia and Indonesia and exported illegally into

the international meat, TCM and pet markets without considering their present survival condition. The main destinations for illegally traded individuals are reported to be Thailand, Hong Kong, Singapore, China and USA (Sabine Schoppe, 2008a).

The Department of Wildlife and National Park Malaysia (PERHILITAN) and Wild Life Malaysia have jointly banned the export of Malayan Box Turtle (MBT) to other countries since 2005 (Schoppe, 2008a). However, evidence was found for continuing export of the species after the Malaysian government imposed the export ban. A survey in Malaysia in 2006 has shown more than 21,884 Malayan Box Turtles (*C. amobinensis*) are illegally exported by 12 suppliers from Sabak Bernam, Malaysia to international export markets (Henderson et al., 2004; Schoppe, 2008). In the recent time, a big illegal shipment seizure report was published on the Traffic website, where the Malaysian Customs Department seized 4.3 tonnes of illegally trafficked reptiles, including Malayan box turtles, lizards, snake and tortoises, at the Malay–Thai border in December 2010 (TRAFFIC, 2010), which reflects the fact that the illegal trade of reptiles is rampant. However, smugglers have not stopped their smuggling activities as Malaysian enforcement further seized more than 2,066 Malayan box turtle at their different operational raids in 2011, 2014 and 2016; these animals were destined for Thailand (Source:http://www.geo.tv/latest.32907-malaysia-seizes-450-protected-snakes-turtles), Anonymous, 2016b) (Figure 2.5). Information from the seizure reports indicate that 98% adult turtles from Peninsular Malaysia and 88% from Sarawak are traded by individuals and most of them were between 170 and 199 mm in median carapace length (Schoppe, 2008c). Mostly illegal shipments were found to enter by chartered air plane from countries in South and Southeast Asia, and by road from neighboring countries through overland routes and border crossings (Figure 2.5) (Henderson et al., 2004). Beyond Malaysia, Thailand customs authority seized 19,000 Tuttle and tortoise species including Malaysian box turtle (*Cuora amboinensis*) at Suvarnabhumi International airport in

Bangkok in the period 2008-2013. The highest exploitation of this species was recorded in Indonesia, where 48,335 live species were seized by the US Fish and Wildlife Service (USFWS) Law Enforcement Management Information System (LEMIS) and those animals had been illegally exported from Indonesia to the US between 1998 and 2002 (Schoppe 2008; Wood et al., 1999). In the period of 2000-2005, Indonesia exported more than 1 million turtles to the international markets but it officially recorded only 18,000 indicating that the majority of these shipments were undeclared (Wood et al., 1999). In addition to those destined for markets in Thailand, animals are also redistributed to other demand centers in Southeast Asia and East Asia (Figure 2.5) (Shepherd, 2014). There are several factors as well as syndicate groups involved that are leading to the decline of this species in Asia.

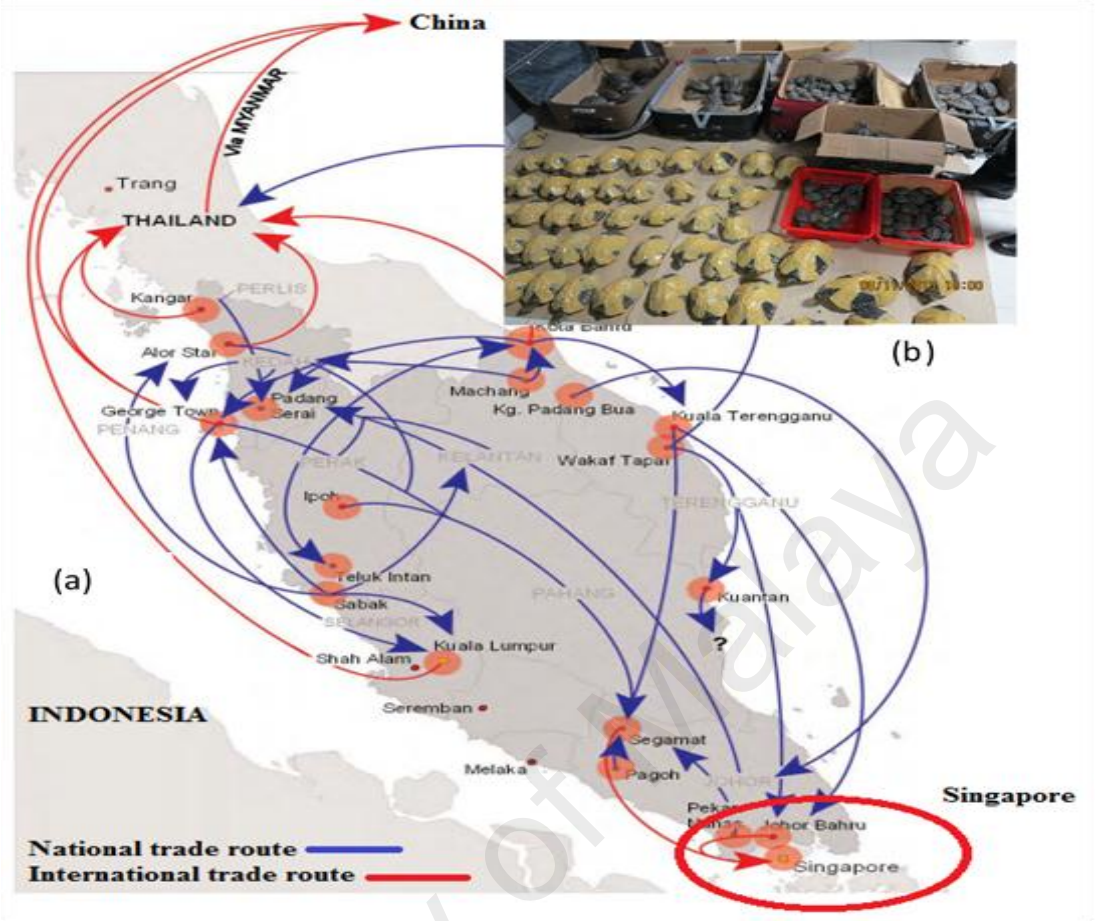


Figure 2.5: Route map of illegal trade in Malaysia and international: blue arrows for domestic route in Malaysia and red arrows for international illegal trade routes. In inset (b), seizure of illegal shipment of the box turtle at Kuala Lumpur airport (KLIA 1) by Malaysia customs department in 2013(Shepherd, 2014).

2.10 Current Species Detection Methods

Adulteration in food and herbal medicines has inflicted substantial concern to the consumers in terms of economic loss, food allergy, religious observance, and food safety (Arslan et al., 2006; Koh et al., 1998; Mane et al., 2009). Therefore, to enhance food security, foodstuff containing even trace amounts of meat must be labeled correctly to ensure food safety and safeguard customers' interest, especially in the case of processed food products where differentiation of the different constituents are extremely difficult. Indeed, falsely labelled food products could be perilous to consumers with chronic illnesses who require specific dietary needs. In Europe, food traceability and

authentication were seriously considered following EC regulations that requires the source of all raw materials in the food to be identified (Stanner, 2008). But several study have discovered that undeclared meat (Table 2.2) including numerous wildlife species are widely substituted in traditional medicine preparations (Coghlan et al., 2012; Premanandh, 2013). Widespread uses of undeclared animal materials in foods and traditional medicines are posing great threats to the food manufacturers, consumers as well as sustainable biological conservations (Nicolai et al., 2009; Quillen & Murphy, 2006; Quinto et al., 2016). In order to enable food and drug control authorities to supervise compliance with labelling requirements, suitable detection methods which could allow unambiguous identification of animal or fish in foodstuff are prudent. The development of methods for species traceability has been the subject of intense research in many countries during the past two decades (Heinz & Hautzinger, 2009). Currently, animal species or their products including highly processed materials are based on identifying either DNA or protein (Nicolai et al., 2009; Calvo et al., 2002; Herman, 2001; Matsunaga et al., 1999; Meyer, et al., 1995; Peter et al., 2004). A summary of fraud events in processed food is presented in table 2.2.

Table 2.2: Food mislabeling and its abundance in meat and meat products

Investigated products	Country	Authentication Issue	Percentage of mislabeling cases (number of analyzed samples)	References
Hamburgers	Brazil	Undeclared soy protein	30.8%	Macedo et al., 2001
Hamburgers	Mexico	Undeclared animal species	39%	Flores et al., 2000
Sausages	Mexico	Undeclared animal species	29%	Flores et al., 2000
Meat products	US	Undeclared animal species	15.9% (raw) 22.9% (processed)	Hsieh et al., 1995
Meat products	Turkey	Undeclared animal species	22%	Ayaz et al., 2006

2.11 Protein based Detection Techniques

Protein biomarkers are widely used in species identification using electrophoretic (Bouwman et al., 2011) and chromatography techniques (Chen et al., 2013) ,or enzyme-linked immunosorbent assay (ELISA) (Asensio et al., 2008) and isoelectric focusing (IEF)(Pergande & Cologna, 2017). All of these proteins based techniques are proposed to identify the specific animals such as cattle, sheep and swine (Marbaix et al., 2016) Hollung et al., 2009; Xu et al., 2009), and poultry(Sentandreu et al.,2010). Numerous protein markers have also been applied in the authenticity assay of food products (Chaze et al., 2006).

2.12 Organ Specific Protein Detection Technique

Some organ specific proteins such as carnosine (ß-alanyl-Lhistidine) (CAR), anserine (ß-alanyl-L-1 methylhistidine) (ANS), and balenine (ß-alanyl-L-3 methylhistidine) (BAL) are found in muscle, liver, kidney, and heart but not available in plants (Aristoy & Toldrá, 1998, 2004). They play vital roles for the generation of meat flavor in food (Aristoy & Toldrá, 1998). Therefore, these biomarkers could be used to measure the animal substituents and sources in animal feeds as animal and fish by-products are main protein sources in feeds preparation for ruminants and aquatic animals (Aristoy & Toldrá, 2004). However, due to the outbreak of bovine spongiform encephalopathy (*BSE)* in Europe, the use of animal byproducts has been banned within the European Union under several regulations. Regulation (EC) 999/2001 and EC 1774/2002 prohibits explicitly the feeding of mammalian byproducts to ruminants or prohibits the feeding of animals with proteins from the same species (Commission, 2001, 2002). Moreover, these biomarkers can't distinguish specific protein in a complex background and are also poorly sensitive in processed foods using HPLC techniques. None the less, it has been reported that species as low as 0.5% can be detected by the

same technique (Aristoy & Toldrá, 1998), but this sensitivity needs to be investigated further. Parvalbumins is known as muscle specific proteins of fish and has been applied to differentiate closely related fish species (Berrini et al., 2006). However, there is need to combine electrophoretic techniques with novel proteomic tools, such as in-gel digestion and mass spectrometry (Martinez et al., 2004; Pischetsrieder & Baeuerlein, 2009). The electrophoretic and proteomics tools are laborious, expensive, demands specialized skills, and also not reliable for the analysis of complex mixtures (Addis et al., 2010; Pischetsrieder & Baeuerlein, 2009). Osteocalcin (OC) (γ-carboxyglutamate containing protein) is another calcium binding protein that is also known as bone protein because it is found as a major constituent of bones. This protein is used to determine the species origin using enzyme-linked immunosorbant assay (ELISA), and highly sensitive MALDI-TOF and Q-TOF mass spectrometry (MS) (Balizs et al., 2011). However, MS is not only expensive but also needs specialized skills for its operation and data interpretation, calling the need for simpler and more convenient methods to perform this job.

2.13 Detection of Species Specific Protein by ELISA

There are several protocols and techniques that are proven to be safe for species authentication studies but the most common among them is the ELISA. Because, of its specificity, sensitivity and low cost, ELISA has become very popular to the regulatory board for the routine analysis of species origin in foods (Asensio et al., 2008; Bellorini et al., 2005; Giovannacci et al., 2004). Various type of commercial available immunoassay kits has been applied for detection of raw and processed samples such as pork (Berger, Mageau, Schwab, & Johnston, 1987; Chen & Hsieh, 2000), chicken (Martín et al., 1991), and minced meats (pork 50%+beef 50%) (Notermans et al., 1983) over the decades. However, ELISA has several drawbacks such as variable affinity, insensitivity to closely related species, it needs extensive purification to eliminate cross-species reactions

(Khadijah et al., 2012) and is often hindered by cross-reactions occurring among closely related species (Fajardo et al., 2010). Additionally, this method is based on soluble proteins but soluble proteins are susceptible to denaturation at heat and are therefore unable to be applied to processed foods (Hsu et al., 1999; Smith et al., 1996). It also requires high titer antisera with specific antibodies for each meat species and its sensitivity is not equivalent to that of DNA-based methods (Fajardo et al., 2010) and also varies significantly in a mixed background of multiple species (Luo et al., 2011). Because, ELISA method less specific and sensitivity and it could detect up to 2% adulteration(Bhat et al.,2015) and was most suitable method for handling numerous samples at a time where DNA based polymerase reaction methods can detect as low as 0.001% of pork (Ali et al., 2012).

2.14 Limitation of Protein Markers for Species Detection

The protein based methods have been reported to be unsuitable for species identification in highly processed meat products due to the denaturation of protein by intensive heating during food processing which in turn lead to modifications in the antigenic activity of molecules and their mobility in electrophoresis (Still, 2003). This changes the ability of the antibody to identify its target protein (Still, 2003). Moreover, protein biomarkers is tissue specific (Adams, 2013) and can't be applied in an authenticity assay in terms of ground and complexed background samples (Anyinam, 1995). Furthermore, as the majority of commercial methods have been designed to detect plasma proteins, it has also been argued that adventitious contamination of meat with blood from other species could lead to spurious results (Robinson & Bennett, 2002). Moreover, cross-reactions of closely related species cannot be ruled out by protein-based methods even though they are specific and sensitive; but these problems can be solved with DNA-based methods especially species-specific PCR which has the potential to reach higher detection sensitivity and specificity (Hsieh et al., 1998). Additionally, DNA is characterized by

more stability under intensive heating, pressures, and chemical processing, has a conserved structure in whole body cells, has a great identification power since they are rely on the recognition of specific DNA segments sequence of a particular tissue or animal (Alves et al., 2013; Ernst & Coon, 2001; Lee et al., 2014).

2.15 DNA based Detection

Three major characteristic of the DNA molecule makes it an extremely useful tool for molecular species identification schemes. First, DNA is an extremely stable and long-lived biological molecule that can be recovered from biological samples that has been under stress conditions (processed food products, coprolites, mummified plant tissues, blood stains, etc) (Ali et al., 2012, 2014, & 2015). Therefore, analyses using nucleic acid are less liable to be disrupted by processing of foodstuffs and medicinal composites. A variety of methods have also been developed and they have shown that DNA molecular markers are more stable and sensitive than proteins (Ali et al., 2014 & 2016). Secondly, DNA is found in all biological cells and tissues, genetically identifiable information can be attained from any sample from the same organism or same sources (saliva, faeces, plant seeds, milk, etc). Finally, DNA can provide more information than proteins due to the degeneracy of the genetic code and the presence of large non-coding stretches. Nowadays, DNA based methodologies are improving rapidly and getting huge attention as suitable techniques by researchers and clinical practitioners for the authentication of target species (Amin et al., 2015).

In order to develop a DNA biomarker, it is important to select a conserve sequence of the target species for primer designing. Conserve DNA sequence from both nuclear and mitochondrial genes have been used to determine or distinguish the species in food and medicinal products. However, nuclear genes DNA have several drawbacks. Because of its high recombination rate and single copy linear DNA in each somatic cell which

make disturbance to perform DNA analysis in species authentication and highly processed samples difficult. Moreover, it is less sensitive in processed samples because DNA breakdown is a common phenomenon in heat treated samples. In contrast, mitochondrial DNA possesses several advantages over nuclear DNA for studies of speciation in meat products. It is relatively more abundant in total nucleic acid preparations than nuclear DNA (more than 1000x copies of nuclear DNA found in each cell), with the copy number of the mitochondrial genome exceeding that of the nuclear genome several fold (Williams, 2007). Mitochondrial DNA also tends to be maternally inherited so that individuals normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided. The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of even closely-related species. It should however be noted that mitochondrial DNA also exhibits a degree of intraspecific variability and so care has to be taken when studying differences between organisms based on single base polymorphisms (Lombardi et al., 2005).

2.16 Cytochrome b (Cytb) Gene, A Potential Candidate Mitochondrial Gene for Species Specific DNA Biomarker

The mitochondrial genome comprises a double-stranded DNA molecule of approximately 16 kb in length and accounts for 1% to 2% of the total DNA in mammalian cells (Figure 2.6). The use of mitochondrial DNA (mt-DNA) genes for species identification may offer a series of advantages which is mentioned in the earlier section. As there are thousands of copies of mitochondrial genes that are present in each cell, this improves the possibility of amplifying template molecules of adequate size among the DNA fragments brought about by heat denaturation. Intraspecific variability of mt-DNA offers the possibility of discriminating breeds currently used in industrial swine

production (Coghlan et al., 2015). Moreover, vast knowledge of its organization, as well as the availability of reported sequences in many species, also makes mt-DNA an easy design of specific primers possible for the direct and specific identification of PCR-amplified fragments (Ali et al., 2012; Rahman et al., 2014; Razzak et al., 2015). Therefore, several types of mt-DNA genes such as 12S rRNA (Park et al., 2007), 16S rRNA (Girish et al., 2007), Cytb (Coghlan et al., 2015), ND5, and a D-loop (Albers, Jensen, Bælum, & Jacobsen, 2013) (Figure 2.6) have been used in molecular techniques to devolve short length DNA biomarkers over the last two decades. Among these, Cytb gene has gained huge attention in animal species authentication tests. Numerous animal species including human to insects have been identified over the decades using complete or partial Cytb sequences (Ali, et al., 2015a; Nicolai et al., 2009; Fajardo et al., 2009; Meyer et al., 1994; Murugaiah et al., 2009). Therefore, of all the mt-DNA genes, Cytb is one of the most frequent target genes used in species identification (Kerry, 2009; Purchas et al., 2007) and is often used for phylogenetic studies and as reference gene in species-specific PCR (IRD, 2006). It contains both variable and conserved regions that are sufficient to resolve divergence at a population level and give high sensitivity (Coghlan et al., 2015). Although, Kocher *et al*. (1989) have mentioned that the primer sequence from 12S ribosomal RNA (rRNA) gene can amplify template DNA obtained from the mtDNA of animals, including mammals, birds, amphibians, reptiles, fish and insects as well as amplify the control region of mtDNA in most mammals and many fish species (Ali et al., 2016). However, the DNA sequencing results obtained from 12S rRNA gene didn't reveal and described by authors (Ali et al., 2015). Besides this, studies on 12S rRNA (BNF, 2005; Razzak et al., 2015), D-loop (Hambræus, 1999; Nathan et al., 1997), subunits of NADH dehydrogenase 2, 5 and ATPase 6, 8 (Haunshi et al., 2009) also provided detection limit ranging from 2% to 0.01% in mixture which offer highly sensitive species detection systems. In contrast, analysis of the sequence of amplicons

from the *cytb* gene revealed inter- and intraspecific base sequence variations showing higher sensitivity (0.1PG) (DIFVR, 2005).

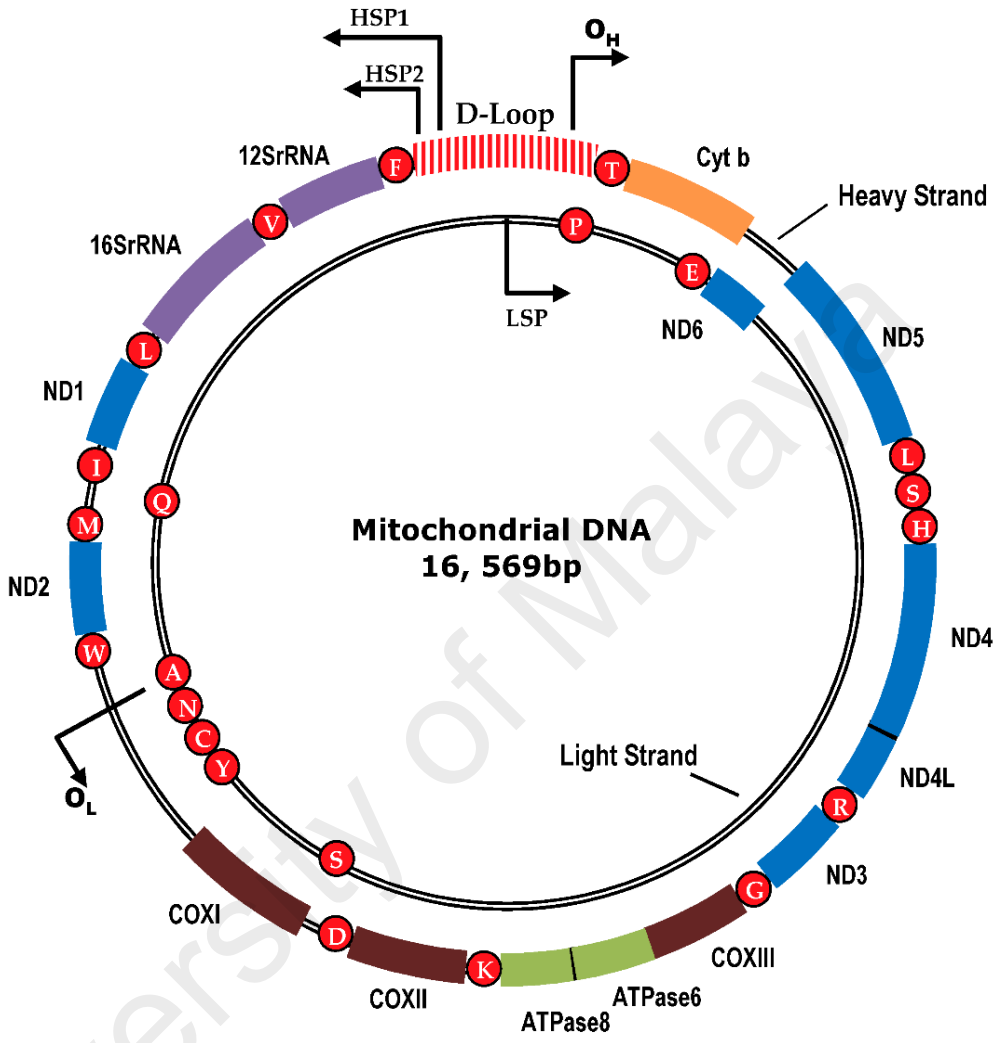


Figure 2.6: Mammalian mitochondrial genome. The gene order is the same in all mammalian species (Yusoff et al., 2015).

2.17 Species-Specific PCR Assay and Short-Length DNA Amplicon

Species-specific PCR assay targeting short length amplicon products obtained from mt-DNA genes has received huge attention in recent years (Coghlan et al., 2015; Fajardo et al., 2010). This technique has shown to be suitable for the detection of species' adulteration because of a specific target sequence that can be detected in sequences of different origin without the need of further sequencing or digestion of the PCR products

with restriction enzymes (Girish et al., 2004; Mafra et al., 2008). The principle of this method is that specific lengths of DNA can be copied multiple times using species-specific primers to provide a sufficient amount of targeted species DNA obtained from analysed samples followed by conventional gel electrophoresis techniques with ethidium bromide staining or via the automated real time qPCR technique being the most frequently used (Do et al., 2010). Several species such as bovine DNA (Maede, 2006), water buffalo and goat DNA (Arslan et al., 2006; Karabasanavar et al., 2011), sheep, pork, horse, donkey (Doosti et al., 2014; Haunshi et al., 2009; Mane et al., 2012), cat, and dog DNA (Ali et al., 2016) have been detected applying this techniques. Numerous studies in meat species authentication reported that species-specific assay can be either simplex or multiplex PCR assays. In a multiplex PCR assay, a single primer is used for the conserved region of the target gene, whilst the second primer is varied depending on the species. Thus, a multiple copy with different length target DNA fragments is amplified and detected in a single PCR assay. Additionally, the multiplex technique is widely accepted because it can detect multiple species in a single run, simultaneously reducing both the cost and time. However, it has some drawbacks such as an inborn complexity, fickle and lower sensitivity and unequal amplification for different-length templates have made them incompatible for target quantification (Credoc, 2003; DIFVR, 2005; Henderson et al., 2003). On the contrary, singleplex PCR assays, even though they cannot detect many species in a single run PCR, they are verified to be more sensitive, accurate, and robust (Ali et al., 2015; Coghlan et al., 2015; Fajardo et al., 2010). Presently, variable sensitivities and stability of singleplex PCR assays with different PCR products lengths are widely reported, because, better sensitivity of this assay is dependent on the reduction of amplicon length. According to Rojas et al. (2010), amplicon length should be kept less than 150 bp for species detection in highly processed foods analysis (Ilhak & Arslan, 2007), because larger amplicons are more susceptible to

breakdown into smaller fragments under harsh environments and often lead to cross-specificity reaction which significantly diminish the assay acceptability (Rashid et al., 2015a).

2.18 DNA Sequencing

DNA sequencing is the most straight-forward, definite and highly informative tool to identify species as the obtained target sequence can directly be compared with the known sequences in the Gene bank database in the National Center for Biotechnology Information (NCBI). Although, species identification by PCR technique is widely accepted, it is costlier and needs to additional preparations and is time consuming. In contrast, the DNA sequencing technique provides more information without further actions such as digestion with restriction endonuclease enzymes or further data analysis (Panduranga, 1996). Commonly mitochondrial genes such as Cytb, 12S and 16S rRNA genes are suitable for species identification because mitochondrial genes are highly susceptible for mutation and provides more variable information, thereby it makes it easier to distinguish between vertebrate animals, birds, fish, reptiles, mammals and amphibians than the closely related species using DNA sequencing techniques (Fajardo et al., 2008; Mutalib et al., 2012; Schoppe, 2008c; Spinks et al., 2012). Different species such as birds, mammals (Mane et al., 2009; Rodriguez et al., 2003), fishes, amphibian and reptiles (Hsieh et al., 2008; Montiel et al., 2000; Moore et al., 2003; Tamura et al., 2011) and some invertebrate species (Rojas et al., 2010) have been identified using the DNA sequencing technique. Abuzinadah et al. (2013) used the direct DNA sequencing method and found 90-98% homology in the DNA sequence to chicken species (*Gallus gallus*) in processed chicken products (Panduranga, 1996). But when they analyzed samples labelled as chicken luncheon they detected 98% homology with turkey meat (*Meleagris gallopavo*). They further used the same techniques and detected adulterants in luncheon, burger, sausage and minced meat products using the 12S rRNA gene

followed by confirmation using species specific primers (Lo et al., 2006). Red deer, roe deer, song thrush, pyrenean ibex, chamois, quail and sparrow have also been identified using partial and fully DNA sequencing techniques (Gans et al., 2012a; Thitika et al., 2014; Wu et al., 2009). Numerous research groups have reported that DNA sequencing is easier and a convenient technique for the identification of target species. However, in terms of short length DNA sequence and processed meat samples, it needs additional preparations, such as a cloning system, multiplication and integration target sequence into vectors, restriction digestion; all of which are costlier, time consuming, require expertise and sensitive protocol procedures. Moreover, their use is further restricted in the analysis of mixed-species meats as the heterogeneous amalgam of sequences from different species hinders interpretation (Fajardo et al., 2010)

2.19 PCR- Restriction Fragment Length Polymorphisms (RFLP) Technique

PCR-restriction fragment length polymorphism (PCR-RFLP) technique is currently used in species authentication including animals to microorganisms, where amplified PCR products obtained from the conserved region of single or multiple species using species specific primers and followed by digestion of those PCR products with selective restriction digestive endonuclease enzymes by incubating for a certain period at a specific temperature (Ball & Ackland, 2000; Davey et al., 2003). The acceptance of this method is more and wider than the species-specific PCR assay, as the PCR-RFLP method is intrinsically more accurate and specific so much so that it can easily discriminate closely related species (Ball & Ackland, 2000; Coghlan et al., 2015; Henderson et al., 2003). In addition, it is a good option to confirm the PCR products compared to DNA sequencing without further use of extensive analytical tools because PCR products can be subjected to incubation for fingerprinting with a wide variety of restriction enzymes. This technique gives flexibility to researchers to verify the specificity without access to direct sequencing facilities (Anyinam, 1995). It has been reported that the PCR-RFLP

technique is used for the identification of a variety of species including meat and fish. Fajardo et al. (2006) have applied the PCR-RFLP technique to the identification of closely related species such as red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), cattle (*Bos taurus*), sheep (*Ovis aries*), and goat (*Capra hircus*) and they digested 712 base pair bp conserve region with MseI, MboII, BslI, and ApoI endonucleases and obtained individual pattern for those species (Frézal & Leblois, 2008). Another group applied PCR-RFLP assay to identify cow, chicken, turkey, sheep, pig, buffalo, camel and donkey species using a 710-bp fragment obtained from all species with Hind II, Ava II, Rsa I, Taq I, Hpa II, Tru 1I and Xba I endonuclease restriction enzymes (Gillian et al., 2000). They were able to distinguish the mentioned species even though all the PCR products where the same but the restriction sites had different lengths. Thus, different levels of polymorphism were detected among samples. PCR–RFLP has also received special interest for meat speciation as it exploits the sequence variation that exists within defined DNA regions, allowing species differentiation of even closely related species by digestion of selected DNA fragments with appropriate restriction enzymes (Fajardo et al., 2008). However, some criteria need to be met when handling the RFLP technique. Usually the method accepts larger size PCR products which are susceptible to breakdown into smaller sized DNA fragments in heat processed samples. Moreover, DNA movement also depends on the concentration of the agarose gel, it is impossible to trace small DNA fragments, especially for sizes smaller than 50 bp (Coghlan et al., 2015). In order to trace out small DNA fragments such as 15 bp, the procedure should be integrated with sophisticated instruments which is costlier and time consuming. Recently, Ali et al. (2012) analysed their small size of restriction digested product (49, 33 and 27 bp) by using a lab on chip- based capillary electrophoresis incorporated in an Agilent 2100 Bioanalyzer (Coghlan et al., 2015). Other similar automated instruments that allows detection of small fragments (≥15 bp) are QIAxcel

capillary 31 electrophoresis system (Qiagen) and Biorad Experion. Generally, lab-on-a-chip is a miniaturized device that integrates onto a single chip one or several analyses, which are usually done in a laboratory; analyses such as DNA sequencing or biochemical detection. Lab on chip based technique has numerous advantages, such as cost efficiency, parallelization, diagnostic speed and sensitivity. The emergence of the lab-on-a-chip mainly focused on human diagnostic and molecular biology analysis. However, this technique cannot give the quantitative detection and is also expensive.

2.20 Real-Time PCR Assay

Real-time PCR has become one of the most widely accepted methods of species specific DNA identification and quantification because it has a large dynamic range, boasts tremendous sensitivity, can be highly sequence-specific, has little to no post-amplification processing, and is amenable to increasing sample throughput (Smithers et al., 1998). Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (Johnson & Walker, 1992). Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence (Rohman, et al., 2011). Generally, two common methods for the detection of PCR products in real-time PCR are: (1) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter and (2) non-specific fluorescent dyes that intercalates with any double-stranded DNA. In a sequence specific real-time PCR system, reporter dye molecules give the fluorescent signals when the probe DNA sequences are completely hybridized with its complementary DNA sequences of the target species (BNF, 2001; Fajardo et al., 2010). Sequence specific technique with various reporter dyes are widely

used based on the instrument and experimental set up. They include TaqMan hydrolysis probe (DIFVR, 2005b; Fajardo et al., 2010; Ilhak & Arslan, 2007) and Molecular Beacon probe (BNF, 2001). Further, TaqMan chemistries are simpler and more reliable than those of molecular beacon probes. This technique has been applied in several studies for the detection of beef, pork, lamb, chicken and turkey meats and the target products were less than 150 bp of regions of the cytochrome b gene. It can detect and quantify as low as 0.1% in raw state and 0.5% in an admixture condition (Fernández et al., 2005). Similarly, it can effectively detect turkey, beef, pork and sheep DNA sequences in complex food products, with the range of detection of 0.02 pg and 0.80 pg and at 1% in-mixture (Matthews & Strong, 2005). However, the drawbacks of this method is that it only allows the amplification of short amplicons PCR (maximum 150bp) (Lenstra, 2010), and there is the possibility of incompatibility of certain platforms with some fluorescent dyes, the restricted multiplex capability and the high cost of most reagents and instrumentation (Pereira et al., 2008). On the contrary, it is cost effective, with no need for additional preparation. Usually, two types such as SYBR green I (NHMRC, 2006) and EvaGreen (Chan, 1995) chemistries bases are used for the detection of target species. Although, it is a nonspecific detection technique as it intercalates with both the amplified and non-amplified ds-DNA and include SYBR green I (NHMRC, 2006) and EvaGreen (Chan, 1995) chemistries. Recently, absolute quantification of viral genomes of infectious hypodermal and hematopoietic necrosis virus, white spot virus, avian leukosis virus and human immunodeficiency virus (HIV) using SYBR Green chemistry has been described (Ali et al., 2016; Mane et al., 2012; Rahman et al., 2015). SYBR Green-based real-time PCR assay does not require probes and, therefore, it is economical and easily adoptable from the conventional PCR system. Furthermore, the SYBR Green-based real-time PCR assay detects the target PCR product accumulation independent of the sequence, as such it allows for the quantification of the viral genome with minor

variations in the sequence (Rodríguez et al., 2004). This may not be possible with probes as they operate in a highly target sequence-specific manner and small changes in target sequence may abrogate probe binding leading to generation of false negative results (Rodríguez et al., 2004)

As can be observed, meat fraud implies many different illegal procedures that need to be controlled by legal authorities through means of robust, accurate and sensitive technical methodologies, in order to ensure that fraudulent or accidental mislabeling of food does not arise. This review will specially deal with the case of meat substitutions as one of the most important sources of meat fraud and how proteomics emerge as a new and powerful tool capable to overcome the limitations of the control methods currently in use to detect these practices.

2.21 Methods for Malayan Box Turtle Detection

Recently, Malayan box turtle (MBT) have been categorized as most vulnerable by the IUCN and listed in Appendix II of the CITES database (Sabine Schoppe, 2008a). However, this species is highly targeted in illegal markets in south and East Asia and exported to East Asian countries for meat and medicinal purposes which causes the potential threat to their population and biodiversity balance. Moreover, turtle materials in any form is a potential haram ingredient in Halal foods as well as a zoonotic threat to human health. Therefore, a sensitive and reliable method to detect trace amounts of turtle materials is also necessary to prevent illegal trafficking of turtle meats and organs in local and international markets as well as to safe guard the religious faith of 1.8 billion Muslims around the world (Ali et al., 2015). Various detection techniques have been addressed based on the morphological, taxonomic approaches to determine MBT turtle species (Schoppe & Das, 2011). However, diagnostic morphological traits do not work when products are degraded or highly treated/processed (Nejad et al., 2014). Turtle

materials are used in highly processed forms in various food items and traditional medicine such as gels, pills and capsules (Hsieh et al. 2006). To overcome this limitation, targeted molecular detection techniques to determine the target species in complex background samples are required. DNA markers have proven to have higher stability, specificity and thus reliability even in environmentally compromised and highly processed samples (Ali et al., 2015; Zhang et al., 2007). Recently, several DNA based methods, such as PCR–DNA sequencing (Lo et al. 2006), PCR-restriction fragment length polymorphism (PCR-RFLP) (Moore et al. 2003), and randomly amplified polymorphic DNA (RAPD) (Saez et al. 2004), have been documented for the detection of MBT (*C. amboinensis*) and other turtle species. Hsieh et al., (2008) detected 14 turtle species including *C. amboinesis* using PCR assay and DNA sequencing (Hsieh et al., 2008). However, all involve very long length of target amplicons which have been assumed to break down when subjected to food processing treatments or compromised environmental conditions such as natural decomposition (Ali et al., 2015). A number of studies have demonstrated that successful analysis of degraded DNA specimens or compromised forensic evidence is improved with the use of smaller-sized PCR amplicons, typically ≤150 bp in length (Turna, 2010). Usually, the smaller the amplicon length, the better the recovery (Ali et al., 2012, 2015).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection and Preparation

Ethical clearance was obtained from the University of Malaya's Ethical Committee for Laboratory Animals as well as the Department of Wildlife and National Parks of Malaysia (PERHILITAN), located at Cheras in Malaysia, to study the occurrence of MBT meat in the food chain and traditional Chinese medicines. Application was made to study 22 turtle and tortoise species including 12 species of the *Cuora* genus, but the Department of Wildlife and National Parks of Malaysia (PERHILITAN) only permitted the study of 5 species, namely, the Malayan box turtle (*Cuora amboinensis*), the pond slider turtle (*Trachemys scripta*), the Malayan soft-shell turtle *(Dogonia suplana)*, the yellow-headed temple turtle (*Heosemys annandalii*) and the elongated tortoise (*Indotestudo elongate)*. Only the Malayan box turtle (*Cuora amboinensis*) samples was found at the authentic shop in various commercial wet markets, Malaysia. Authentic raw meat samples of Malayan box turtle(MBT) (*Cuora amboinensis* accession: FJ763736.1) and Chinese edible frog (*Hoplobatrachus rugulosus*) accession: NC_019615.1), and common meat species (chicken (*Gallus gallus* accession: KP269069.1), cow (*Bos taurus* accession: GU947021.1), goat (*Capra hircus* accession: KR059217.1), pig (S*us scrofa domestica* accession: AP003428.1), pigeon (*Columba livia* accession: KP168712.1), sheep (*Ovis aries* accession: NC_001941.1), duck (*Anas platyrhychos* accession: EU755253.1), buffalo (*Bubalus bubalis* accession: NC_006295.1)), fish (giant river prawn (*Macrobrachium rosenbergii* accession: NC_006880.1), cod (*Gadus morhua* accession: NC_002081.1), salmon (*Salmo salar* accession: NC_001960.1), carp (*Cyprinus carpio* accession: KU050703.1)) and plants (wheat (*Triticum aestivum* accession: NC_007579.1) and cucumber (*Cucumis sativus* accession: NC_016005.1)) were purchased from Pasar Borong, Pudu Raya and Selangor on three different days to

increase the genetic diversity of the samples. Venison (*Odocoileus virginianus* accession: KM612279.1) meats from three different animals were obtained in triplicate from the Faculty of Veterinary Science, University of Putra Malaysia located at Serdang in Selangor, Malaysia. Stray dog *(Canis lupus familiaris* accession: KF907307.1), cat (*Felis catus* accession: NC_001700.1) and rat (*Rattus rattus* accession: NC_012374.1) muscle meats were donated by Kuala Lumpur City Hall (KLCH) or Dewan Bandaraya Kuala Lumpur (DBKL), Air Panas, Kuala Lumpur and monkey (*Macaca fascicularis* accession: NC_012670.1) meat was a gift from the Department of Wildlife and National Park Malaysia (PERHILITAN/DWNPM), Cheras, Kuala Lumpur. DBKL routinely killed these animals for population control and public security in the town area and sufficient amount of muscle tissue samples of these species were taken from them following institutional and country laws. Information of all collected samples is compiled in Table 3.1. The identities of all of the collected samples of animal, fish and plant species were authenticated and confirmed by veterinary, fisheries and botanical taxonomy experts at the University of Malaya. All samples were transported under ice-chilled conditions and cut into small pieces for storage in a freezer at -20 °C until further use to prevent the further degradation of the tissues and DNA. On the other hand, 273 traditional Chinese medicinal samples for detoxification, antipyretic, nocturnal-urination, coughing, anti-inflammation, anti-cancer and sex-stimulator drugs and others were collected from 15 different shops of Chinese medicines across Kuala Lumpur and Selangor, Malaysia (Tables 4.6 and 4.13). Similarly, 189 samples (21x9) of 21 different branded commercial meat products (Table 4.6), namely, chicken and beef meatball, burger and frankfurter products were purchased from 10 different wholesale food shops in Kuala Lumpur and Selangor. All collected samples were kept in the samples' chamber of the freezers according to the manufacturer's guideline.

Table 3.1: Information of collected samples

No	Species	Sources	Geographic coordinates of the sources	Number of samples
1	Malayan box turtle	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	30
2	Frog	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	15
3	Chicken	Tesco supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	30
4	Cow	AEON supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	30
5	Goat	AEON supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	30
6	Pig	Tesco supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	25
7	Pigeon	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	20
8	Sheep	AEON supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	20
9	Duck	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	25
10	Buffalo	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	20
11	Prawn	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	30

Table 3.1, continued

No	Species	Sources	Geographic coordinates of the sources	Number of samples
12	Cod fish	Wet market	Paser Borong, Kuala Lumpur, Malaysia	25
13	Salmon fish	AEON supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	25
14	Carp fish	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	25
15	Wheat	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	30
16	Cucumber	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	20
17	Dog	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	15
18	Cat meat	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	15
19	Rat meat	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	15
20	Venison	Veterinary Department	Kuala Lumpur, Malaysia	15
21	Monkey meat	Wildlife and National Parks (DWNP)	Cheras, Kuala Lumpur, Peninsular Malaysia,	15

3.2 Preparation of Binary and Ternary Meat Mixtures for Specific PCR and PCR-RFLP Assay

To simulate the complexity of the matrices, two types of binary mixtures, i.e., MBT-beef and MBT-goat, were made in a total volume of 100 g of specimen by mixing MBT meat in a proportion of 10%, 1%, 0.5%, 0.1% and 0.01% with an adjusted amount of beef and goat meat (Ali et al., 2012, 2014a, 2015; Rashid et al., 2015). Two types of ternary admixtures were compiled following the procedure by Ali et al., (2012), i.e., MBT-chicken-wheat flour and MBT-beef-wheat flour were made by mixing 10%, 1%, 0.5% 0.1%, and 0.01% of turtle meat into chicken, beef and wheat flour, at a ratio of 20:80:100, 2:98:100, 0.04:99.60:100, 0.2:99.8:100, and 0.02:99.98:100. Finally, the required amount of deionized water was added to the admixtures and briskly ground with a blender (Pensonic Super Blender-PB-3205, 13600 Prai, Penang, Malaysia) to obtain a homogenous, semi-solid slurry. The admixture samples were autoclaved at 120 °C under 45 psi pressure for 2.5 h. All prepared admixture meat samples were kept at -20 °C for further DNA extraction. To prevent contamination, each of the mixtures was made and tested on separate occasions in triplicate, and all of the prepared admixtures were stored at -20 °C prior to DNA extraction.

3.3 Sample Preparation for Real Time PCR Assay

For the determination of the lower limit of detection (LOD) and lower limit of quantification (LOQ) either for Malayan box turtle or eukaryotic control content, approximately 50-60 g of MBT, chicken and beef pure muscle meat were sterilized individually at 120 °C under 45-psi pressure for 2.5 hours on three different days. Once the autoclaving was completed, samples were kept to cool at room temperature and finally stored in -20 °C or had the DNA extracted immediately for further analysis. To evaluate the performance of the real time PCR assay in complex matrices, two types of binary

mixtures, namely, Malayan box turtle-chicken and Malayan box turtle-beef were made into a total amount of 100 g specimen by spiking of MBT meat at a proportion of 10%, 1%, 0.1%, 0.01% and 0.001% with an adjusted amount of ground chicken and beef meat. All binary admixed meats were made following the procedures of Ali et al. (2012, 2015) and Rashid et al. (2015). Sufficient (50-100 ml) sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM Na_2HPO_4. 12 H_2O, and 2.6 mM KCl, pH 7.2) was added to the admixtures and briskly grinded with a blender (Pensonic Super Blender-PB-3205, 13600 Prai, Penang) to obtain a homogenous semi solid-slurry. To prevent contamination, every mixture was taken out separately using separate materials and a separate blender on three different days by three independent analysts. Fifty to sixty grams of each type of mixture were sterilized at 120 °C under 45 psi pressure for 2.5 h to check the thermal effect on the target species. Once the tissues were autoclaved, the samples were left to cool at room temperature inside a lamina flow cabinet with positive air flow. Finally, all raw and heat processed binary admixture samples were used directly for DNA extraction or stored at -20 °C for later DNA extraction.

3.4 Preparation of Chicken and Turtle Meatball for Specific PCR Assay

Meatballs were prepared according to Rahman et al. (2014) with a well-adjusted amount of ground chicken and turtle meat with culinary salt, garlic and other ingredients (Table 3.2). To obtain MBT meat contaminated meatballs, 1%, 0.5%, 0.1%, and 0.01% of turtle meats were added with 100 g of deboned chicken. All other ingredients were properly mixed with meats by vigorous blending and the emulsified homogenous meat mixtures were then given a meatball shape. To simulate cooking and extensive heat treatment effect, the prepared meatballs were subjected to autoclaving at 120 °C at 45 psi pressure for 2.5 h. All samples and preparations were stored at −20 °C for further DNA extraction.

Table 3.2: Ingredients used in meatball preparation

Ingredient	Chicken meatball	Turtle meatball
Minced meat	100 g[a]	100g
Breadcrumbs	7.5g	7.5 g
Chopped onion	5g	5 g
Ginger freshly chopped		1.5 g
Cumin powder		1.25 g
Garlic powder	1.25 g	1.25 g
Black pepper	0.14	0.14 g
Milk	0.011	0.011
Butter	3.28 g	3.28 g
Tomato paste		2.5 g
Salt	0.05 g	0.05 g

Notes: [a]1%, 0.5, 0.1% and 0.01% of deboned turtle meats were mixed with a balanced amount of deboned chicken to obtain in total a 100 g specimen, chopped, mixed and minced prior to making the meatball.

3.5 Preparation of Reference Meat Products for PCR-RFLP and Real Time PCR Assay

Reference/Dummy chicken and beef meatball, burger and frankfurter products were prepared following Ali et al. (2012 & 2015), Rahman et al. (2014) and Razzak et al. (2015), and the negative controls of meat products (chicken and beef meatball (≥ 50 g/piece), burger (≥ 100 g/piece) and frankfurter(≥ 80 g/piece)) were prepared on three different days using pure ground chicken and beef meat (blended by Pensonic Super Blender- PB-3205, 13600 prai, Penang, Malaysia) along with typical amounts of fats and other culinary ingredients as found in commercial mixtures (Table 3.3). Similarly, the positive controls were made by spiking 10%, 1%, 0.1%, 0.01%, and 0.001% of MBT meat into the chicken and beef meat, which was used to make various dummy meat products (Table 3.3). As presented in Table 3.3, culinary salt, garlic and other ingredients

were added to the mixtures and blended vigorously until a homogenous mush was obtained, and the emulsified homogenous mixtures were then shaped into meatball, burger, and frankfurter products (Razzak et al., 2015). All samples were prepared in triplicate on three different dates by three independent analysts. All of the prepared meat products were individually subjected to autoclaving at 120 °C under a pressure of 45 psi for 2.5 h, after which, all of the meatballs were stored at -20 °C for further DNA extraction.

Table 3.3: Formulation of ready-to-eat model meat products

Ingredients	Meatball (≥50 g/piece)		Burger (≥100 g/piece)		Frankfurter (≥80 g/piece)	
	Beef	Chicken	Beef	Chicken	Beef	Chicken
Minced meat (g)[a]	33	33	70	70	55	55
Soy protein(g)[b]	5	5	10	10.	10	10
Starch/breadcrum(g)[b]	6	6	8	8	7	7
Chopped onion(g)[b]	2	2	4	4	2	2
Chopped ginger(g)[b]	0.2	0.2	0.4	0.4	0.2	0.2
Cumin powder(g)[b]	1	1	1	1	1	1
Garlic powder(g)[b]	0.5	0.5	1	1	0.5	0.5
Black pepper(g)[b]	0.15	0.15	0.3	0.3	0.2	0.2
Tomato paste(g)	1.5	1.5	2.5	2.5	2	2
Butter(g)	1.5	1.5	2.5	2.5	2.5	2.5
Egg(g)			1	1		
Salt(g)	SA	SA	SA	SA	SA	SA
Others(g)[c]	SA	SA	SA	SA	SA	SA

Notes: [a]10%, 1%, 0.1%, 0.01% and 0.001% of MBT meat was added to a balanced amount of minced chicken and beef to make ≥ 50-g, 100-g, and 80-g specimens of each meatball, burger, and frankfurter, respectively. [b]Amounts are approximate, and some items were measured in teaspoons. [c]Enhancers and flavoring agents. SA-Suitable amount.

3.6 Sample Preparation for Target DNA Stability Test

All of the MBT meat samples were individually cut into smaller pieces, and approximately 5-6 g of each sample were cooked at 100 °C for 60, 90, 120 and 150 min to simulate traditional cooking. Secondly, all meat samples were autoclaved at 120 °C under 45 psi for 60, 90, 120 and 150 min. Finally, microwave cooking was performed at 600, 650 and 700W (watt) for 30 min, and all of the heat-treated samples were stored at -20 °C until further use. Total DNA was extracted from 30 mg of the heat-treated samples using a Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan).

3.7 DNA Extraction of Animals and Plants Samples

Total DNA was extracted from 30 mg of muscle tissue of each animal species, heat processed or admixed samples as well as from the binary, ternary mixtures of turtle meat spiked models and commercial meat products using the Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd, Taipei, Taiwan) following the manufacturer's instruction. DNA from plant sources was extracted following Ma et al. (2000) using traditional CTAB (Cetyl Tri Methyl Ammonium Bromide) extraction kits. The purity and concentration of all of the extracted DNA was determined using a Biodrop UV-VIS spectrophotometer (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK) based on absorbance at 260-280 nm in the calculating ratios of A260/280. The purified genomic DNA was kept at - 20 °C until further use.

3.8 DNA Extraction of Traditional Chinese Medicines (TCM)

DNA extraction from traditional Chinese Medicines was carried out with a slight modification. Total genomic DNA was extracted from 30 mg of traditional Chinese medicine (TCM) samples using Yeastern genomic DNA (animal tissue DNA) mini kits

(Yeastern Biotech Co., Ltd. Taipei, Taiwan). Firstly, the powder was ground with a micro pestle and dissolved properly with 400 µL GT buffer. A high amount of GT buffer (400 µL) was used to properly dissolve the powder samples and ensure the proper dissociation of the sample tissues and contaminants. Due to the over dried form of TCM and Chinese herbal jelly powder, twice the normal amount of GT (400 µL) and GBT (400 µL) buffer were added to these samples to avoid the formation of solid glue or to allow settling down of the target samples and kept for a prolong incubation (60 minutes) period for tissue lysis. Prolong incubation increases the DNA yield and dissociates the unwanted materials from the target samples.

To lyse the protein molecules in the target samples, thirty (30) µl (10 mg/mL) of protein kinase enzyme was added, shaken vigorously and incubated for 60 minutes at 60 °C to ensure the samples lysate clearly. Afterwords, 400 µL of GBT buffer were added into the sample, shaken vigorously and incubated for 60 minutes at 60 °C which confirmed clear lysis and dissociation of polysaccharides and phenol from the samples. At this stage, protein molecules undergo breakdown and DNA in chaotrophic salt is bound to the glass fibre matrix of the spin GD column. To remove the insoluble materials, samples (phenol, chloroform, polysaccharides or any possible remaining proteins) were centrifuged (Allegra X-30R centrifuge, CAT A99471, Backman Coulter, Inc, California, U.S.A) for 3 minutes at 16,000 X g where after the supernatant was transferred to a new 1.5 mL micro-centrifuge tube and checked for any sediment. 200 µL of absolute ethanol was added in the supernatant to clear the lysate and immediately shaken vigorously for 10 second and again centrifuged for 2 minutes at 16,000 X g to remove the precipitate. Finally, the clear solution was transferred into a GD column containing a fiber matrix and centrifuged for 2 minutes at 16000 X g where dissociated DNAs are bound into the fiber matrix in the GD column. The resulting bound DNA in the fiber matrix in the GD column was rinsed twice with 400 µL of W1 buffer (ethanol) and 600 µL of wash buffer and

centrifuged at 16,000 X g for 3 minutes to remove the non DNA molecules or impurities. Finally, 100 µL pre-heated DNA elution buffer was added into the purified DNA which was bound onto the fiber matrix and left for 10 minutes until dissolving of the fibre bound DNA into the elution buffer was completed. Once properly dissolved, purified DNAs were eluted by low salt elution buffer. The yield and quality of extracted DNA was determined with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies Inc. Montchanin, DE, UK) based on absorbance at 260 nm and purity ratio (A260/280).

3.9 Design of Oligonucleotide Primers

A pair of MBT-specific primers (MBT-F and MBT-R) targeting a 120 bp site of the mitochondrial cytochrome b (mt-cytb) gene were designed using publicly available primer3 Plus software (www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi). To check species specificity, the designed primers were aligned against the similar sites of the 28 different species mentioned above (Table 3.1) using MEGA 5 software. They were blasted in the National Center for Biotechnology Information (NCBI) by the basic local alignment search tool (BLAST) against non-redundant nucleotide sequences. The designed primers were synthesized and supplied by the 1st Base Laboratories Sdn. Bhd. (Selangor, Malaysia). For internal control, a 141-bp conserved fragment of eukaryotic 18S rRNA gene was amplified from all species using the Eukaryotic primers (Euk-F and Euk-R) described elsewhere. For the comparison study, another universal primer pairs set (L1373-F and H1478-R) of 165 bp length for multiple turtle including *Cuora amboinesis* species detection was used (Lo et al., 2006). All primer sequences and target product-sizes used are given in Table 3.4.

Table 3.4: Oligonucleotide primers used in this study

Target gene	Primer	Sequence (5′-3′)	Amplicon (bp)	Reference
Cytb	MBT-F	AGCCCTTCTAACATCTCTGCTC	120	
	MBT-R	CTCACCAGACATCTCACTAGCA		
18S rRNA	Euk-F	GGTAGTGACGAAAAATAACAATACAGGAC	141	Rojas et al., (2010)
	Euk-R	ATACGCTAT TGGAGC TGGAATTACC		
12S rRNA,	L1373-F	CGCTCGAGAGAAATGGGCTACATTTTCT	165	Lo et al., (2006)
	H1478-R	TGACTGCAGAGGGTGACGGGCGGTGTGT		

3.10 Specific PCR Assay Optimization

To confirm the theoretical specificity determined by the BLAST analysis (source: http://blast.ncbi.nlm.nih.gov/Blast.cgi), the designed primers were challenged against 20 different non-target species (Frog, chicken, cow, goat, pig, pigeon, monkey, rat, cat, dog, sheep, duck, water buffalo, deer, giant river prawn, cod fish, salmon fish, carp fish, wheat, cucumber) by performing an optimized PCR assay against the template DNA of the said species. Additionally, *in silico* analysis was performed and a phylogenetic tree was constructed using MBT-specific 120 bp site of the cytochrome b gene against 66 reptile species. Finally, theoretical deigned primer set was optimized with the following modifications; three annealing temperatures such as 58, 59 and 60 °C and different concentration of PCR master mix (4-7 unit µ/L of Tag DNA polymerase, and 130-160 µM each dNTP and 1-1.30 mM $MgCl_2$), 20 ng of extracted DNA concentration were mixed with 150 nM of each primer to determine the optimized PCR amplification. Generally annealing temperature chosen for a PCR product depends directly on length and composition of the primers. In each reaction a negative template control of PCR reaction (a PCR reaction mixture without template DNA and replaced with 18.2 Ω Millipore-nuclease free water) and internal control (18S rRNA gene) with similar

concentration were included to ensure the purity of the PCR reaction mixture from contaminating DNA. All steps of optimization were recorded and analyzed and compared results in each other. Finally optimized PCR assay was performed in a 20 µL reaction mixture holding 1× PCR master mix (Promega, Corp., Madison, WI, USA) composed of 5 unit µ/Lof Taq DNA polymerase (supplied in a proprietary reaction buffer pH 8, 150 µM each dATP, dGTP, dCTP, dTTP and 1.25 mM MgCl2), 150 nM of each primer and 20 ng of extracted DNA. PCR cycling was done in Veriti 96-well Thermal Cycler (Applied Biosystems Inc., Foster, CA, USA), using an initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. Negative template control of PCR reaction (a PCR reaction mixture without template DNA and replaced with 18.2 Ω Millipore-nuclease free water) and eukaryotic internal control (18S rRNA gene) primer pair with similar concentration were included to eliminate false positive results as well as ensure the purity of the PCR reaction mixture from contaminating DNA. The separation of PCR products was performed in 2% agarose gel (1st base Laboratories, Pte. Ltd, Selangor, Malaysia) in 1× TBE buffer at a constant voltage of 120 V for 60 min, pre-stained with 6× loading dye and using a 100 to 1000 bp DNA marker (Promega Corporation, 2800 Woods Hollow Road · Madison, WI 53711-5399, USA) as reference standard. PCR product on agarose gel was visualized using a gel image documentation system (AlphaImager HP, Santa Clara, CA, USA).

3.11 DNA Sequencing and Data Analysis

To establish the assay validity and PCR products, target amplicon (120 bp) was subjected into DNA sequencing through the following steps. Firstly, PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics, GmbH, Roche Applied Science 68298, Mannheim, Boehringer, Germany) and ligated into a plasmid vector (PJTI1.2) using the TOPO^{TM} TA Cloning Kit (Invitrogen, Carlsbad,

CA, USA) following the manufacturer's instructions. DH5α-T1 *Escherichia coli* (Invitrogen) were added into recombinant plasmids for the transformation reaction and incubated overnight at 37 °C on agar plate containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). About forty colonies were selected from each agar plate, and plasmid DNAs were isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen Gmbh, Hilden, Germany). Finally, cloned target DNA sequencing was carried out with universal LpJET1.2 forward primer (5′-CTGCTTTAACACTTGTGCCTGA-3′) and LpJET1.2 reverse primer (5′-TACGATTGGTGGAGTAGTCCTT-3′) provided by the cloning kit manufacturer and analyzed with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Perkin-Elmer/Cetus, Norwalk, Connecticut, USA). Chromatogram sequencing files where inspected with Chromas 2.2 software (Technelysium) (http://technelysium.com.au/wp/).

3.12 Sensitivity Tests for Specific PCR and PCR-RFLP Assay

The sensitivity of the assay was determined using serially diluted DNA extracted from pure meat tissues (10, 1, 0.1, 0.01, 0.001, 0.0001 ng. μ/L) as well beef-turtle and goat-turtle binary and chicken-turtle-wheat flour ternary mixtures prepared by spiking 10%, 1%, 0.5%, 0.1% and 0.01% (w/w) of turtle meat into an adjusted amount of beef, goat and chicken in a 100 g mixture, whereas, 1%, 0.5%, 0.1% and 0.01% turtle meat was spiked in chicken meatballs for specific PCR assay. Similarly, sensitivity tests of admixed for PCR-RFLP assay was designed with the following samples from a binary mixture, ternary mixture and dummy meat products (chicken and beef meatballs, burgers and frankfurters). All of the admixed meat samples were made by spiking the beef, chicken and goat meat with 10%, 1%, 0.1%, and 0.01% MBT meat (Table 3.3). The lower limit of detection (LOD) was determined by amplification of 10-fold serially diluted (10, 1, 0.1, 0.01, 0.001, 0.0001 ng μL^{-1}) target species DNA in nuclease-free water. Total DNA was extracted as described above.

3.13 Comparison of Target DNA Sensitivity and Stability

To check the comparative stability and sensitivity of the newly developed (120 bp site of the cytochrome b gene of MBT) and previously documented shortest target (165 bp site of the 12S rRNA gene)(Lo et al., 2006) PCR assays, PCR was performed with 20 ng MBT DNA template extracted from turtle meats after boiling at 120 °C for 60, 90, 120 and 150 min; autoclaving at 120 °C for 60, 90, 120 and 150 min at 45 psi pressure and microwave cooking at 600, 650 and 700 Watt for 30 min. In addition, the sensitivity of the newly designed and published targets was tested by a 10-fold serial dilution of extracted DNA from pure raw meats.

3.14 Enzymatic Digestion for PCR Product Authentication with PCR-RFLP Assay

To authenticate the specific PCR product, the MBT-specific PCR product (120 bp) was digested with *Bfa1*-restriction endonucleases (New England Biolabs, Ipswich, MA, USA) in a 30 µL reaction mixture containing 12 µL of PCR product, 1 µL of restriction enzyme (1 FDU), 2 µL of 10x digestion buffer supplied with enzyme and 15 µL of distilled water. All of the mixtures were mixed properly and kept in a water bath at 37 °C for 30 min, and DNA digestion was stopped by placing the mixture in another water bath at 80 °C for 20 min. For the RFLP analysis, 1 µL of digested PCR product was applied to a microfluidic lab-on-chip well using a 1 K DNA analysis kit (Experion, Bio-Rad, Inc., USA), and the desired fragments were separated in a Bio-Rad Automated Electrophoresis station (Experion, Bio-Rad, Inc., USA). The Experion automated electrophoresis system employs Lab Chip microfluidic technology to automate nucleic acid electrophoresis and this system is widely used for the applications: DNA analysis-quantitation and sizing of restriction digests (15 to 1500bp), amplified DNA, microsatellites, and amplified fragment length polymorphisms (AFLPs). It integrates

separation, detection, and data analysis within a single platform. Using much smaller sample and reagent quantities than standard analysis methods, the Experion system accomplishes analysis in a single 30–40 minute, automated step. Thereby, lab-on-a-chip technology provides more convenient for smaller DNA fragment analysis and productive way to gather and store experimental data.

3.15 PCR-RFLP Analysis of Admixed and Processed Samples

The PCR products from the binary mixture, ternary mixture and commercial meat products (chicken and beef meatballs, burgers and frankfurters) were digested with *Bfa1*-restriction endonucleases (New England Biolabs, Ipswich, MA, USA). All of the admixed meat samples were made by spiking the beef, chicken and goat meat with 10%, 1%, 0.1%, and 0.01% MBT meat (Table 3.3). Further, all types of PCR products of the heat processed (boiling, autoclaving and microwave cooking) samples were digested with *Bfa1*-restriction endonucleases (New England Biolabs, Ipswich, MA, USA). Further RFLP analysis was carried out with Bio-Rad Automated Electrophoresis station (Experion, Bio-Rad, Inc., USA). The total procedure is described in 3.14 section

3.16 SYBR-Green Duplex Real Time PCR Assay Optimization

The real-time PCR assay was optimized and executed in a 20 µL reaction mixture (20 µL per reaction) containing 10 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA; Thermo Fisher Scientific, Foster, CA, USA), 1.5 µL forward primer (150 nM), 1.5 µL reverse primer (150 µM) and 3 µL template DNA (10 ng) and 4 µL of nuclease-free water. Same concentration (150 nM) of eukaryotic (18S rRNA) primer pairs as the endogenous positive control were combined into the reaction mixture to verify the target DNA amplification and ensured the presence of good quality DNA extract obtained from all eukaryotic samples (Rojas et al., 2010). A negative

template control was made by using nuclease-free water (PCR reaction mixture without template DNA and replaced with 18.2 Ω Millipore water). The PCR reaction was run on a QuantStudio® 12 K Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Cycling conditions were 10 min initial denaturation at 95 °C and 40 cycles at 95 °C for 15 s, 58 °C for 1 min and 72 °C for 30 s. The melting curve analysis was programmed to form a slope between 70 and 94 °C by raising 1 °C at each step. The program waits for 118 s of pre-melt conditioning on the first step and 5 s for each step afterwards. Each reaction was performed in triplicate using two different reagents and different analysts.

3.17 Melting Curve Analysis of SYBR Green Real Time PCR Assay

The melting temperatures (*Tm*) of the amplified DNA targets were determined by melting curve analysis using ExpressionSuite software (version 1.0.4., Life Technologies, Thermo Fisher Scientific, USA). The specific location of the *Tm* was obtained by plotting the variation in fluorescence (dF/dT) against the temperature of the reaction products.

3.18 Construction of Standard Curve

Three types of standard curves namely (i) pure meat DNA (ii) binary admixtures (MBT-chicken and MBT-beef) and (iii) reference meat products (percentages) were separately constructed. The first calibration curve was constructed using the DNA extract obtained from pure muscle meat and adjusted to concentration 100 ng μL^{-1} and diluted with nuclease free water at a ratio of 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$. Other standard curves for admixture (MBT-chicken and MBT-beef) and reference meat were constructed with 10-fold serially diluted DNA obtained from the binary admixtures of 10 ng (10%) MBT DNA spiked into 90 ng (90%) DNA of beef and chicken and six types of reference meat products, respectively. Initially, the extracted total DNA of the

binary mixtures was made into 100 ng/µl (100%) and then serially diluted using nuclease-free water in ratios of 1:10, i.e., 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$. All standard curves were applied for the quantification of the target DNA in unknown samples.

The linear calibration curves for MBT and total meat (eukaryotic) of pure and, binary admixtures (MBT-chicken and MBT-beef) and references meat products were obtained by plotting the respective Ct values against the logarithm of the concentration of MBT DNA, and total meat DNA, respectively. For quantification, the concentration of MBT DNA and total meat DNA was calculated using equation (1) and (2) and the content of MBT meat or recovery of MBT meat in an unknown sample was calculated according to equation (3) following Druml et al. (2015b).

$$\text{DNA (MBT)(ng/µL)} = 10^{*[(\text{Ctspec-dspec})/\text{slope(spec)}]} \quad \text{Eq. 1}$$

$$\text{DNA (Total meat) (ng/µL)} = 10^{*[(\text{Ceuk-deuk})/\text{slope(euk)}]} \quad \text{Eq. 2}$$

Where Ct_{spec} and C_{euk} are Ct values obtained with MBT specific and eukaryotic (18S rRNA gene) systems real time PCR assay, respectively; d_{spec} and d_{euk} are intercepts from the standard curves; and $slope_{spec}$ and $slope_{euk}$ are the slopes of the standard curves for the MBT-specific and eukaryotic PCR systems, respectively.

$$\text{MBT } meat\ content(\%) = \frac{MBT\ DNA\ (\frac{ng}{\mu L})}{total\ meat\ DNA(\frac{ng}{\mu L})} X100 \quad \text{Eq. 3}$$

3.19 Amplification Efficiency (E %)

The amplification efficiency (E) of the real time PCR assay was determined by analyzing the DNA extract obtained from MBT in various concentration ranges from 100 ng μL^{-1} to 0.1 pi µ/L.

Amplification efficiency was extrapolated from the slope of the best fitted line in the standards curve and also calculated using equation (4).

$$E\ [\%] = [10^{\ [-1/Slop]} - 1] \times 100 \qquad \text{Eq. 4}$$

3.20 Limit of Detection (LOD) and Quantification (LOQ), Repeatability and Assay Robustness in the Real Time PCR Technique

The lower limit detection of the assay was determined using serially diluted DNA (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng and 0.00001 ng) extracted from pure meat tissues, binary admixtures (MBT-chicken and MBT-beef), and various known percentage of MBT tissue (10%, 1%, 0.1%, and 0.001% (w / w))added into lab made reference meat products (chicken and beef meatballs, burgers and frankfurters)(Table 3.3) and commercial meat products (chicken and beef meatballs, burgers and frankfurters) (Table 4.4). The LOD was defined as the lowest concentration that resulted in an increase in fluorescence signal by at least seven out of eight replicates within 38 cycles of the PCR. Quantification was performed based on an MBT target and eukaryotic control system DNA corresponding to the MBT and total meat. In order to determine the lower limit quantification (LOQ), the real-time PCR assay was calibrated with the calibration standard curves of two types of binary admixtures (MBT-chicken and MBT-beef) and six types of reference meat products containing 10% MBT DNA in 90% chicken and beef meat DNA and reference meat product DNA, respectively. Finally, the quantity of the target DNA in the samples (binary admixtures and reference meat products) was determined using a normalized Ct value fitted into a calibration curve; and finally the target DNA content was determined by equation 1 to 3. Data obtained from an SYBR Green duplex real-time PCR assay was analyzed by Expression Suite software (version 1.0.4., Applied Biosystems, Thermo Fisher Scientific, USA) and Microsoft Office Excel

2007. Microsoft Excel software and One-way analysis of variance (ANOVA) using SPSS software (version 16.0, IBM, Chicago, IL, USA) was performed to assess the significant differences in the mean values of the different samples. Relative standard deviation of the targets in complexed background samples was calculated and determined following the good laboratory practice of the Food and Agricultural Organization guidelines (FAO-CAC/GL 74-2010)(FAO, 2010). Thus, under experimental conditions, the LOQ reflected the lowest concentration of the target in complex matrices (Table 4.10-4.13) at which the relative standard deviation was <25% (FAO-CAC/GL 74-2010)(FAO, 2010). The assay reproducibility and recovery of the targets were confirmed by analyzing 150 reference meat samples using two different DNA extraction kits: (1) Yeastern Genomic DNA Mini Kit(animal tissues) (Yeastern Biotech Co., Ltd, Taipei, Taiwan), and (2) NucleoSpin® Extraction Kit (Macherey-Nagel, Düren, Germany), at PCR instruments (QuantStudio® 12 K Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA) with two different analysts on five different days in the same laboratory using three replicates in each reaction.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 DNA Extraction

Total genomic DNA was extracted from pure and admixed (binary, ternary and MBT-mixed) meat products under raw and processed (boiled, autoclaved and microwaved) states and traditional medicines using Yeastern Genomic DNA Mini Kit (animal tissues) (Yeastern Biotech Co., Ltd, Taipei, Taiwan). The specimens were prepared on three different dates by three independent analysts. The concentration and purity of the extracted DNA were determined, and the 260/280-nm absorbance of all of the samples was 1.7–2.0 which indicates good quality DNA (Adams, 2013). DNA yield was higher from the heat-treated MBT meat samples (220–350 ng μL^{-1} from boiled and autoclaved 400-450 ng μL^{-1} from microwaved samples) than from the raw meat samples (210–275 ng μL^{-1}) probably due to sample dehydration, which increases the effective number of cells and, thus, the number of analytes per unit weight of the treated samples (Fairbrother et al., 1998). In contrast to the meat and meat products, the concentration of the extracted DNA from medicinal samples was 18–35 ng $\mu L^{-}1$ and the purity of the genomic DNA was 1.75–1.80; this might be due to the multiple components such as polysaccharides within the medicinal samples that may not contain DNA. Furthermore, the various plant and animal materials might involve inhibitors and also the series of processing procedures such as drying and stewing that definitely degrade DNA to variable extents will cause these lower concentrations. Initially, difficulties were encountered in dissolving some of the herbal jelly medicinal powder samples (~15 samples) which frequently precipitated during the DNA extraction process (without modification). This was not a surprise since TCM preparations often involve a decoction method that extensively modifies the natural composition and textures of the source ingredients,

bringing in many excipient species. Therefore, the DNA extraction method was modified by increasing the lysis time in the GT buffer and prolonging the binding time to the nitrocellulose membrane in the GD column using a GBT buffer (Yeastern Biotech Co., Ltd. Taipei, Taiwan). This modified procedure resulted in 120 of 135 (Tables 4.6 & 4.13) jelly powder samples being dissolved and DNA extraction being successfully carried out. However, the concentration of the DNA from these jelly powder samples was 18-27 ng μL^{-1}, which was far less than that from the other medicinal samples that yielded 30–35 ng μL^{-1} by the self-modified extraction protocol system. Without modification of extraction protocol (manufacturer's protocol), the yielded DNA concentration was very low and ranged from 2.0 to 5.0 ngμL^{-1} and the purity was at 1.65 to 1.7 at 260/280 nm, suggesting possibly less dissolved target samples in the buffer solution or an inappropriate amount of buffer solution or less dissociated DNA from the samples. TCM is based on multiple types of herbal materials and sources and contains impurities such as polysaccharides, polyphenols, and other substances which may hinder the extraction. Under the experimental condition, 200 µL of GT buffer and 200 µL GBT buffer (according to the manufacturer protocol) volume were insufficient to properly dissolve the jelly medicines powder and consequently it formed a solid glue that settled down. Thus, adding more buffer solution (double the amount) and prolonging the incubation period (60 minute at 60 °C in water bath) accelerated the dissolving and thus dissociated the DNA from the target samples. Finally, extracted DNA concentration and purity obtained from TCM and herbal jelly powder were determined using Biodrop UV-VIS spectrophotometer (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK) based on absorbance at 260-280 nm in the calculating ratios of A260/280 and the concentrations ranged between 18~35 ng μL^{-1} and purity ranged between 1.75-1.80 ratio. All the purified genomic DNA were kept at -20 °C until further use.

4.2 Development of Short DNA Biomarker and Malayan Box Turtle Specific PCR Assay

There are increasing events at global level which represent the illicit labeling, and food and medicine adulteration. The trade of food and medicine is frequently disturbed by anomalies of product safety and quality (Ali et al., 2015; Coghlan et al., 2015, 2012). Recent horse meat scandal in food chain in Europe and animal materials adulteration in Chinese medicines in Australia have brought a lot of control and supervises more frequently in connection with the authenticity of the products(Ali et al., 2015; Coghlan et al., 2015, 2012). Generally it is difficult for the consumer to identify target species and for processed products this also constitutes a challenge for control and enforcement authorities. Consequently, it is need a reliable method for regulatory body as well as industries authority to authenticate the food and medicinal products. At a globe level, several analytical methods have been highlighted in order to test the authenticity of different food and medicinal products. The most important techniques have been proved to be those which are based on the DNA analyses using electrophoresis methods.

Nowadays, species specific DNA biomarker plays an important role in the polymerase chain reaction studies for species authentication in food and medicine samples. It has gained enough attention among the scientists as well as regulatory departments; because, it gives efficient and accurate results in forensic sample analysis. Although, obtaining target PCR products depend on the primer sequence selection from conserve region of the target species genome. Therefore, primer sequence selection is among the most critical input factors in species-specific polymerase chain reaction (PCR) assay optimization and PCR assay efficiency. Because, a quality primer pairs depends on the several key factors such as primer size, GC content (%), melting and annealing temperature for assay efficiency and unambiguously DNA amplification. An inaccurate designed primers may interrupt the assay by forming secondary structure, as well as non-

specific amplification and reduce the PCR efficiency at normal annealing temperature (Abd-Elsalam, 2003). Generally, an ideal primer length and GC content(%), should be (18-30) nt and 40 to 60 % and 3 or more G or C content at 3′-end should be avoided because it has adverse effect to the primer amplification (Ali et al., 2014; Rychlik et al., 1990). Besides, selection of amplicon DNA size especially short amplicon DNA is also important factor that reflects the real content in molecular analysis; because, its extensive stability enhance the assay validity in forensic case study. Moreover, several research group have proved that short target amplicon DNA more suitable than the longer target DNA sequence due to longer DNA more susceptible to breakdown into smaller DNA fragments at harsh environment or in highly decomposed samples (Ali et al., 2012; Rojas et al., 2010). Due to extensive sensitivity and stability of the shorter DNA biomarker, it has vast application in forensic analysis, biochip and biosensor deployment. Considering all these factors, a pair of MBT-specific primers for short length PCR product (120 bp) was developed in this study and carefully analysed both theoretically and experimentally. In theoretical analysis, a whole mitochondrial DNA sequence of MBT (NC014769.1) was retrieved from the NCBI and designed a set of primer with Primer3Plus bioinformatics software to amplify unambiguously a short PCR product. Melting temperature and GC content (%) and amplicon size were also properly checked by TM calculator and Primer3Plus bioinformatics software. Theoretically, designed primer set and its amplicon(120 bp) DNA sequence were aligned with 29 potential non-target species including eight closely related species of the *Cuora* genus using a ClustalW multiple and Molecular Evolutionary Genetic Analysis Version 5 (MEGA 5) alignment program (Table 4.1). Although a total of 12 species, all of which are critically endangered (Spinks et al., 2012), belong to the *Cuora* genus, the genetic sequences of only nine species were available in NCBI. Mismatch analysis, reflects very close matching among these nine species of the *Cuora* genus. Thus, there is a high chance of *Cuora* genus detection using

the MBT specific assay and there was a need of practical cross-testing among *Cuora* species. However, due to lack samples and regulatory restriction, practical evaluation could not perform. Close observation of the sequence reflected 1–4 nt mismatches among the *Cuora* species. These few numbers of mismatch nucleotides (1–4 nt) at the primer region were probably due to habitat loss or modification, animal translocations and wildlife farming that lead to increased rates of anthropogenic hybridization and introgression between native and introduced animals within the same genus (Spinks et al., 2012). Although even a single-nt mismatch at the 3′ end of primer may prevent successful PCR amplification (Wu et al., 2009), most of the mismatches (1–4 nt) among *Cuora* species were found at the middle position in the reverse primer (Table), which have little probability of hindering the PCR amplification. This evidenced that all *Cuora* species might be detected by the MBT-specific primers. Instead of compromising interest in the assay, these findings have increased the scope of the assay by many fold since all species of *Cuora* genus are critically endangered and finding a universal PCR assay for their detection should be highly appreciated. Among the 20 tested non target species, 100% match was obtained only with the cytochrome b gene of MBT and multiple mismatches (5–33 nt) were found with the non-target species (Table 4.1). A BLAST study against non-redundant nucleotide sequences (data not shown) in NCBI reflected similar outcomes.

Further, to determine the genetically distances, a 3D plot(Figure 4.2) and pairwise table(Table 4.3) were also constructed using XLSTAT and Molecular Evolutionary Genetically Analysis (MEGA 5) software and reflects the clear distance between MBT with other species. Later, in order to observe the genetic relationship between MBT and other reptile species, a phylogenetic tree was constructed by neighbor joining method(Tamura et al., 2013) using similar sites of cytochrome b genes of 66 reptile species, of which 44 were tortoise and turtles, 10 were snakes, 8 were crocodiles, and 5

were lizards (Figure 4.1). The constructed genetic tree demonstrates a close relationship among the nine species of *Cuora* genus and a specific distance between MBT and other reptile species. This reflected the probability of *Cuora* genus detection, but eliminated the chances of other reptile detection. Because, mismatch, pairwise distance, 3D plot and phylogenetic tree results reflects the very close matching among the nine *Cuora* species (Figure 4.1 & 4.2) (Table 4.1 & 4.3)

To validate the assay, a most useful universal oligo primer set (Euk-F and Euk-R) for 141 bp length amplicon site of the eukaryotic gene (18S rRNA) was applied as internal control in this study (Rojas et al., 2010). Although, all tested samples including *Cuora* genus were belongs to eukaryotic species and applied into mismatch analysis with eukaryotic 18S rRNA gene. However, there was a challenge due to the unavailability of genetic information of the 18S rRNA gene of most the selected species in NCBI, thereby mismatch analysis result of the 18S rRNA was not reflected to the real experiment. However, among the 20 tested non target species, above 98.14-100% match was obtained only with the complete 18S rRNA gene sequence of *Cyprinus carpio, Macrobrachium rosebergii Gallus gallus, Bos taurus, Macaca fascicularis Rattus norvegicus, Ovis aries, Anas platyrhynchos,Gadus morhua, Salmo salar* sequence obtained from complete sequenced DNA, and multiple mismatches (4–29 nt) were found with the non-target species such as *Cucumis sativus, Triticum aestivum, Felis catus Sus scrofa domesticus, Capra hircus* and *Odocoileus virginianus*(Table 4.2). However, most of the mismatches were found in the middle area of the primer binding region and genetic information were partially sequenced retrieved from NBCI gene bank that reflects the importance of complete 18S rRNA genes sequence to do theoretically analysis. Finally, ClustalW, MEGA 5 and BLAST analysis results were experimentally authenticated in a practical PCR run using 20 ng templates DNA of target and non-target species extracted from raw and various processed meats. A 120 bp PCR product was obtained only from MBT and

other species did not yield any PCR products (Figure 4.2a). Amplifiable DNA in non-target species was confirmed from an endogenous control which amplified 141 bp PCR product from all species using a set of universal eukaryotic primers of 18S rRNA gene. This results reflects the quality DNA extracted from all tested eukaryotic species. The MBT-specific 120-bp PCR product was purified (Montiel-Sosa et al., 2000), sequenced, pairwise distance measured (Table 4.3) among the closely matched species, and was constructed using a maximum composite likelihood method (Ali et al., 2014; Tamura et al., 2013). The minimum distance was observed between MBT and Chinese box turtle, Yellow headed box turtle, Pan's box turtle, Zhou's box turtle, Vietnamese box turtle, Indochinese box turtle, Bourret's box turtle, Chinese three-striped box turtle, chicken, buffalo (0.06–0.37), and the maximum distance was found between MBT and cucumber (0.73). These data reflects a large genetic distance, demonstrating the unlikelihood of cross-species amplification in a practical PCR run. The number of mismatched bases in the primer-binding sites of the studied species were between 11.4% and 75%or 5 and 33 nucleotides, which made cross-species detection improbable (Table 4.1). The phylogenetic tree (Figure 4.1) constructed by a neighbour-joining method undoubtedly reflected a high degree of discrimination among MBT and other animal, fish and plant species. Several methods such as PCR (Lo et al., 2006) (12 S rRNA, 165 bp and cytochrome b, 376 bp), PCR-RFLP (Moore et al., 2003) (cytochrome b, 876 bp) and PCR product sequencing (Hsieh et al. 2008) (cytochrome b, 405 bp) have been documented for the detection of different turtle species including MBT. However, all the documented assays are based on long-size amplicons (≥ 150 bp), which frequently fragmented under food processing treatments and thus may not be suitable for meat-species detection (Ali et al., 2012; Arslan et al., 2006). Additionally, target stability of these assays was not tested under food processing conditions. Here the specificity of the newly developed PCR assay was tested under pure and admixed samples such as binary, ternary and chicken

meatball products under raw and processed states and MBT specific 120 bp product was realised under all conditions, suggesting a robustness and precision of the assay even under harsh conditions where the shortest target (165 bp) among the published report was degraded(Figure 4.4-4.6)

Table 4.1: The mismatch comparison of the Malayan box turtle specific amplicon (120bp) against tested species and eight *Cuora* species

Species name	Mismatch analysis of *Cuora amboinensis* specific amplicon(120 bp) site of cytochrome b gene		Primer Mismatch	
	Forward Primer	Reverse Primer	Forward	Reverse
Cuora amboinensis	AGCCCTTCTAACATCTCTGCTC	TATGAAACTTCGGATCATTACTAGGCACCTGCCTAATCCTTCAGATCACCACAGGAATCTTCCTGGCAATACATTA	CTCACCAGACATCTCACTAGCA	0	0
Cuora aurocapitata	.....T	.....C..T.....G.....A.....T...C...G.....A.....C..	.....	1	0
Cuora pani	.....T	.....C..T.....G.....A.....T...C...G.....A.....C..	.....	1	0
Cuora zhoui	.....T	.....C.....G.....A.....T...C.....C..	.....	1	0
Cuora picturata	.....C.....T	.....C.....T.....A.....T...C.....C..	T.....G.....	2	2
Cuora galbinifrons	.....C.....T	.....G...C.....A.....T...T.....C..	T.....T.....	2	2
Cuora bourreti	.....C.....T	.....C.....T.....A.....T...C.....C..	T.....G.....	1	2
Cuora trifasciata	.....C.....T	.....C.....A.....T...C.....C..	.....C.....	2	1
Cuora flavomarginata	.....T.....T	.....T.....C..T.....G.....T.....A.....T...C.....C..	C	2	1
Hoplobatrachus rugulosus	GC...AG.....C.T..AT.CT	.....T..T..CC.T.....ATTA.....G...A....A..TG....C..CC.A..T.....T.....C..	TA..G.T..T.CA..C.....C	9	9
Gallus gallus	GC...A..C.....TG	.....T.....C..CC..T...CAGT.....C..GACC..A...CT...C..CC.AC.A..A..C..G..C..	.A..G.....CA..C.....C	5	6
Ovis aries	GCT..A..A..T..T..AT.AT	G.....T..C..TC.C.....TT..T....TT.A.....TCTA.....CC.A.....A.....C..	TA....T....CAA..AC....	11	8
Caprus hicus	.C...A..A.....AT.AT	G.....T.....CC.C.....A.TT.....T.A..A...CTG.....CC.A.....A.....C..	TA..T.C..T.CAAT.AC....	7	10
Bos taurus	GC...A..A.....T..AT.AT	G.....T.....T..CC.C..G..A.T.....A..A...CT.....CC.A.....A.....C..	.A..T.C....CAA..AC....	9	8
Bubalus bubali	GCT..A..A.....AT.AT	G.....T..C..TC.C.....T.....G..A...CT...C..CC.A.....A.....C..	.A..T.C....CAA..AC....	9	8
Odocoileus virginianus	GC...A..A.....T..AT.AT	G.....C..TC.G.....A.TT...T.....A..A..TCTT..T..TC.A.....A.....	TA..T.C....CAAT.AC....	10	10
Sus scrofa domestica	GC...C..A.....AT.AT	G.....T..CC.CT.....T.....T.G..A...CTA.....CC.G...T.A.....	.A..T.....CAA..AC...T	8	8
Columba livia	.C...C..A.....C..CT	G...G.....T..G..CC.....TT...T.GC.AAC...A...CTA..C..CT.AC.A..C..CGC.....	.A.TG.T....CTA.C.....C	6	8
Anas platyrhychos	GCA..C.....T.....CT	G.....TC.G..C.C..T.....GGC.ACA..A...CT.....CC..C.A.....T..G..C..	.A.CG.....CA..C..T..T	7	8
Rattus rattus	GC...A.....AT.AT	G.....T..TC.....AGTA.....C..AG.A..A...CT.....CT.A.....A.....C..	.A.GT.T..T.C.AT.AC....	7	10
Macaca fascicularis	.C...GC.C...C.T..CATAT	G.....C...C.T..CACAG....T....C.T.A..A....T.....CC.AC....A.....C..	.....C...CTCC..C	11	6
Canis lupus familiaris	GCG..G.....TG	G...G.....C.....AGTA...T.G..T..A.....TCTA.....TT.A...T.A..T..G..C..	TA..T.G....CAG.CAC...T	5	11
Felis catus	GC...A.....A..ATG	.....C..CC.T.....AGT.....C.T.A..A...CT...C..CC....TT....C.....C..	.A..T.....CAAT.ACC..C	6	10
Salmo salar	GCA..A.....A.T.TG	.....T..C...C.CT.....CTA..T...GC.ACC..A...CTT..C..GC.....A..C.....C..	.A.CT.C..T.....AC...T	7	8
Gadus morhua	GC...C..C..T.....A.TATG	.....T..T..C..TC.T.....CTT...T....TAC...AC.TCTA.....C.A..T..A..C.....C..	TA.CT.....GAGAC...C	9	10
Cyprinus carpio	.CA..A..C.....A..ATG	.....T.....CC.C.....ACTA...T....TACC..A..TTTA..C..CC.A.....A..C.....C..	.A.CT.....ACC...	7	6
Macrobrachium rosenbergii	.AT..AG.....T...AT..	.....T..T..T..TC.....TA...T....AG....A...G.A...A..C.A..TT.AA.T.....C..	.A..G..A.TG.AGATT.....	7	10
Cucumis sativus	.C...GAGC..TC.TAG.TA.TGG	.GGGG.....TC.G...GC...TCTT..TT..G..A.....AGTG..T..CC.T..TT.A..T..G.....	.A....TC.TG.GGAT.....T	19	14
Triticum aestivum	.C...GAGC..TC.TAG.TA.TGG	.GGGG.....T..G...GC...T.TT..TT..G..A.....AGTG..T..CG.T..TT.A..T..G...C.	.A....TC.TG.GGAT.....T	13	10

Table 4.2: The mismatch comparison of the eukaryotic internal control (18S rRNA gene) specific amplicon (141bp) against tested species and eight *Cuora* eight species

Species name	Forward primer	Eukryotic internal control specific amplicon(120 bp) site of 18S rRNA gene	Reverse primer	Primer Mismatch	
				Forward	Reverse
18S rRNA(Eukryotic)	GGTAGTGACGAAAAATAACAATACAGGAC	TCTTTCGAGGCCCTGTAATTGGAATGAGTCCACTTTAAATCCTTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGC	GGTAATTCCAGCTCCAATAGCGTAT	0	0
Cuora amboinensis(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora aurocapitata(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora pani(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora zhoui(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora picturata(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora galbinifrons(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Cuora bourreti(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Gallus gallus(CS[b])	....	....	....	0	0
Bos taurus(CS[b])	....	.......G..C............	....	0	0
Capra hircus(PS[c])	T.....T..A.CGCC.CG.TTA..CAC..	C.C.A..G.AGA.A.C.G.GAC..AA.T.AAG.CA....CGA...G..T.A.CCATG....TT..T..A...C........CA....	...C..A.G.TTAA.CCA...TA.C	15	13
Sus scrofa domesticus(PS[c])	......C.TA.CGCC.TG.TCA..CAC..	C.CCA..G.AAA.A.C.G.GAT..AA.T.AAG.CA.G..CGA...G..T.A...TA...A.....T..A...C........CA....	...C..A.T.A.C.A....ATAG..	15	10
Columba livia(CS[b])	....	............................CG..............G.G..	....	0	0
Macaca fascicularis(PS[c])	...................GGA..--.TT	...CCATGA.A.GC..T.CC....C..TCGAG.CG..C..AAG..CGGCTAA...T...............................	....	5	0
Rattus norvegicus(CS[b])	....	....	....	0	0
Canis lupus familiaris(PS[c])	A.....C.TA.C.CC.TG.T.AG.CAC..	C.CCA..G.ATA.A.C.G.GAT..AA.T.AAG.CA....CGA...G..T.A.CCAT.C.AA.T..T..A.T.C........CA....	...C..A.G.TTAA.CCA.A.TA..	15	13
Ovis aries(CS[b])	....	....CCG..	....	0	0
Anas platyrhynchos(CS[b])	....	....G.G..	....	0	0
Bubalus bubalis(CS[b])	....	....CCG..	....	0	0
Odocoileus virginianus(PS[c])	C.....C.T..CGCC.TG.TCAG.CAC..	C.CCA..G.AGA.A.C.G.GAT..AA.T.AAG..A....CGA...G..T.A.GCTA...A.G...T..A.T.C........CA....	...C..A.G.TTAA.CCA..TTA..	16	13
Macrobrachium rosebergii(PS[c])	......................G.G....	....C....T.TGC..............CA................G.A.CA.C.A...............................	....	2	0
Gadus morhua(CS[b])	....	.............................GT..............GG.C..T.C.A...............................	....	0	0
Salmo salar(PS[c])	....	.............................A...	....	0	0
Cyprinus carpio(PS[c])	...........................T.	............................CGT.TCC....C..A.GGG......C.................................	....	1	0
Cucumis sativus(CS[b])	.........A.T............C..G.	....C--GA.T.TG...............A..A.C.......C............A...............................	....	4	0
Triticum aestivum(CS[b])	.........A.T............C..G	.G.A..-AGT.T.TG...............A..A.C.......C........ .. ???????????????????????????????	?????????????????????????	4	0
Hoplobatrachus rugulosus(SNF[a])	?????????????????????????????	???	?????????????????????????	?	?
Felis catus(PS[c])	.C.T..CT.A..G.T...C..G..T.T.	.A.ACGC.C.G.TG....G..A..C..A.G.T.A.......AG...TG.TTCC.TTGG.C.CTC..TCC...CC.A.TT.T.A.T.T	........T..AG.T.....A.GCC	11	8

Note: CS[b]: Complete sequence. SNF[a]: Not found, PS[c]: Partial sequence

Table 4.3: Pairwise distances of the Malayan box turtle (MBT) specific amplicon (120 bp) site of cytochrome b gene against corresponding sites of 29 different species by maximum composite likelihood method

	MBT	YHBT	PBT	ZBT	VBT	INDCBT	BBT	CTSBT	CBT	Chicken	Cow	Goat	Piq	Pigeon	Monkey	Rat	Dog	Sheep	Cat	Duck	Buffelo	Deer	Carp	Frog	Prawn	Cod	Salmon	Cucumber	Wheat
MBT	0.00																												
YHBT	0.09																												
PBT	0.09	0.00																											
ZBT	0.06	0.03	0.03																										
VBT	0.09	0.07	0.07	0.04																									
INDCBT	0.09	0.08	0.08	0.05	0.04																								
BBT	0.09	0.07	0.07	0.04	0.00	0.04																							
CTSBT	0.07	0.05	0.05	0.03	0.03	0.04	0.03																						
CBT	0.11	0.05	0.05	0.04	0.09	0.10	0.09	0.07																					
Chicken	0.44	0.33	0.33	0.36	0.38	0.41	0.38	0.36	0.33																				
Cow	0.38	0.34	0.34	0.34	0.33	0.34	0.33	0.32	0.39	0.29																			
Goat	0.41	0.37	0.37	0.37	0.33	0.36	0.33	0.34	0.44	0.37	0.11																		
Piq	0.37	0.34	0.34	0.37	0.35	0.35	0.35	0.33	0.39	0.33	0.13	0.16																	
Pigeon	0.50	0.50	0.50	0.49	0.46	0.49	0.46	0.44	0.52	0.31	0.44	0.39	0.38																
Monkey	0.41	0.35	0.35	0.35	0.40	0.36	0.40	0.35	0.39	0.36	0.32	0.38	0.39	0.59															
Rat	0.41	0.34	0.34	0.34	0.36	0.37	0.36	0.35	0.40	0.32	0.16	0.16	0.22	0.41	0.39														
Dog	0.44	0.39	0.39	0.39	0.37	0.41	0.37	0.39	0.43	0.34	0.32	0.32	0.32	0.43	0.51	0.30													
Sheep	0.45	0.41	0.41	0.41	0.37	0.38	0.37	0.38	0.46	0.42	0.16	0.14	0.19	0.41	0.42	0.23	0.29												
Cat	0.41	0.34	0.34	0.31	0.34	0.35	0.34	0.31	0.35	0.26	0.19	0.20	0.19	0.44	0.31	0.21	0.33	0.29											
Duck	0.42	0.41	0.41	0.38	0.37	0.39	0.37	0.34	0.39	0.25	0.39	0.44	0.37	0.36	0.42	0.43	0.40	0.44	0.32										
Buffelo	0.37	0.31	0.31	0.31	0.30	0.33	0.30	0.29	0.38	0.30	0.09	0.12	0.13	0.38	0.38	0.18	0.35	0.13	0.19	0.37									
Deer	0.44	0.40	0.40	0.41	0.37	0.37	0.37	0.38	0.48	0.39	0.12	0.13	0.20	0.43	0.42	0.20	0.29	0.14	0.23	0.47	0.13								
Carp	0.44	0.37	0.37	0.37	0.39	0.41	0.39	0.39	0.45	0.31	0.27	0.24	0.30	0.38	0.42	0.28	0.33	0.26	0.27	0.42	0.24	0.30							
Frog	0.46	0.46	0.46	0.43	0.40	0.44	0.40	0.42	0.46	0.32	0.31	0.31	0.39	0.40	0.45	0.33	0.40	0.35	0.31	0.40	0.32	0.35	0.37						
Prawn	0.43	0.48	0.48	0.48	0.48	0.46	0.48	0.48	0.53	0.51	0.47	0.47	0.50	0.56	0.64	0.45	0.50	0.45	0.55	0.61	0.45	0.51	0.50	0.38					
Cod	0.52	0.50	0.50	0.50	0.47	0.47	0.47	0.50	0.49	0.38	0.36	0.33	0.34	0.40	0.50	0.36	0.39	0.26	0.33	0.45	0.32	0.31	0.22	0.44	0.43				
Salmon	0.45	0.35	0.35	0.38	0.38	0.41	0.38	0.39	0.41	0.35	0.34	0.32	0.31	0.48	0.47	0.32	0.44	0.38	0.29	0.36	0.25	0.36	0.21	0.42	0.55	0.30			
Cucumber	0.73	0.73	0.73	0.75	0.75	0.77	0.75	0.77	0.75	0.71	0.75	0.73	0.69	0.76	0.81	0.78	0.64	0.70	0.80	0.82	0.80	0.70	0.80	0.62	0.60	0.70	0.73		
Wheat	0.71	0.70	0.70	0.74	0.72	0.74	0.72	0.74	0.74	0.73	0.75	0.73	0.69	0.74	0.83	0.78	0.64	0.70	0.82	0.82	0.80	0.70	0.86	0.64	0.60	0.75	0.78	0.03	0.00

9 species of *Cuora*

Note: *Cuora amboinensis*-Malayan Box Turtle(MBT), *Cuora flavomarginata*-Chinese Box Turtle(CBT), *Cuora aurocapitata*-Yellow-headed box turtle(YHBT), *Cuora Pani*-Pan's Box Turtle(PBT), *Cuora zhoui*-Zhou's Box Turtle(ZBT), *Cuora picturata*-Vietnamese Box Turtle(VBT), *Cuora galbinifrons*-Indochinese Box Turtle(INDCBT), *Cuora bourreti*-Bourret's Box Turtle(BBT) and *Cuora trifasciata*-Chinese Three-striped Box Turtle(CTSBT).

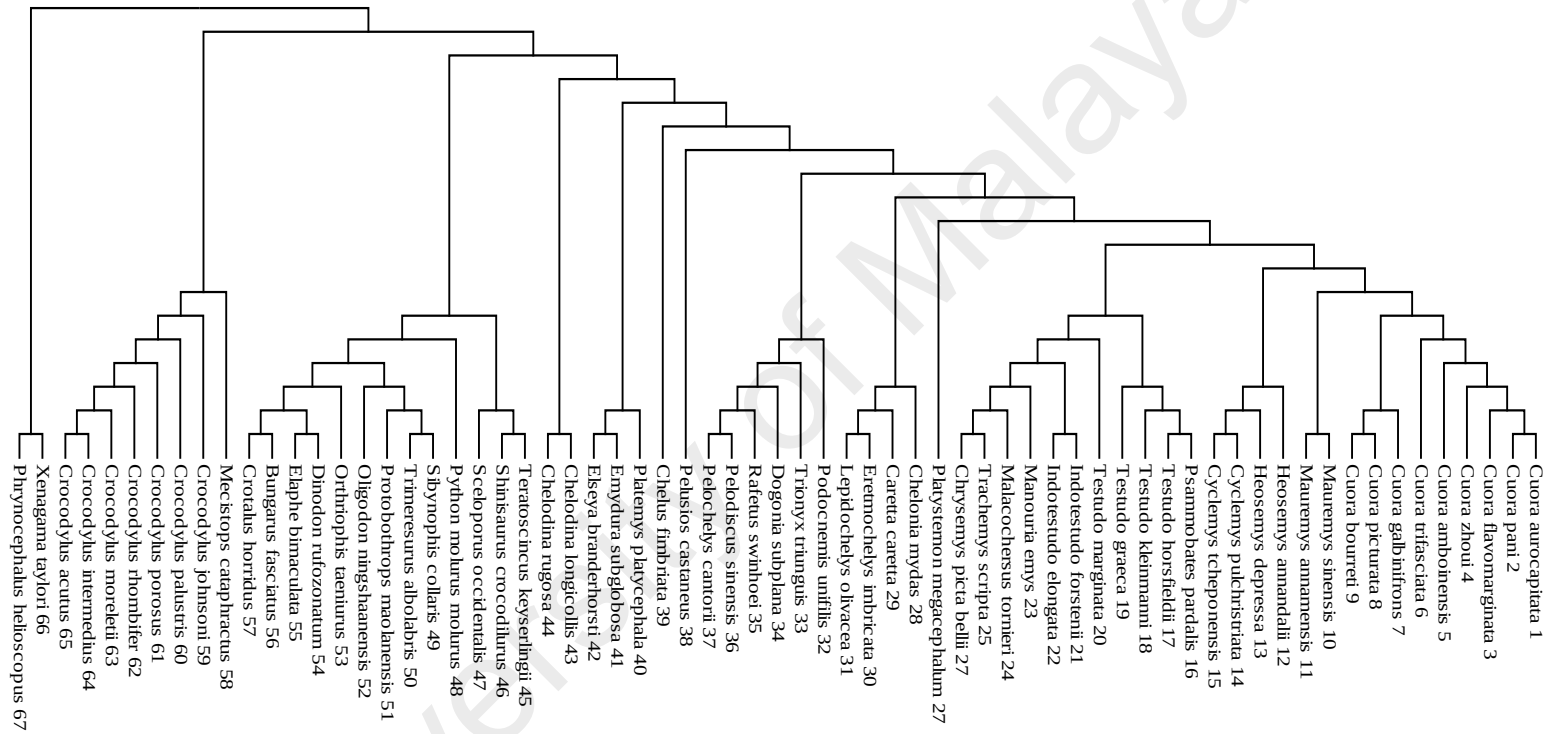


Figure 4.1: Evolutionary distance between Malayan box turtle and reptiles by neighbour joining method. Shown are 1-44: Turtles/Tortoise; 48-57: Snakes; 58-65: Crocodiles and 45-47 & 66-67: Lizards. **).** While a very close genetic gap was found among the nine species of *Cuora* genus, wide genetic distances to distinguish MBT from other reptile species was observed.

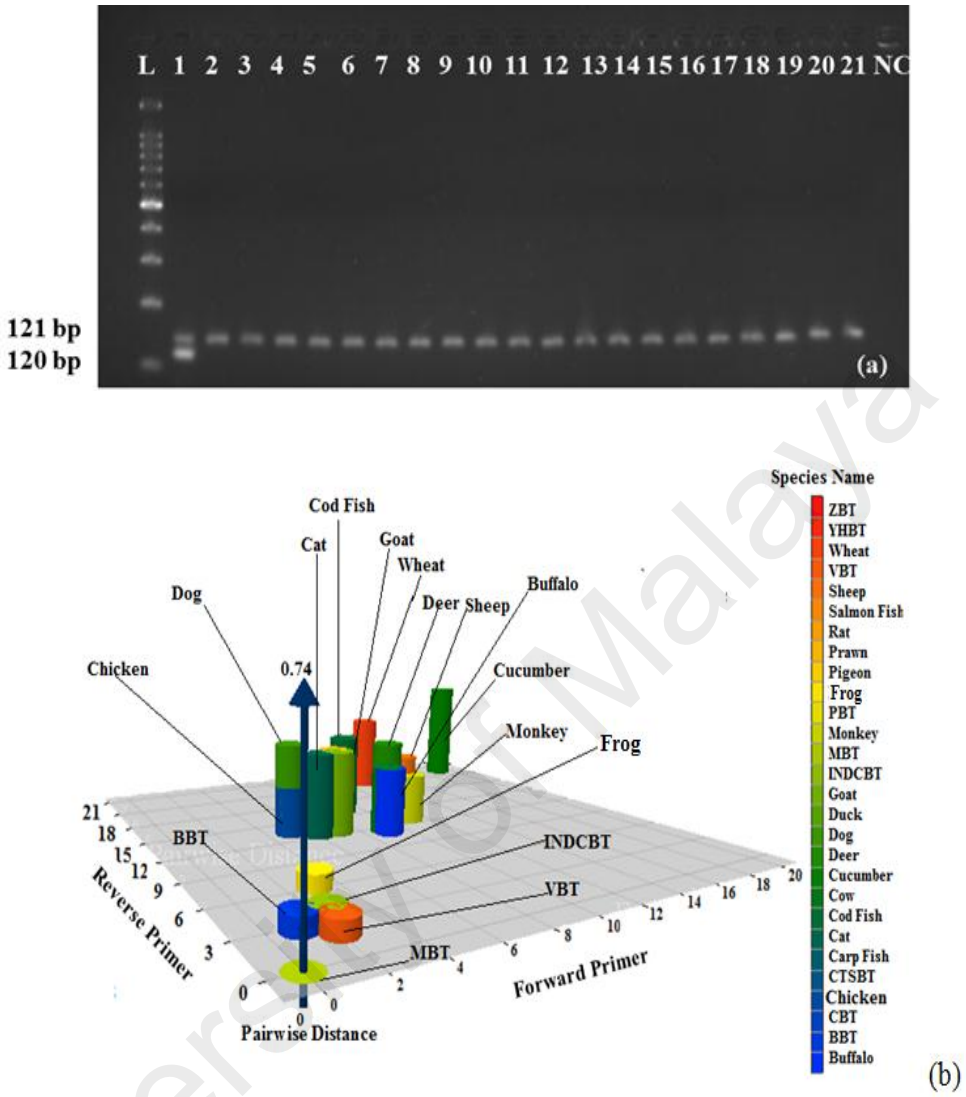


Figure 4.2: Specificity of Malayan box turtle specific primers against 20 different species and 3D plot: (a) PCR products from MBT and other 20 species; and (b) 3D plot of primer mismatch and pairwise distance of 29 species including nine *Cuora* species. In (a), *lane L*: ladder DNA (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and1100 bp); and *lanes 1–21* : PCR products from Malayan box-specific target (120 bp) and eukaryotic endogenous control (141 bp). Malayan box turtle-specific product was amplified only from Malayan box turtle (*lane 1*), but endogenous control was amplified from all species (*lanes 1–21*): Malayan box turtle, frog, chicken, sheep, goat, cow, water buffalo, deer, pig, duck, pigeon, dog, monkey, cat, rat, salmon fish, carp fish, cod fish, prawn, wheat and cucumber, respectively.

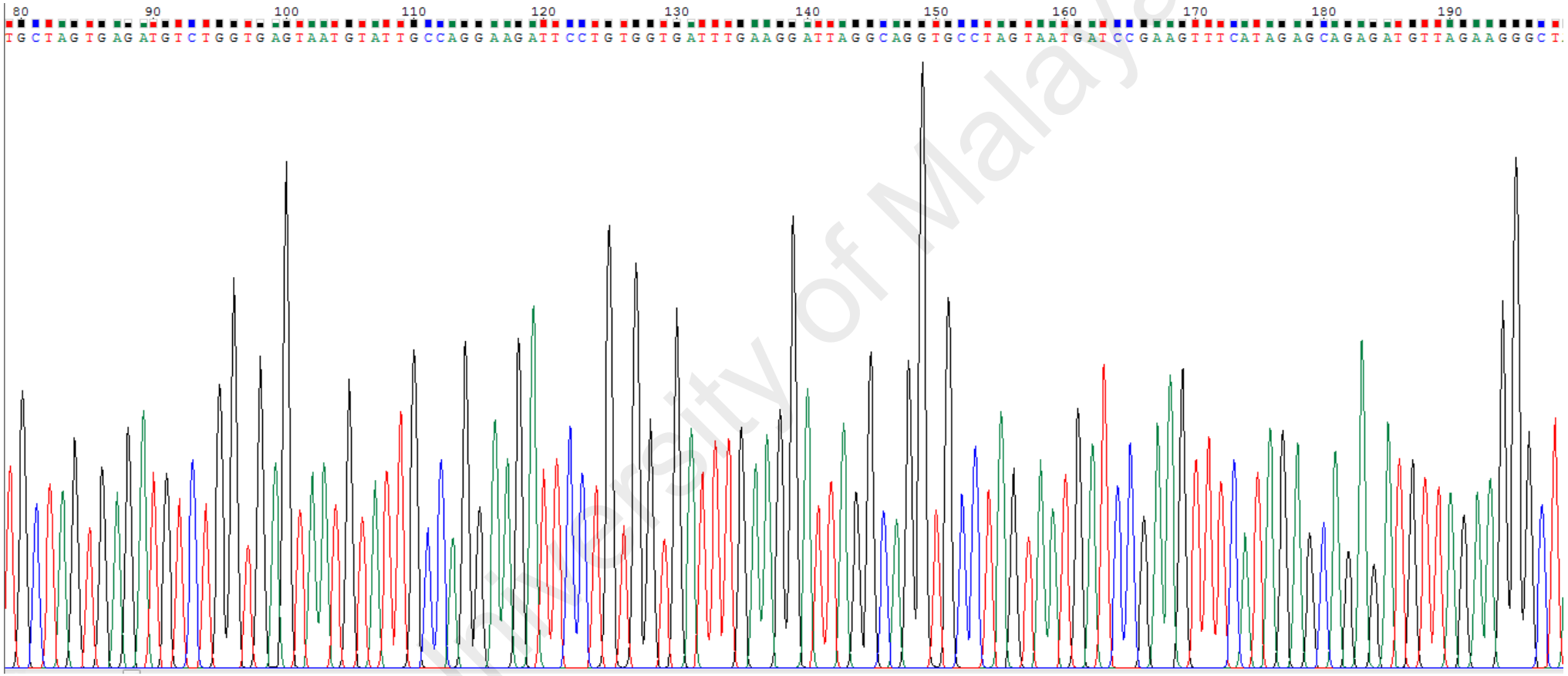


Figure 4.3: Target (MBT) PCR product (120 bp) DNA sequence and its corresponding eletropherogram

4.3 Sensitivity Test for Pure, Admixed and Commercial Meatballs and Comparison Study for Species Specific PCR assay

In food adulteration studies, beef (Mane et al., 2012), chicken (Mane et al., 2009), turkey (Rodriguez et al., 2003), goat (Karabasanavar et al., 2011) lamb and pork (Girish et al., 2004) among the livestock, and deer (Fajardo et al., 2008) and wild boar (Mutalib et al., 2012) among the wild animals have been extensively studied. However, to the best of our knowledge, no detection technique has been documented or articulated for MBT species detection under a complex background or as commercial foods. The previously published MBT-specific PCR–DNA sequencing assays were used to study evolutionary (Spinks et al., 2012), taxonomic (Schoppe, 2008a, and b) and phylogenetic relationships (Lo et al., 2006) among the closely related species. Detection sensitivity was not the main focus of the earlier assays and hence the lower limit of detection (LOD) or assay sensitivity was not defined (Spinks et al., 2012). For the first time, the present research determined PCR assay sensitivity using two approaches. Firstly, extracted DNA concentration was measured by UV-VIS spectrophotometer (Biochrom Libra S70, Biochrom) and then DNA solutions of various concentrations were prepared using 10-fold serial dilution (10, 1, 0.1, 0.01, 0.001, 0.0001 ng) with nuclease-free water starting from a 10 ng concentration of target DNA templates extracted from raw and pure meats (Figure 4.4). This method has been used by several researchers to determine PCR sensitivity for pork (Karabasanavar et al., 2014), dog (Ali et al., 2014), game birds (Fajardo et al., 2010) and many other species (Ali et al., 2014). In this study a comparative analysis of target sensitivity and stability was performed between newly designed (120 bp) and previously documented (165 bp) targets under extreme food processing treatments (Figures 4.4 and 4.6). While the new target (120 bp) was amplified from as low as 0.0001 ng DNA template, the lower limit of detection (LOD) for the published target (165 bp) was limited to 0.001 ng DNA (Figure 4.4), reflecting a better sensitivity

of the newly designed target. Secondly, a real process of meat adulteration was simulated using four sets of base-adulterated meat mixtures (BAM) (Ali et al., 2012). 1%, 0.5%, 0.1% and 0.01% (w/w) of turtle meats in an adjusted amount of beef, goat and chicken in a 100 g mixture, whereas, 1%, 0.5%, 0.1% and 0.01% turtle meat was spiked in chicken meatballs (Table 4.4). Figure 4.5(a–c) shows PCR products from turtle–beef and turtle–goat binary as well as turtle–chicken–wheat ternary admixtures and turtle meat spiked chicken meatballs. The MBT-specific PCR assay was highly sensitive since it identified as low as 0.01% (w/w) MBT meat in a mixed species background. The intensity of the PCR product obtained from the 0.01% MBT admixed suggested that the assay could detect levels much lower than 0.1%. However, since less than 0.1% adulteration is unlikely to be done for profit-making purposes (because of the huge risk of defamation and loss of goodwill), and also because it was difficult to prepare admixes below 0.1% (Razzak et al., 2015), the LOD was tested up to 0.1%. Previously, Ali et al. (2012) detected up to 0.0001 ng of swine DNA in pure state formats and 0.01% (w/w) spiked pork under a mixed background. Karabasanavar et al. (2011b) obtained 0.1% sensitivity for mutton with a 0.001 ng LOD. Mane et al. (2012) detected less than 1% adulteration of beef in admixed meat and meat products. Thus, the LOD, obtained in this study was far below those of previously reported assays.

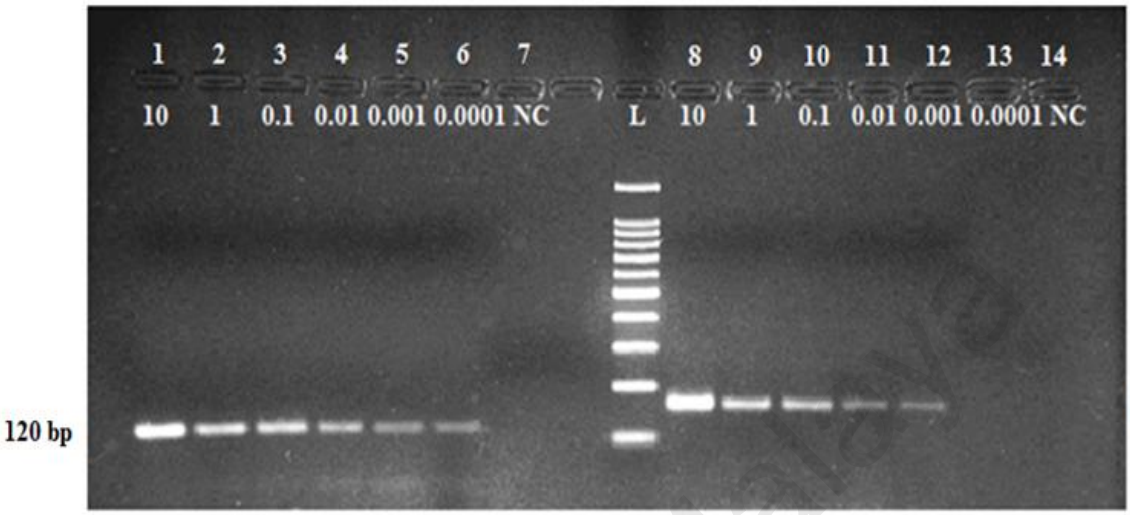


Figure 4.4: PCR sensitivity test with the newly designed 120 bp target and previously published primers with the shortest amplicon (165 bp) under raw states**.** *Lanes 1–6*: 120 bp PCR products from 10, 1, 0.1, 0.001 and 0.0001 ng DNA from box turtle meats; and *lanes 8–13* : 165 bp PCR products under identical conditions. *Lane L*: ladder DNA (DNA marker, from 100 -1100 bp); and *lane* NC: negative control. Tested LOD was 0.0001 and 0.001 ng DNA for 120 and 165 bp targets, respectively.

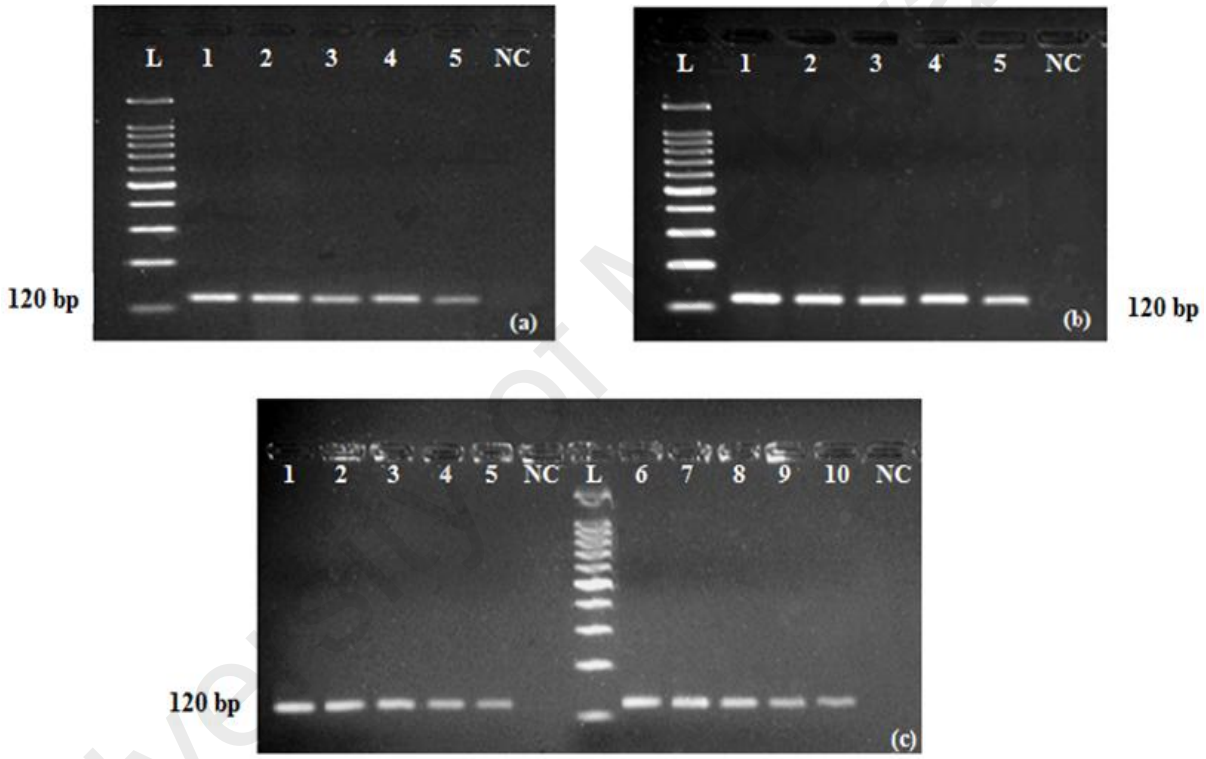


Figure 4.5: Specificity and sensitivity test of the newly designed primers from binary (Malayan box turtle and beef (a) and Malayan box turtle and goat (b)) and ternary mixtures (Malan box turtle, chicken and wheat flour) (*c: lanes 1–5*), and commercial chicken meatball products (*c: lanes 6–10*). *Lanes 1–5*: PCR products from 10%, 1%, 0.5%, 0.1% and 0.01% binary (a, b) and ternary (c) mixtures; *Lanes 6* : pure turtle meatball; *lanes 7–10*: chicken meatballs with 1%, 0.5%, 0.1% and 0.01% spiked turtle meats; *lane* NC : negative control; and *lane L* : ladder DNA(DNA marker, from 100 -1100 bp).

Table 4.4: Analysis of binary, ternary and commercial meat products using the Malayan box turtle (MBT) specific PCR assay

Samples and code of commercial meat product items	Day 1	Day 2	Day 3	MBT DNA detection	Detection Accuracy (%)
Turtle-beef binary mixture	3	3	3	9/9	100
Turtle-goat binary mixture	3	3	3	9/9	100
Turtle-chicken-wheat ternary mixture	3	3	3	9/9	100
Pure chicken meatball	3	3	3	0/9	100
Pure turtle meatball	3	3	3	9/9	100
Turtle meat spiked with chicken	9	9	9	27/27	100
Commercial chicken meatball					
A	3	3	3	0/9	100
B	3	3	3	0/9	100
C	3	3	3	0/9	100
D	3	3	3	0/9	100
E	3	3	3	0/9	100

4.4 Effect of Processing Treatments and Comparison Study for Species Specific PCR Assay

Meat samples were treated to study the effect of different thermal processing treatments on target DNA stability or degradation (Arslan et al., 2006; Haunshi et al., 2009; Ilhak & Arslan, 2007). In this study, three different heat treatment schemes, namely, boiling, microwave cooking and autoclaving, were performed. Boiling is a traditional way of cooking; while microwaving is a modern technique to heat food within a short time. Autoclaving, on the other hand, is the most appropriate method to simulate

the steaming and canning process since it cooks at very high temperature (up to 300 °C) under pressurized conditions to kill any potential microbes present in the samples.

Figure 4.6 demonstrates that DNA extracted from all the heat-treated sample was successfully amplified by MBT-specific PCR. Comparison was done between the designed primers for the 120 bp target and published primers for 165 bp targets at various processed states. Meat samples were boiled at 100 °C for 60, 90, 120 and 150 min, but no adverse effect was found for the 120 bp target, whereas the 165 bp target failed to amplify from 150 min-boiled meat samples (Figure 4. 6(a)). Previously, Haunshi et al. (2009); Karabasanavar et al. (2011) and Mane et al. (2012) studied the effect of autoclaving on DNA by treating various types of domestic meat at 121 °C for 15–30 min and found their samples were stable at this condition (Haunshi et al., 2009; Karabasanavar et al., 2011; Mane et al., 2012). Further MBT meat was autoclaved at 120 °C for 60, 90, 120 and 150 min for extensive treatment) and found 120 bp PCR products under all conditions. However, primers for 165 bp length were unable to produce target PCR product (165 bp) at 150 min of autoclaving treatment (Figure 4.6(b)). Finally, turtle meat was cooked at extreme microwaving conditions at 600–700 Watt (W) for 30 min and still 120 bp PCR products were obtained from 20 ng template DNA, whereas the published 165 bp target could not be detected at 700 W treatments (Figure 4.6(c)). This clearly reflected the fact that the new target was the better choice over the published targets (Figure 4.6). According to Rojas et al. (2010), longer than 150 bp of amplicon targets are not stable under different physical and chemical processes during food preparation (Ilhak & Arslan, 2007). Meat cooked above 700 Watt for 30 min appeared to be dried, burnt and thus was no longer suitable for consumption (almost ashes powder). Arslan et al. (2006) pan-fried beef at 190 °C for 80 min and found no PCR product at this regime when cooking was performed under non-aqueous conditions. However, the DNA extracted from the ashes was sufficiently amplified for the 120 bp MBT target, suggesting its validity even for

degraded specimens (Arslan et al., 2006). In all these assays, MBT specific 120 bp product was realised under all conditions, suggesting a robustness and precision of the assay even under harsh conditions whereas the shortest target (165 bp) among the published report was degraded.

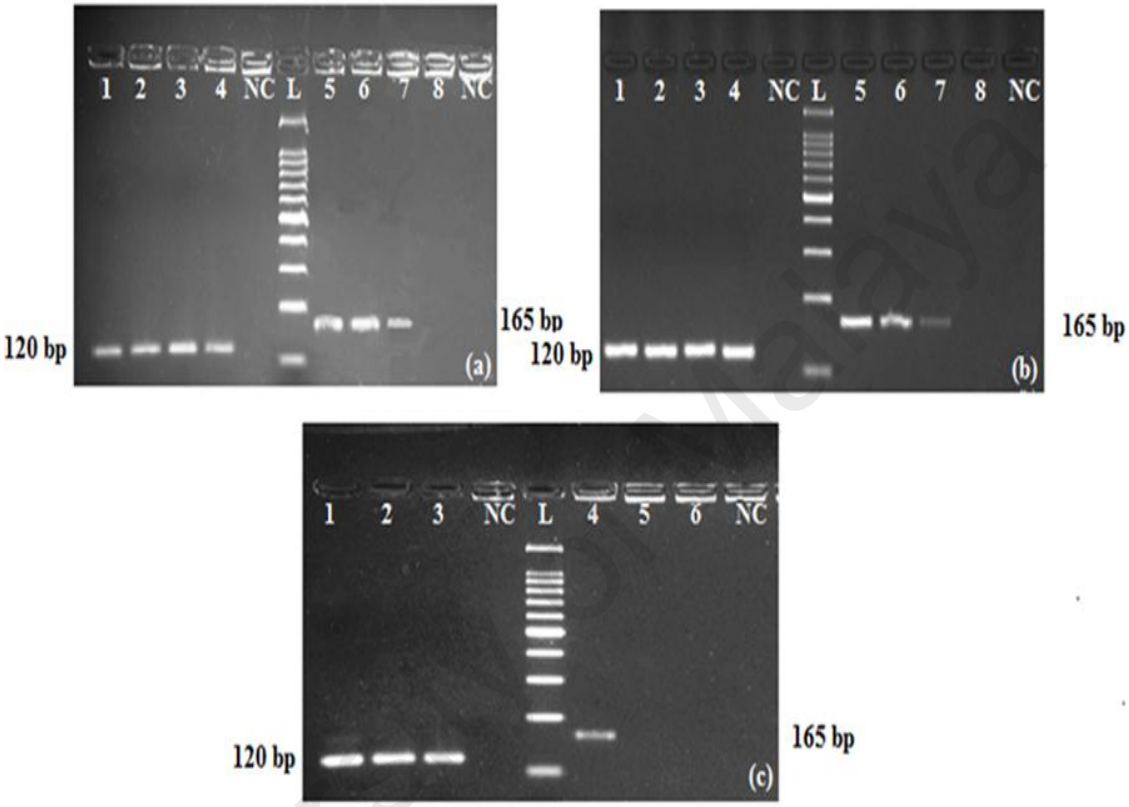


Figure 4.6: Stability test of the newly designed (120 bp) and published shortest (165bp) targets under boiling (a), autoclaving (b) and microwave cooking (c) treatments**.** In (a), *lanes 1–4 and 5–8*: boiling at 100 °C for 60, 90, 120 and 150 min, respectively. In (b), *lanes 1–4 and 5–8*: autoclaving at 120 °C for 60, 90, 120 and 150 min, respectively. In (c), *lanes 1–3 and 4–6*: microwave cooking at 600, 650 and 700 W, respectively, respectively. *Lane L*: ladder DNA (DNA marker, from 100 -1100 bp); and *lane* NC: negative control.

4.5 Target Authentication by PCR-RFLP Assay

Current species identification schemes are mainly based on DNA analysis due to some of its inherent features, such as the universal information content and excellent stability of the DNA molecule itself (Fernández et al., 2010). DNA-based approaches such as species-specific PCR is known as simple, low-cost, reliable and conclusive

techniques. It has been widely used for the authentication of species both in simplex (Karabasanavar et al., 2011) and multiplex (Ali et al., 2014; Kitpipit et al., 2014). However, it has yet to be considered a definitive analytical method due to certain "hard-to-control" features of the amplification process (Yang et al., 2005). For example, it sometimes produces artifacts due to contamination by alien DNA at a minute scale (Doosti et al., 2014), but these ambiguities or doubts could be eliminated by the verification of the amplified product through at least one of three different methods, namely, PCR-RFLP assay, probe hybridization, or target product sequencing (Maede, 2006). Probe hybridization is an attractive technique because it can detect multiple species in a single experimental run through the use of multiple labeled probes (Do et al., 2010), but this procedure requires purified DNA and is also laborious, expensive and time-consuming (Rashid et al., 2015b). In contrast, DNA sequencing is a more efficient and reliable tool, but it requires an expensive laboratory set-up and is often not suitable for the analysis of processed food under complex matrices, because co-extracted food ingredients can complicate the results (Albers et al., 2013). In contrast, the PCR-RFLP assay can overcome all of these limitations and has been widely used to authenticate the original PCR product amplified from a particular target (Fajardo et al., 2009; Girish et al., 2007). It comprises the generations of a specific fragment profile through restriction digestion with one or two endonucleases. A carefully selected restriction endonuclease cleaves the PCR product at specific recognition sites, producing a set of DNA fragments of different lengths that could be separated and visualized by gel electrophoresis (Nicolai et al., 2009), so it distinguishes the artificial PCR product from the original through the analysis of the restriction fingerprints (Davey et al., 2003; Doosti et al., 2014; Meyer et al., 1994). In this study, the restriction site of target DNA sequence was determined *in-silico* from the online-available website (http://nc2.neb.com /NEBcutter2/) by inserting the target sequence and the enzymes were selected based on the; i) restriction site in the

sequence, and ii) ability to cut at proper fragment size (at least 10 bp different)(Table 4.5)(Figure 4.7). From the analysis, *Bfa1* cutting site was verified where the enzymes that met these criteria and digested the 120-bp MBT-specific PCR product by the *Bfa1*-restriction endonuclease enzyme (New England Biolabs, http://nc2.neb.com/NEBcutter2/) because *in silico* analysis showed two restriction sites for the *Bfa1* enzyme with unique fragment lengths: 72 bp, 43 bp and 5 bp (Table 4.4) (Figure 4.7). The digested products (72 bp, 43 bp and 5 bp) obtained from the MBT-specific PCR products (120 bp) were separated and visualized by a micro-fluidic chip-based automated electrophoresis station (Bio-Rad Laboratories, Inc., USA) (Figure 4.7).

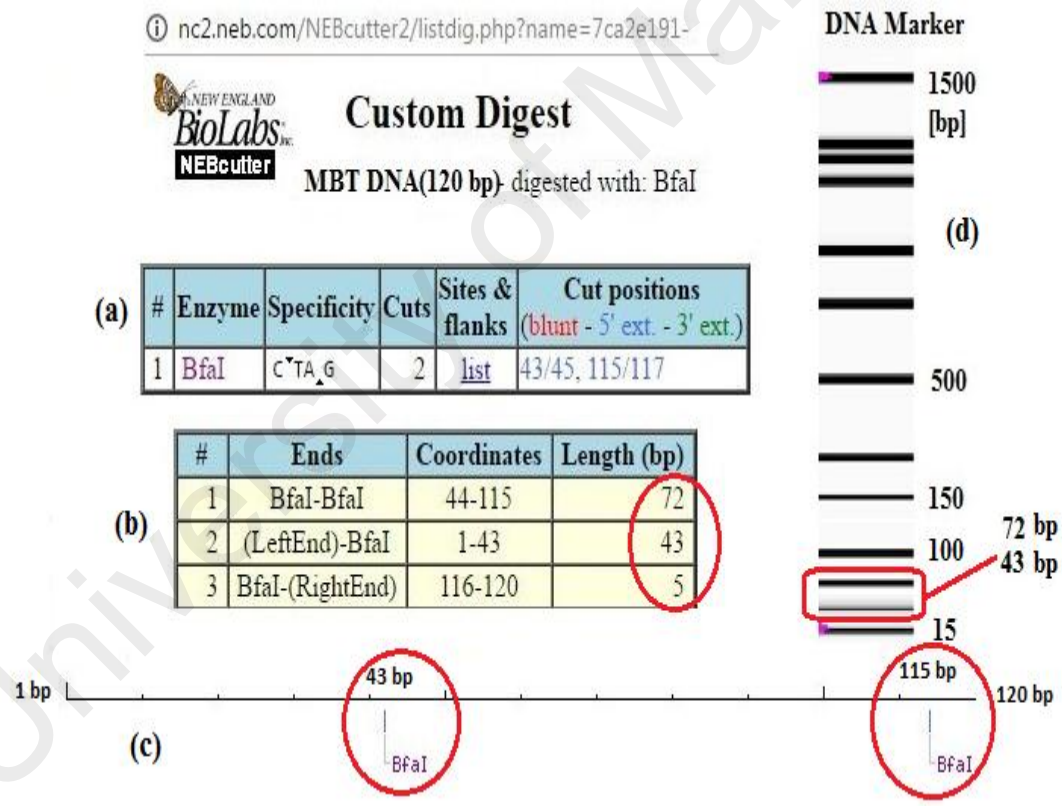


Figure 4.7: *In Silico* digestion of MBT species specific PCR (120 bp) product with *Bfa1* restriction enzyme using online (NEBcutter2 tool (http://nc2.neb.com/NEBcutter2/). In (a and c) *Bfa1* cutting cites in MBT species specific amplicon and its fragment number. In (b) fragments base pair after *Bfa1* digestion. In (d) DNA marker in gel image.

Table 4.5: In silico analysis of the MBT-specific primers against twenty study species eight species of the *Cuora* genus with Bfa1-restriction sites

Bfa1 cutting site

Species	1bp	10 bp	43 bp	99bp	115bp	120bp
MBT[a]	A G C C C T T C T A A C A T C T C T G C T C	T T T A C T A G G C A C C T	C T C A C C A G A C A T C T C A C T A G C A			
CBT[b]	. . . . . T . . . . . T	. C . . T . . . . . G . . .	 C			
YHBT[c]	. . . . . T	. C . . T . . . . . G . . .	. . . . .			
PBT[d]	. . . . . T	. C . . T . . . . . G . . .	. . . . .			
ZBT[e]	. . . . . T	. C . . . . . G . . .	. . . . .			
VBT[f]	. . . . . C . . . . . T	. C . . . . . T . .	T . . . . . G			
INDCBT[g]	. . . . . C . . . . . T	. C	T . . . . . T			
BBT[h]	. . . . . C . . . . . T	. C . . . . . T . .	T . . . . . G			
CTSBT[i]	. . . . . C . . . . . T	. C	. . . . . C			
Frog	G . . . . . C . . . T T C . . C A . C . . T	. . . . T . . . . T . . T .	T A . . G . T . . T . C A . . C C			
Chicken	G C . . . . A . . C . . . . . T	. C . . T . . . C A G T . .	. A . . G . . . . . C A . . C C			
Lamb	G C T . . A . . A . . T . . T . . A T . A T	A C . . C . . . . T T .	T A . . . . T . . . . C A A . . A C			
Goat	. C . . . A . . A . . . . . A T . A T	A C . . C . . . . . A . T T .	T A . . T . C . . T . C A A T . A C			
Cow	G C . . . A . . A . . . . . T . . A T . A T	A C . . C . . G . . A . T . .	. A . . T . C . . . . C A A . . A C			
Buffalo	G C T . . A . . A . . . . . A T . A T	A C . . C . . . . . . T . .	. A . . T . C . . . . C A A . . A C			
Venison	G C . . . A . . A . . . . . T . . A T . A T	A C . . G . . . . . A . T T .	T A . . T . C . . . . C A A T . A C			
Pig	G C . . . C . . A . . . . . A T . A T	A C . . C T . . . . . T . .	. A . . T . . . . . C A A . . A C . . . . T			
Pigeon	. C . . . C . . A . . . . . C . . C T	C C . . . . . T T .	. A . T G . T . . . . C T A . C C			
Duck	G C A . . C . . . . . T . . . . . C T	C C . G . . C . C . . T . .	. A . C G . . . . . C A . . C . . T . . T			
Rat	G C . . . A . . . . . A T . A T	A C . . . . . A G T A .	. A . G T . T . . T . C . A T . A C			
Monkey	. C . . . G C . C . . . C . T . . C A T A T	A C . T . . C A C A G . . .	. . . . . C . . . C T C C . . C			
Dog	G C G . . G . . . . . T	. . . . . A G T A .	T A . . T . G . . . . C A G . C A C . . . T			
Cat	G C . . . A . . . . . A . . A T	A C . T . . . . . A G T . .	. A . . T . . . . . C A A T . A C C . . C			
Salmon	G C A . . A . . . . . A . T . T	. C . C T . . . . . C T A .	. A . C T . C . . T . . . . . A C . . . T			
Cod	G C . . . C . . C . . T . . . . A . T A T	A C . T . . . . . C T T .	T A . C T . . . . . G A G A C . . . C			
Carp	. C A . . A . . C . . . . . A . . A T	A C . C . . . . . A C T A .	. A . C T . . . . . A C C . . .			
Prawn	. A T . . A G . . . . . T . . . A T . .	. C . . . . . T A .	. A . . G . . A . T G . A G A T T			
Cucumber	. C . . . G A G C . . T C . T A G . T A . T	. . . . G C . . . T C T T .	. A . . . . T C . T G . G G A T . . . . . T			
Wheat	. C . . . G A G C . . T C . T A G . T A . T	. . . . G C . . . T . T T .	. A . . . . T C . T G . G G A T . . . . . T			

4.6 Authentication of Limit of Detection (LOD) Assay by PCR-RFLP Assay

The limit of detection (LOD) of an assay is a critical aspect of the determination of marginal-level targets in adulterated foodstuffs, and the LOD values for several types of animal species, such as beef (Mane et al., 2012) chicken, turkey (Mane et al., 2009; Rodriguez et al., 2003), goat (Karabasanavar et al., 2011), lamb and pork (Girish et al., 2004), deer (Fajardo et al., 2008) and wild boar (Mutalib et al., 2012), have been defined for food authenticity studies. However, the Malayan box turtle is a relatively new species in food chains, so it's LOD has not been defined under various food matrices. All of the previous assays for the detection of MBT and other turtle species have described the evolutionary origins (Spinks et al., 2012), taxonomy (Schoppe, 2008b) and phylogenetic (Lo et al., 2006) relationships among the closely related species, so this study addressed this research gap by determining the LOD in two different ways. Firstly, the concentration of the extracted DNA was measured by UV-VIS spectrophotometer at a relatively high concentration (100 ngμL^{-1}) (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK), and then various concentrations (10, 1, 0.1, 0.01, 0.001, 0.0001 ng) were made by dilution in nuclease-free water because inaccuracies and inconsistencies have been observed in spectrophotometric readings using low concentrations. A 10-fold serial dilution method has been used by several studies to determine the PCR sensitivity for porcine, mutton (Karabasanavar et al., 2011), monkey (Rashid et al., 2015) and cat species (Amin et al., 2015), and in this study, the amplified PCR product was found from an amount as low as 0.0001 ng DNA extracted from pure meat (Figure 4.8). Previously, Ali et al. (2012) and Raifana et al. (2015) detected 0.0001 ng of porcine and monkey DNA in pure meat. The sensitivity of this newly designed PCR assay for MBT detection indicated that the actual LOD was higher than those in published reports (Ali, et al., 2015a).

The aim of the study was to detect a minimal amount of adulterated MBT in raw and processed meat products, therefore two sets of binary mixed-meat products (MBT-beef and MBT-goat) and one set of a ternary mixed-meat product (MBT-chicken-wheat) were made to emulate the most likely forms of adulteration in processed foods. In the admixtures, 10, 1, 0.1 and 0.01% of the MBT meats were spiked in a balanced amount of deboned beef and goat, whereas 10, 1, 0.1 and 0.01% of MBT meat were added to chicken and wheat flour at ratios of 20:80:100, 2:98:100, 0.2:99.8:100 and 0.02:99.98:100 (Figures 4.8 and 4.9). Figure 4.8 shows the MBT-specific PCR products from the binary (MBT-beef and MBT-goat) and ternary admixtures (turtle-chicken-wheat flour), and all of these results clearly supported the high sensitivity and specificity of the MBT-specific primers developed in this study because they amplified the specific target product from admixtures containing MBT meat in concentrations as low as 0.01% (w/w) under complex matrices. From a practical point of view and also based on published reports, it is clear that meat products that are adulterated by less than 0.1% do not yield remarkable profits in the food industry, and it is very difficult to prepare admixed samples with less than 0.1% contamination. However, the findings obviously suggested that this assay could be used to detect adulterations much lower than 0.1% (w/w). Previously, Rashid et al. (2015) detected 0.1% (w/w) monkey DNA under various admixed states, and Ali et al. (2012) identified up to 0.01% (w/w) pork under different food matrices. In contrast, Karabasanavar et al. (2011) obtained PCR product from 0.1% (w/w) mutton mixed with cattle, buffalo, goat pig, and chicken and Mane et al. (2012) found less than 1% beef adulteration in admixed meat and meat products. Thus, the LOD established in this study was far below those from the published reports. Furthermore, to verify the specificity, the PCR products from the 0.01% (w/w)-admixed samples were restriction digested with *Bfa1*-restriction endonuclease, and distinctive DNA fragments (72, 43 & 5 bp) or identifiable MBT fingerprints were obtained (Figures 4.8 and 4.9). All these digested

results by PCR-RFLP analysis strongly reflects the assay validity. Due to the inability of the Bio analyzer to detect the 5 bp fragment, only 72 bp and 43 bp were found in the gel image. The endogenous control specific to eukaryotic 18S rRNA does not contain the *Bfa1* cutting site, so a clear 141-bp product was detected in all admixture samples.

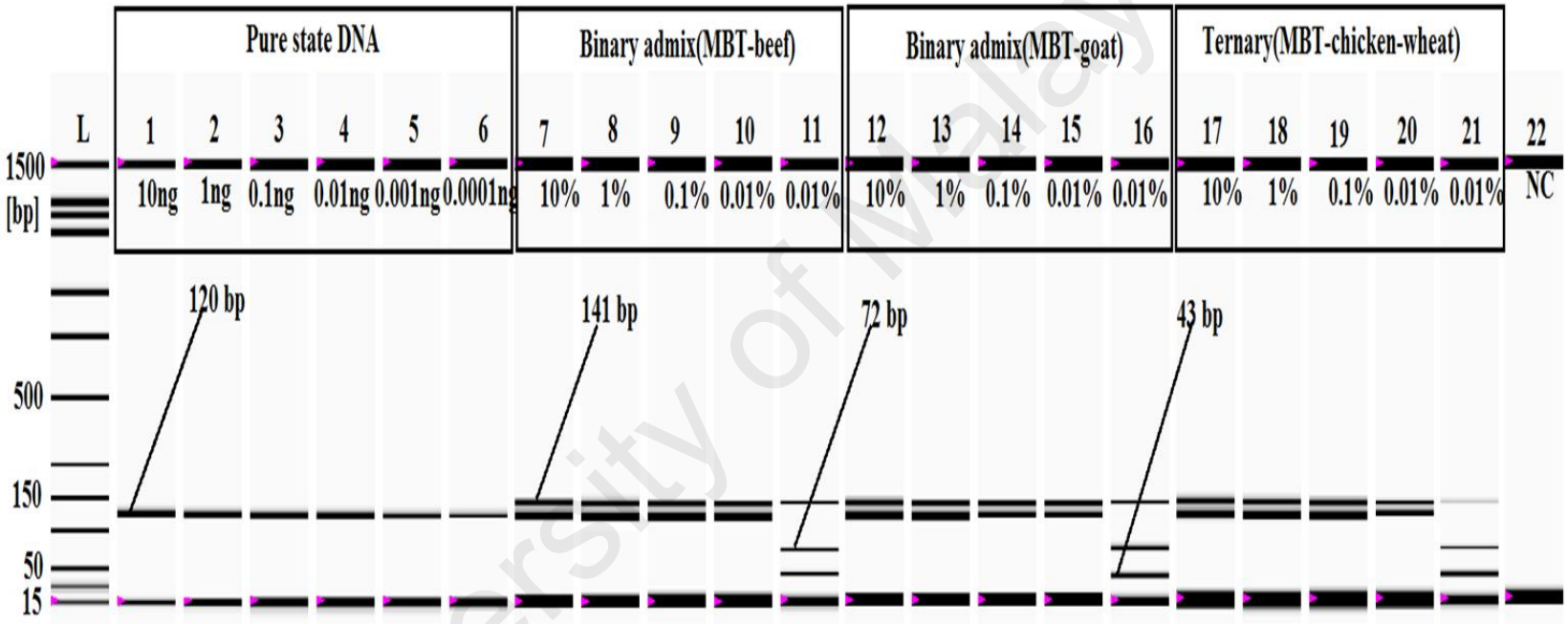


Figure 4.8: Sensitivity analysis under pure, binary and ternary admixtures. In the gel image, *lanes 1–6:* PCR products from 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng MBT DNA, respectively. *Lanes 7-10:* (MBT and beef) and *lanes 12-15:* (MBT and goat) represent PCR products from 10, 1, 0.1, and 0.01% MBT-adulterated binary admixtures, respectively. The *Bfa1* digestions of the MBT-specific PCR product realized from 0.01% MBT admixed with beef and goat are shown in *lanes 11 and 16*, respectively. In the gel image, *lanes 17-20:* represent PCR products from the ternary mixture (MBT, chicken and wheat flour) containing 10, 1, 0.1, and 0.01% MBT meat, and *lane 21* shows the *Bfa1* digestion of the MBT-specific PCR products obtained from the ternary admix containing 0.01% MBT. *Lane L*: ladder DNA, and *lane 22*: negative control (NC).

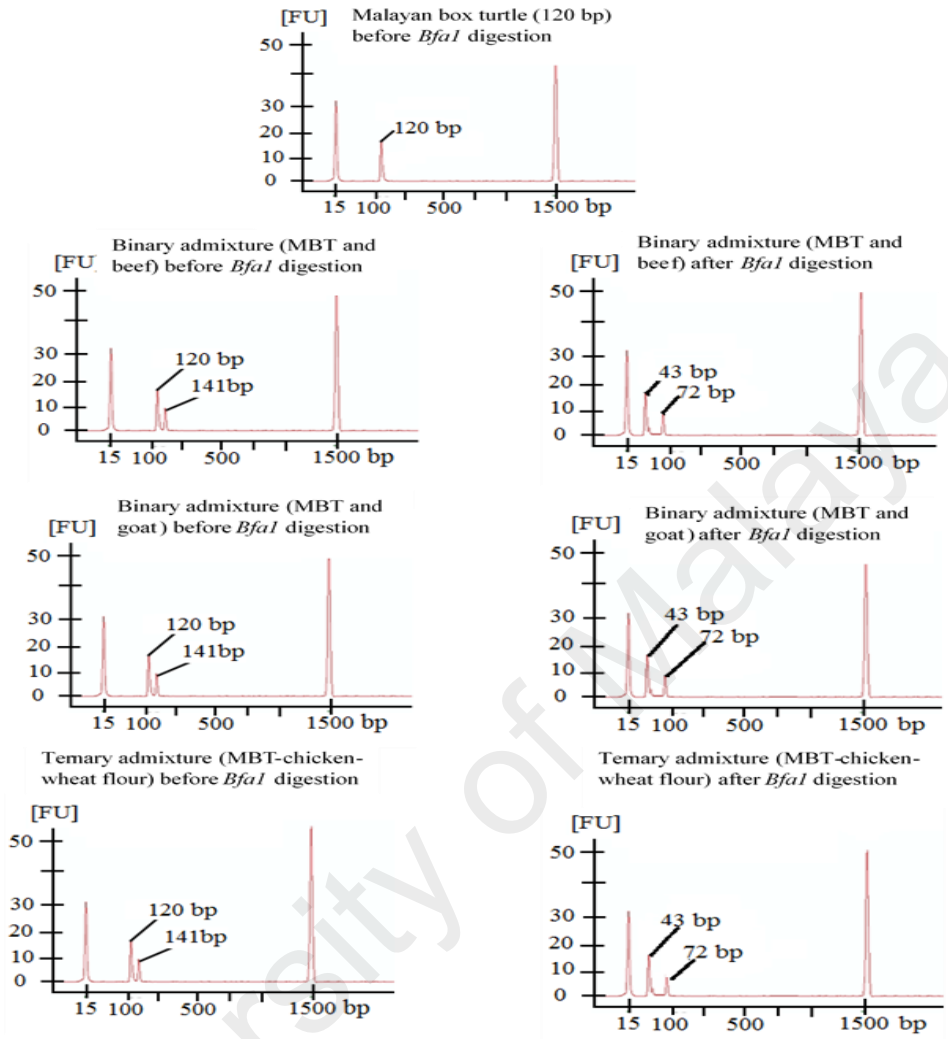


Figure 4.9: Sensitivity analysis under pure, binary and ternary admixtures using MBT specific 120 bp by PCR-RFLP assay are shown in electropherogram are demonstrated by respective labels.

4.7 Validation of Stability Assay by PCR-RFLP Assay

Target stability is a key point to consider in the validation of any analytical results, especially for forensic samples where less stable analytes are frequently decomposed resulting in false negative identifications. Published reports reflect the significant effort that scientists have put into the development of short-length DNA targets, which are thermodynamically more stable than the longer targets and hence persist through extreme stresses that often break down longer DNA markers (Ali et al., 2015). In several instances, DNA barcoding has failed to recover longer targets from degraded specimens because of

the longer and fragile attributes of the targets, which are often more than 600 bp in length (Frézal & Leblois, 2008). Furthermore, the interference of nuclear integrated mitochondrial pseudogenes (numt) seriously compromises the reliability of DNA barcoding in species authentication (Song et al., 2008), so instead of targeting universal markers, species-specific short amplicon-length PCR assays and short-length barcode markers (Kress et al., 2005) have been prioritized over the years. In food forensic analysis, various thermally treated samples have been used to benchmark target biomarker stability (Arslan et al., 2006; Haunshi et al., 2009), but because there are very few PCR assays available for the analysis of MBT in the food chain, there is a gap in the evaluation of the stability and robustness of the MBT-specific targets under various food processing conditions. This study confirmed the stability of the MBT target through three different thermal treatment schemes, namely, boiling, microwave cooking and autoclaving. Boiling simulates traditional cooking, in which meat is cooked in boiling water at 100 °C for a fixed amount of time (Ali et al., 2015a), and over the years, steam cooking or boiling have increased in popularity over pan frying for health reasons (Figure 4.6). In contrast, microwave cooking is a modern technique that heats and cooks food through exposure to electromagnetic radiation in the microwave spectrum (Rashid et al., 2015), while autoclaving is the most appropriate method to simulate steaming and canning-based meat processing because it cooks at a high temperature (121 °C) under pressurized conditions to kill any potential microbes in the samples. Extreme autoclaving (2.5 h at 120 °C and 45 psi) has been used as a benchmark for target DNA stability in several studies (Ali, et al., 2012, 2014 and 2015). In this study, the MBT-specific target was obtained from all of the thermally processed samples (Figures 4.10 and 4.11); when MBT meat was boiled at 100 °C for 60, 90, 120 and 150 min, no adverse effects on the amplification cycle were found (Figures 4.6 and 4.10). Previously, Ali et al. (2012) detected a 109-bp porcine target after boiling for 2.5 h, and Haunshi et al. (2009), Karabasanavar et al. (2011) and Mane

et al. (2012) identified target species after autoclaving various types of domestic meat at 121 °C for 15–30 min. Here, MBT meat was autoclaved at 120 °C for 60, 90, 120 and 150 min (extensive treatment) under 45 psi and obtained targeted PCR products from all of the treated samples (Figures 4.6). Finally, extreme microwave cooking was done at 600-700 W for 30 min, and clear a band for the desired product (120 bp) was obtained (Figures 4.6 and 4.10). This study showed that short-length targets are more stable than longer ones, but when meat samples were cooked in a microwave at 700 W for 30 min, the meat turned into ash that was no longer suitable for consumption. Previously, Arslan et al. (2006) failed to amplify the target product from pan-fried beef meat at 190 °C for 80 min, but in this assay, target amplification from ash-like specimens was a clear indication that this method could be used to detect the MBT target from highly decomposed specimens, which are frequently found in forensic samples. Furthermore, to verify the specificity and stability assay, the PCR products from the boiled (150 min), autoclaved (150 min) and microwave cooked (700 Watt) samples were restriction digested with *Bfa1*-restriction endonuclease, and distinctive DNA fragments or identifiable MBT fingerprints were obtained (72, 43 bp) (Figures 4.10 and 4.11). This evidence reflects the higher specificity and stability of the short length DNA amplicon in harsh environments and the designed primers also showed a higher sensitivity.

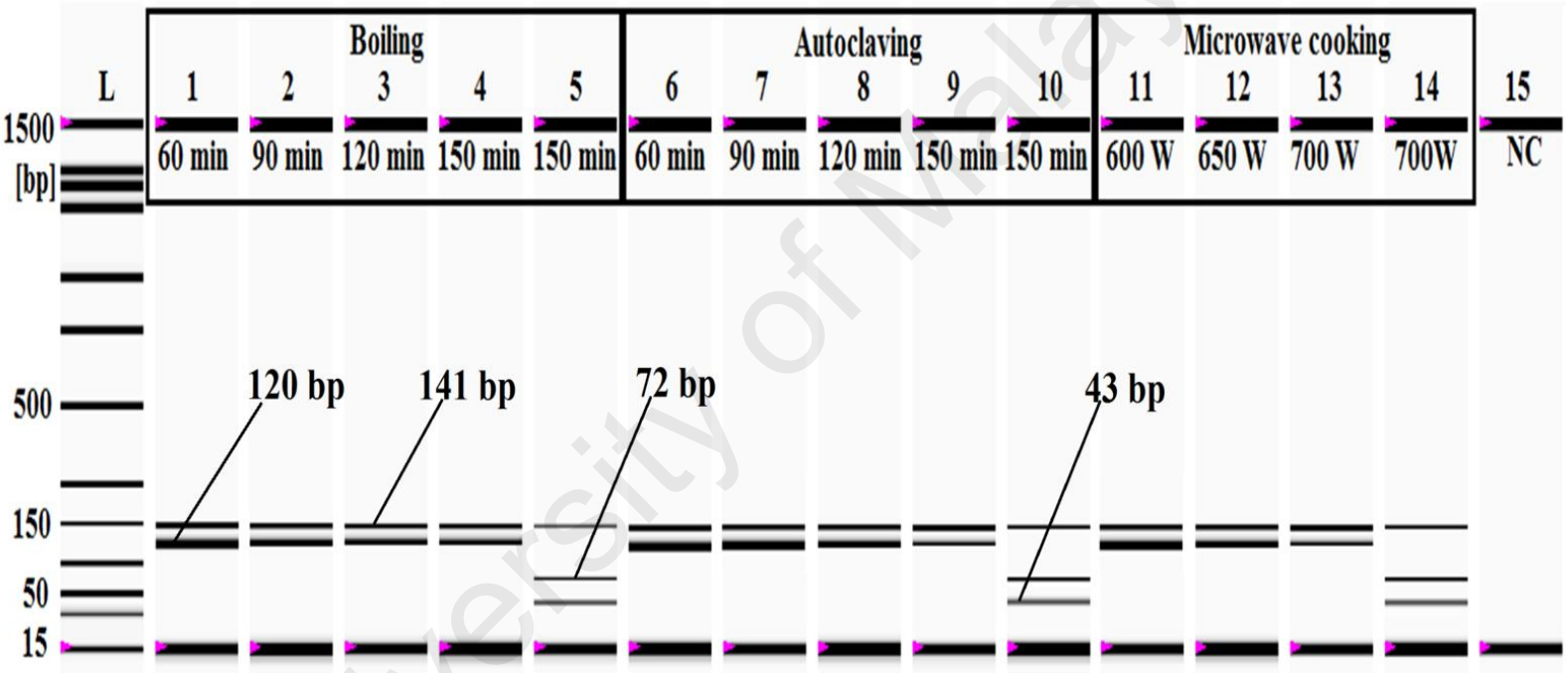


Figure 4.10: Stability analysis of the MBT-specific target DNA (120 bp) under boiling, autoclaving and microwave cooking. In the gel image, *lanes 1-4:* are boiling at 100 °C for 60, 90, 120 and 150 min, respectively, and *lanes 6-9* are autoclaving at 121° C for 60, 90, 120 and 150 min under a pressure of 45 psi. *Lanes 5* and *10:* represent *Bfa1* digestion from samples boiled (*lane 5*) and autoclaved (*lane 10*) for 150 min, respectively. In the gel image, *lanes 11-13:* represent microwave cooking at 600, 650 and 700 W for 30 min, respectively, and *lane 14* is the *Bfa1* digestion of the microwave cooked sample at 700 W. *Lane L*: ladder DNA, and *lane 15*: negative control(NC).

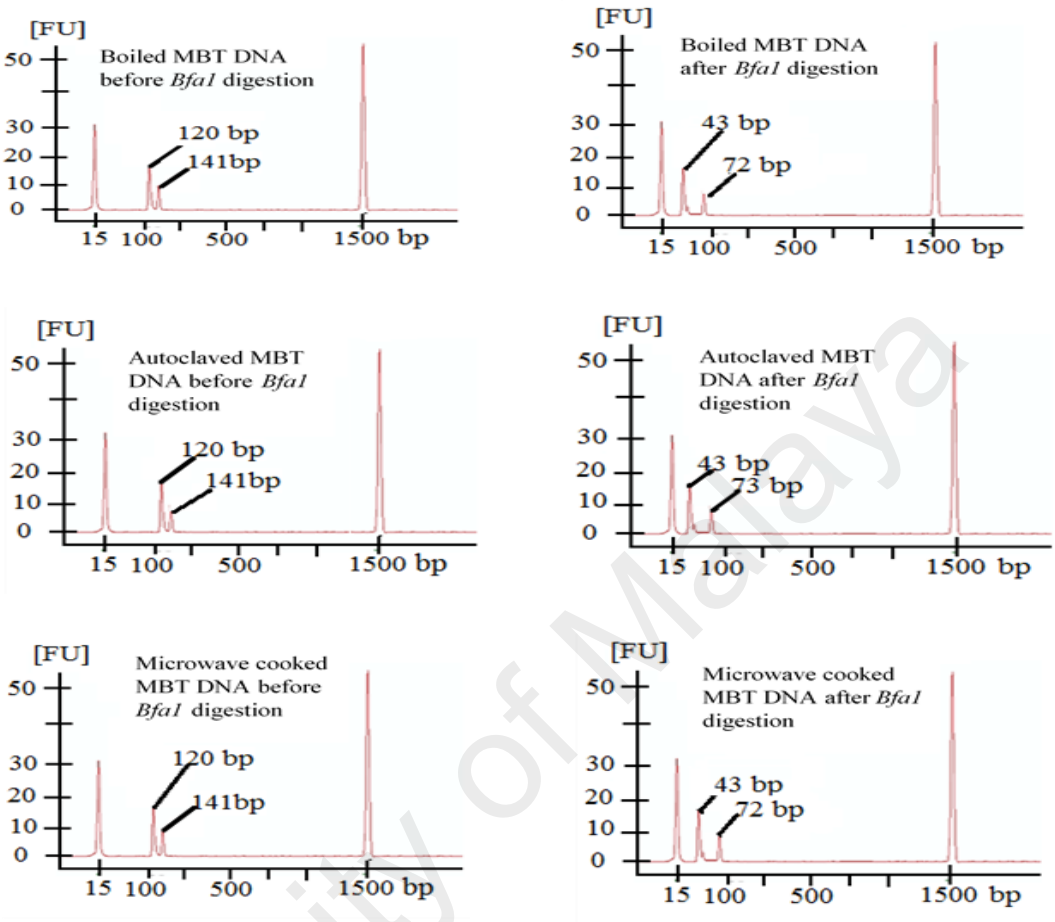


Figure 4.11: Stability analysis of the MBT-specific target DNA (120 bp) under boiling, autoclaving and microwave cooking are shown in electropherogram are demonstrated by respective labels.

4.8 Evaluation of Meat Products and Commercial Products Screening

To establish the validity, stability and reliability of the developed PCR-RFLP assay, various commercially available meat products including beef and chicken meatballs, burgers and frankfurters, which are widely consumed across Malaysia, Indonesia, China and most of the world (Rahman et al., 2015; Rahman et al., 2014; Razzak 2015; Rohman et al., 2011) were experimentally screened. Eight (8) commercially available Halal-branded chicken and beef meatballs (A-H), seven (7) chicken and beef burgers (I-O) and eight (6) chicken and beef frankfurters (M-T) were collected from four different Malaysian outlets located in the states of Kuala Lumpur and

Selangor in Malaysia on three different days (Table 4.6). The adulteration of meat products was simulated in dummy commercial meat products following Ali et al. (2012), Rahman et al. (2014), and Razzak et al. (2015), in which dummy meatball, burger and frankfurter products were spiked with 10, 1, 0.1 and 0.01% of ground MBT meat. Additionally, the 1% MBT-spiked meat products were subjected to autoclaving for 2.5 h at 120 °C under 45 psi (Figure 4.12a, b and c) because this treatment is known to break down DNA. The MBT-specific PCR product was amplified from all levels of adulteration including the 1% autoclaved samples. However, no MBT-specific PCR product was observed from the meat products collected from commercial sources (Table 4.6). To confirm the origin of the amplified target, the PCR products were digested with *Bfa1*-restriction endonuclease enzyme, and distinctive MBT fingerprints composed of 72, 43 and 5-bp oligo fragments were obtained from all of the meat products, but only the 72 and 43-bp products were detected in the gel image due to the inability of the currently available analytical machine to detect a 5-bp product (Figures 4.12 a, b and c and 4.13). These digestion results completely matched the *in silico* digested fragments, so the findings strongly supported that the PCR-RFLP assay was specific to MBT and suitable for identifying less than 0.01% (w/w) MBT meat adulteration in commercial food. The endogenous control specific to eukaryotic 18S rRNA does not contain the *Bfa1* cutting site, so a clear 141-bp product was detected in all samples. Thus the analysis of commercial products screening are also presented in Table 4.6. The endogenous control specific to eukaryotic 18S rRNA does not contain the *Bfa1* cutting site, so a clear 141-bp product was detected in all samples (Ali et al., 2015). All of the experiments were carried out in triplicate by three independent analysts on three different dates to confirm the reproducibility of the results (Ali et al., 2012). The experimental and theoretical specificity, stability and sensitivity of the developed assay indicated that it was a reliable and rapid technique for the authentication of MBT adulteration in the food chain.

Malaysia is committed to developing a Halal hub industry and to being a competitive partner in the global Halal food business, so the absence of MBT meat in Malaysian food was quite encouraging.

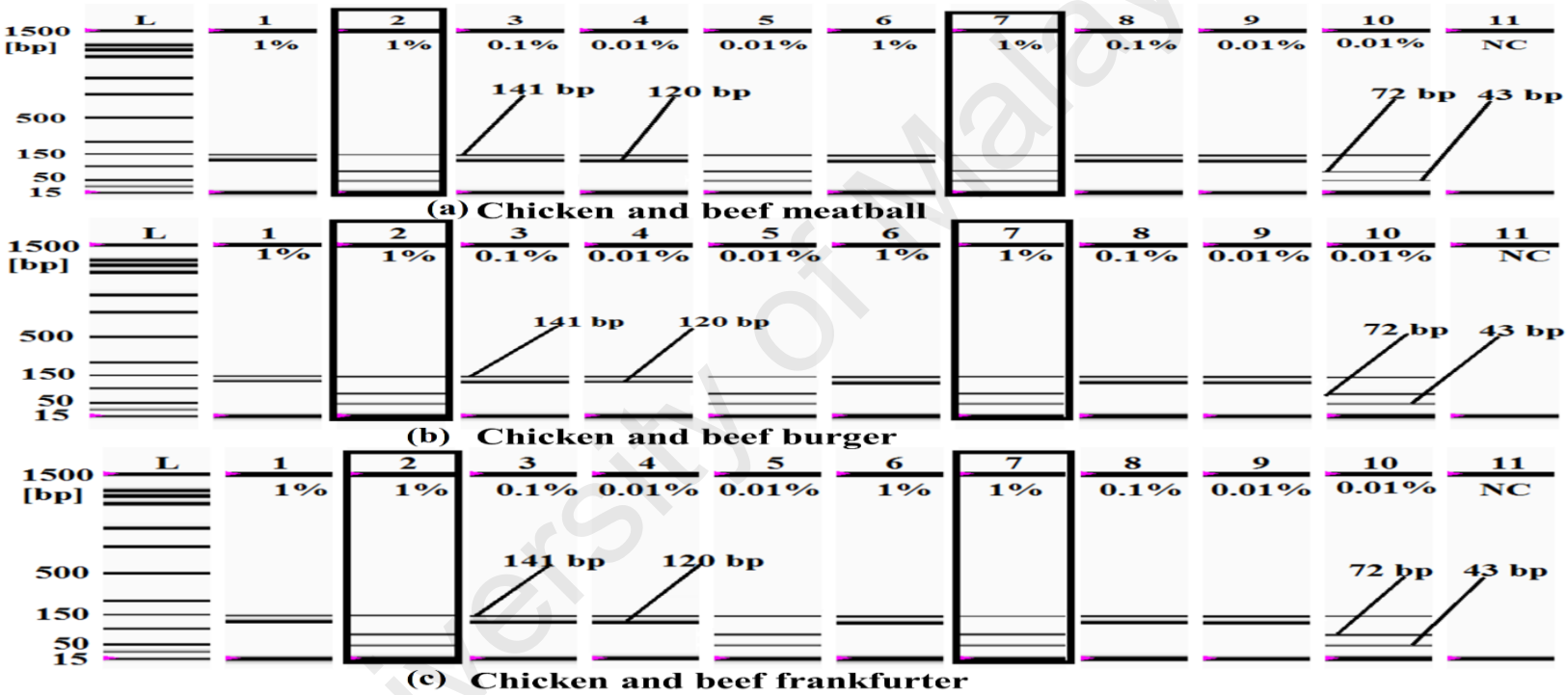


Figure 4.12: MBT meat screening in model meat products. In all figures (a-c), *lanes 1, 3, 4 and 6, 8, 9* are PCR products from 1%, 0.1% and 0.01% MBT meat spiked chicken (*lanes 1, 3 and 4*), beef (*lanes 6, 8, 9*) meatballs (a), burgers (b), frankfurters (c), respectively. *Lanes 5 and 10:* represent *Bfa1* digest of PCR products obtained from of 0.01% MBT meat spiked chicken and beef meatballs (a), burgers (b), frankfurters (c). *Lanes2 and 7* are the *Bfa1* digest of PCR products obtained from of 1% MBT meat spiked chicken and beef meatballs (a), burgers (b), frankfurters (c) after autoclaving treatment. *Lane L*: DNA ladder and *lane11*: Negative control (NC).

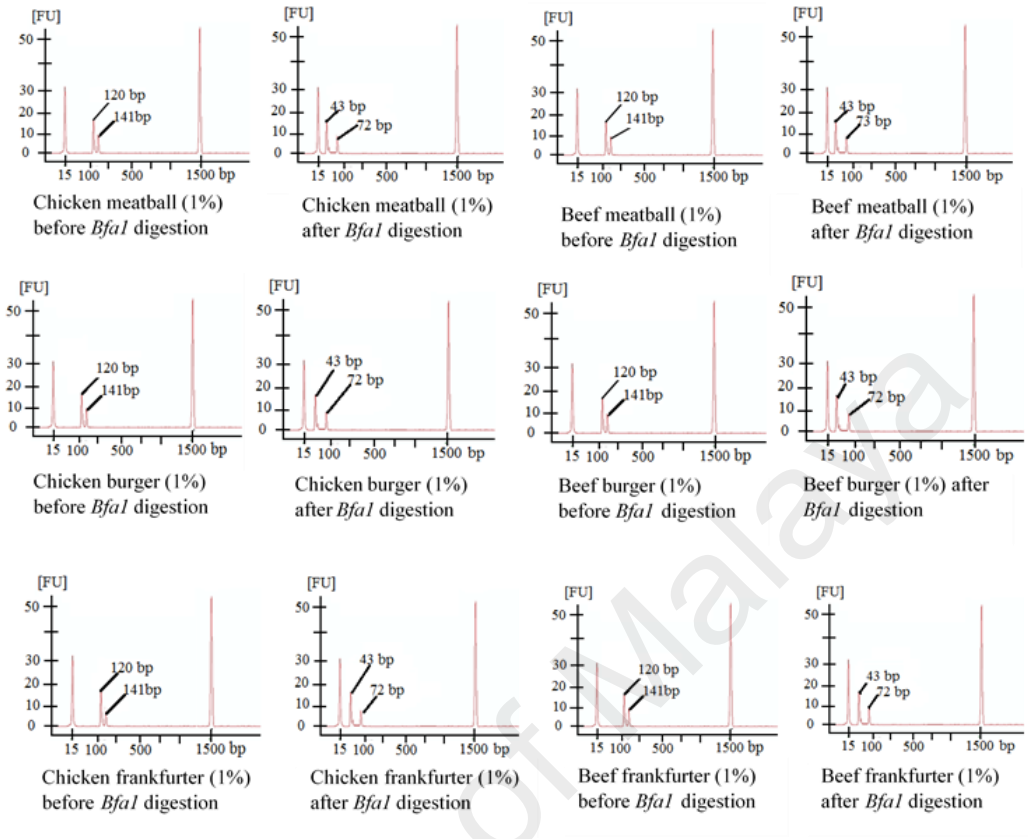


Figure 4.13: MBT meat screening in model meat products and validated by PCR-RFLP assay, showing 120 bp PCR product before and after *Bfa1* digestion

4.9 Product Authentication by PCR-RFLP Assay

In this study, MBT-specific PCR product was digested by the *Bfa1*-restriction endonuclease enzyme (New England Biolabs, see http://nc2.neb.com/NEBcutter2/) because *in silico* analysis showed two restriction sites for the *Bfa1* enzyme with unique fragment lengths: 72, 43 and 5 bp. The digested products (72, 43 and 5 bp) obtained from the MBT specific PCR products (120 bp) were separated and visualized by a micro-fluidic chip-based automated electrophoresis station (Bio-Rad Laboratories, Inc., USA) (Figures 4.8–4.13), but because the 5-bp fragment size was below the resolution capacity of the instrument (bioanalyzer), it remained undetected in the gel image (Figures 4.8–4.13). The endogenous target (141 bp) was amplified from all of the analyzed samples, which indicated the presence of good quality DNA and thus ruled out the probability of any false

negative results. Two sets of binary mixed-meat products (MBT-beef and MBT-goat) and 1 set of a ternary mixed-meat product (MBT-chicken-wheat) were made to emulate the most likely forms of adulteration in processed foods. In the admixtures, 10, 1, 0.1 and 0.01% of the MBT meats were spiked in a balanced amount of deboned beef and goat, whereas 10, 1, 0.1 and 0.01% of MBT meat were added to chicken and wheat flour at ratios of 20:80:100, 2:98:100, 0.2:99.8:100 and 0.02:99.98:100 (Figures 4.8 and 4.12). The MBT-specific PCR product (120 bp) was obtained from all forms of adulteration at a tested limit of detection of 0.01% MBT meat; all of the PCR products were validated by digestion with the *Bfa1* enzyme (Figures 4.8 and 4.12).We further screened for MBT DNA in various types of food products using laboratory prepared dummy meat products and commercially available meat products (Tables 4.5). Lower-value meats are commonly used to replace higher-value meats to increase economic profit (Ali et al., 2012), so the most popular meat products, such as chicken and beef meatballs, burgers and frankfurters, were prepared following Ali et al. (2012), Rahman et al. (2014) and Razzak et al. (2015). All of these meat products were spiked with 10, 1, 0.1 and 0.01% MBT meat to demonstrate the common forms of adulteration in the commercial food industry. However, mixing with less than 1% of low-value meat does not yield any significant economic benefit relative to the great risk of defamation (Razzak et al., 2015). Consequently, we determined that the sensitivity of this assay using 1% MBT-adulterated autoclaved dummy meats was suitable for the detection of trace level adulteration in processed food. The RFLP results indicated clearly unique fragment patterns specific to MBT in 1% MBT-adulterated meat products (Figure 4.12). Finally to establish the validity, commercially available meat products including beef and chicken meatballs, burgers and frankfurters, which are widely consumed across Malaysia, Indonesia, China and most of the world, were experimentally screened (Table 4.6). No positive amplification signals from these commercial meat products were observed with the MBT-

specific primers. Thus we could not extend further analysis against the collected meat products samples.

Table 4.6: Analysis of reference and commercial meat products using MBT specific PCR assay

Items and code of product items	Number of samples	Malayan box turtle DNA detection	Detection accuracy (%)
Meat products			
Pure chicken meatball	9	0/9	100
Pure beef meatball	9	0/9	100
MBT-spiked chicken meatball	27	27/27	100
MBT-spiked beef meatball	27	27/27	100
Pure chicken burger	9	0/9	100
Pure beef burger	9	0/9	100
MBT-spiked chicken burger	27	27/27	100
MBT-spiked beef burger	27	27/27	100
Pure chicken frankfurter	9	0/9	100
Pure beef frankfurter	9	0/9	100
MBT-spiked chicken frankfurter	27	27/27	100
MBT-spiked beef frankfurter	27	27/27	100
Commercial chicken meatball			
A	9	0/9	100
B	9	0/9	100
C	9	0/9	100
D	9	0/9	100
Commercial beef meatball			
E	9	0/9	100
F	9	0/9	100
G	9	0/9	100
H	9	0/9	100
Commercial chicken burger			
I	9	0/9	100
J	9	0/9	100
K	9	0/9	100
L	9	0/9	100
Commercial beef burger			
M	9	0/9	100
N	9	0/9	100
O	9	0/9	100
Commercial chicken frankfurter			
P	9	0/9	100
Q	9	0/9	100
R	9	0/9	100
Commercial beef frankfurter			
S	9	0/9	100
T	9	0/9	100
U	9	0/9	100

4.10 Traditional Chinese Medicines Screening and Validation by PCR-RFLP Assay

For thousands of years, traditional Chinese medicines (TCM) have been used in China. Despite of great advance of western medicines, TCM is widely exploited as healing methods for many countries in Asia and 80% of the developing countries rely on traditional medicines. With it broad-spectrum effects, TCM have been recognized as suitable medication for modern diseases such as cancer, cardiovascular disease, asthma and chronic illness. Moreover, due to increasing global demand, various types of health care products have been developed from TCM to meet the contemporary trend of "back to nature". However, substituents and adulterants of TCMs materials are often introduced intentionally or accidentally, thus not surprisingly, there are persistent signs that consumers may face real risks to their health from using these products. Although most of the TCM claim to be made from plant products, this is not always true. As a result, several highly endangered species, such as rhinos, crocodiles, turtles, tigers and elephants which are enlisted in the CITES Appendix I and II, have continued to be killed to supply the raw materials of these medicines which has a huge market in the Southeast Asia and Chinese communities around the world (Lee et al., 2014). So, consumers are completely at the mercy of the manufacturer's own quality assurance process that must confirm the presence of the desired substance and also the absence of contaminating substances. Therefore, we attempted to screen MBT materials in 153 (9X17) traditional Chinese medicinal products of 17 different brands sold in various Chinese medicine shops across Malaysia (Table 4.7) 40% of the total(153 smaples) or Sixty two sample (62) of the 153 tested products and up to 33-66% contaminants were found to be MBT positive, reflecting the wide spread consumption and uses of MBT materials in these medicines. Most of this information was not indicated in the product labels (Table 4.7). A study reported in the

US, 7% undeclared animal materials were found in 260 Asian traditional medicines (Ko, 1998).

To verify the authenticity, the amplified PCR products of the MBT positive samples were digested with *Bfa1*-restriction endonuclease enzyme and distinctive MBT fingerprints (72 and 43 bp) were obtained (Figures 4.14 and 4.15) (Table 4.7). Since herbal products are regarded as low-risk and natural sources of cure for many diseases, traditional medicines are not under the stringent labelling regulation what the medicine contains. In this regard, the TCMs studied here clearly reflect that such declarations are not correct at all times; the most worrisome concern is that MBT ingredients were not declared on the labels and that most of these preparations were claimed to be plant products to prove that there are no religious obligations since plant products are permitted in all religions. Thus 62 of the total 153 tested TCM samples in this study provided a 100% matching of DNA materials with *Cuora amboinensis* species, reflecting a clear break of wildlife conservation law in the preparation and selling of TCMs (Adeola, 1992; Alves & Rosa, 2005; Angeletti et al., 1992) (Table 4.7). This is also incompatible with the regulation of the US Food and Drug Administration (FDA) and European (Bennett et al., 2002; Robinson & Bennett, 2000, 2002) laws, which require a mandatory declaration of the product ingredients. Moreover, turtle species are considered as zoonotic agents and heavy metals reservoirs, thus the possibility of threat to the public health should be seriously considered. Between 1990 and 1992, more than 100 women in Belgium and France were found to have extensive interstitial fibrosis of the kidneys after using a weight-loss regimen involving TCM (Liu et al., 2015). Thereby, it is the responsibility of the manufacturer to ensure safety before a product is marketed.

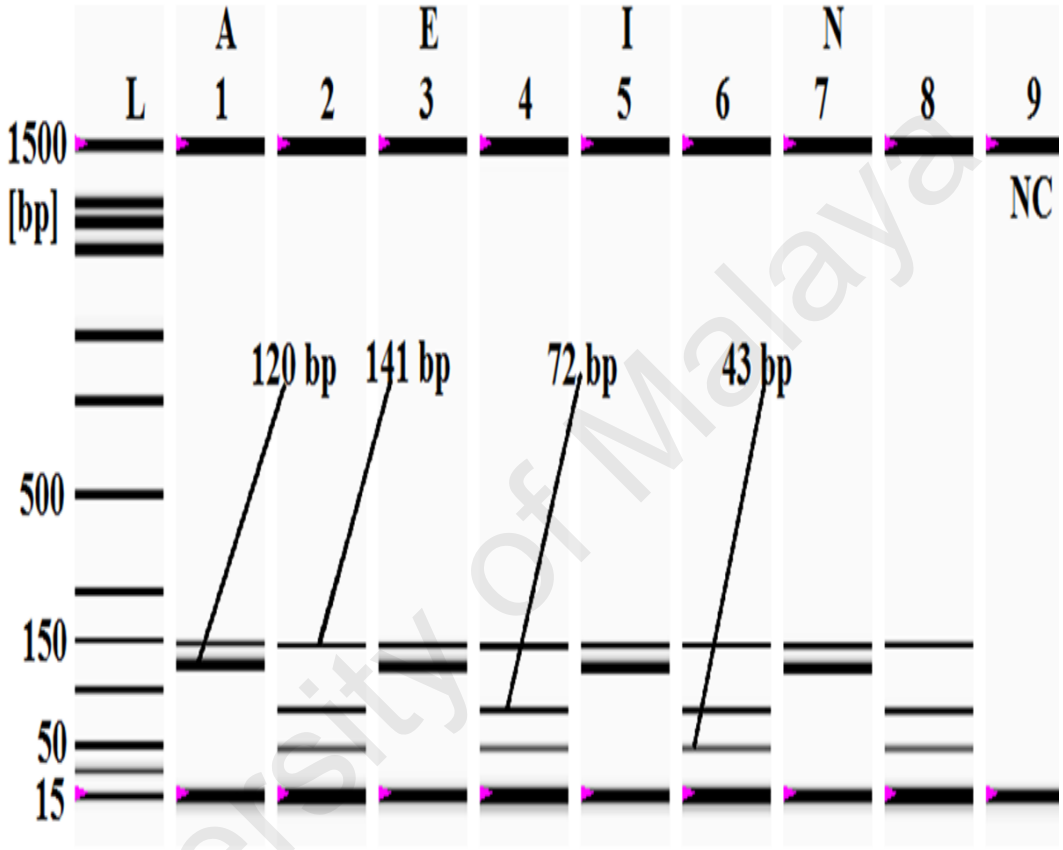


Figure 4.14: Traditional Chinese medicines analysis. In the gel image, *lanes 1, 3,5 and 7*are *for A* (Chinese herbal jelly) *E* (Anti inflammation) *I* (Sex stimulator) & *N* (Leukorrhea) medicines respectively, and *lanes 2,4* and *6* represent *Bfa1* digestion from samples *A E I* & *N* respectively. *Lane L*: ladder DNA, and *lane 9*: negative control (NC).

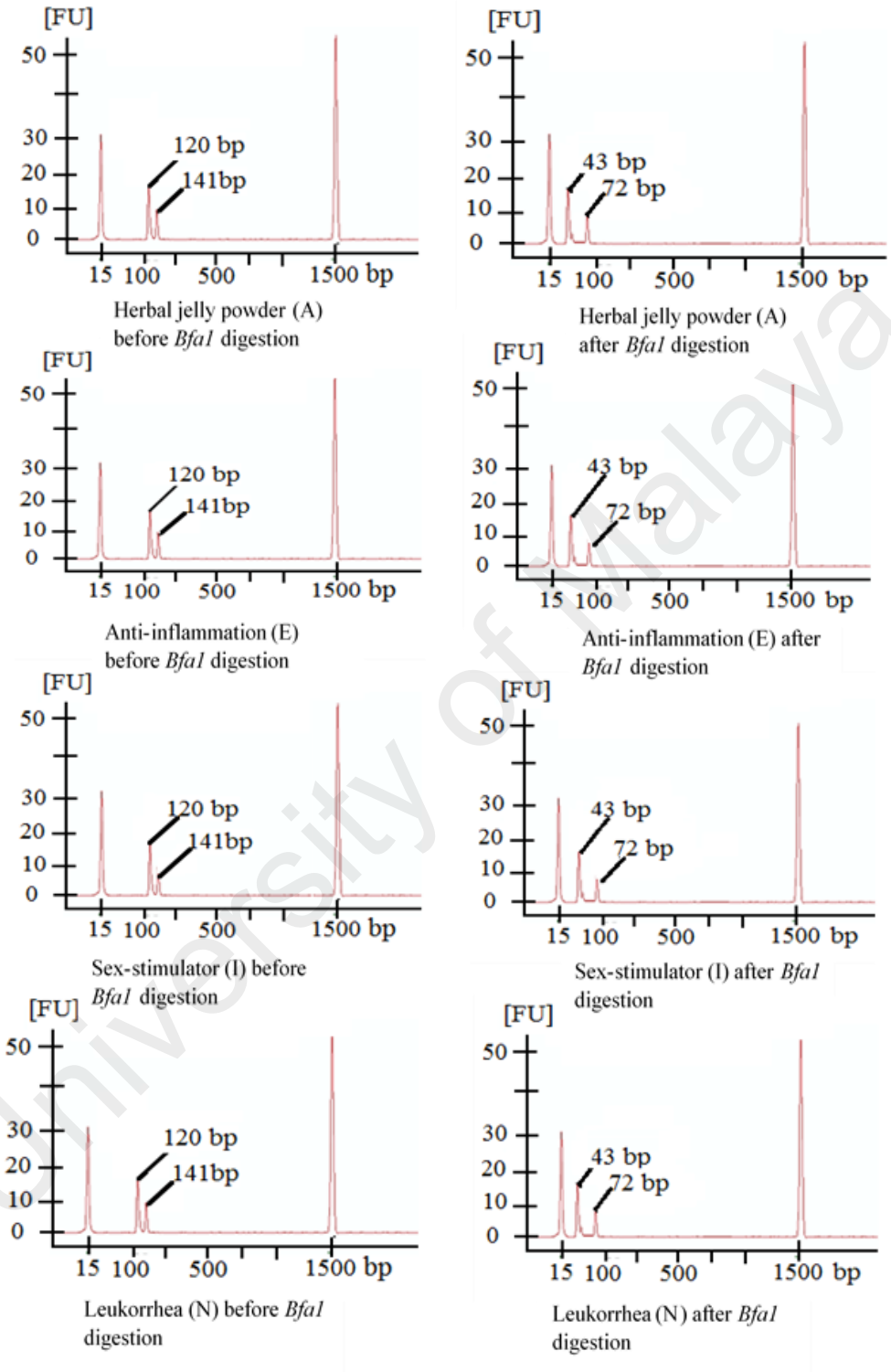


Figure 4.15: Screening of traditional Chinese medicines by PCR-RFLP assay and showing 120 bp PCR product before *Bfa1* digestion and after *Bfa1* digestion of medicinal products are shown in the electropherograms and demonstrated with labels in insets.

Table 4.7: Analysis of traditional Chinese medicines using MBT specific PCR assay

Code of medicinal items	Information of the products labeling	Product applications	Number of samples	Malayan box turtle DNA detection	Malayan box turtle contamination (%) in individual product
A	Chinese herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation,	9	5/9	55.55 %
B	Chinese herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation	9	4/9	44.44 %
C	Herbal jelly powder	Nocturnal enuresis, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Anti-inflammation	9	4/9	44.44 %
D	Herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Anti-inflammation	9	3/9	33.33 %
E	Semen nulumbinis, Mel, Frutus Lycii, Radix ginseng, Cordyceps, Radix astragali, Radix codonopsis pilosulae, Radix morindae	Anti-inflammation, Pumpils, Blood Circulation, Male fertility, Strength of knees, Gall bladder, Hepatitis, Herpes, Shingles, Otitis meida, Cystitis, Hyperthyroidism, Migraines and Jaundice	9	3/9	33.33 %
F	Rhizomz dioscoreae, Fructus ziziphi jujubae, Radix ginseng, Cordyceps, Radix astragali, Radix codonopsis pilosulae, Radix morindae	Anti-inflammation, Pumpils, Gall bladder, Hepatitis, Herpes, Shingles, Otitis meida, Cystitis, Hyperthyroidism, Migraines and Jaundice	9	4/9	44.44%

Table 4.7, continued

Code of medicinal items	Information of the products labeling	Product applications	Number of samples	Malayan box turtle DNA detection	Malayan box turtle contamination (%) in individual product
G	Semen nelumbinis, Radix ginseng, Cordyceps, Radix astragali, Radix codonopsis pilosulae, Radix morindae	Gall bladder, Hepatitis, Herpes, Shingles, Otitis meida, Cystitis, Hyperthyroidism, Migraines and Jaundice, Anti-inflammation, Pumpils	9	5/9	55.55 %
H	Radix ginseng, Cordyceps, Radix astragali, Radix codonopsis pilosulae, Radix morindae	Gall bladder, Hepatitis, Herpes, Shingles, Otitis meida, Cystitis, Hyperthyroidism, Migraines and Jaundice, Pumpils	9	0/9	0.00 %
I	Tongkat ali powder	Stimulate libido, Promote semen quality, Muscle growth, Pumpils. Blood circulation, Male enhancement	9	6/9	66.66 %
J	100% Tongkat ali capsule powder	Stimulate libido, Promote semen quality, Muscle growth, Anti-inflammation, Blood circulation, Male fertility	9	3/9	33.33 %
K	Tongkat ali capsule powder	Stimulate libido, Promote semen quality, Muscle growth, Anti-inflammation, Blood circulation, Male fertility	9	5/9	55.55 %
L	100% Tongkat ali capsule	Stimulate libido, Promote semen quality, and Muscle growth, Anti inflammation, Blood circulation, Male fertility	9	4/9	44.44 %

Table 4.7, continued

Code of medicinal items	Information of the products labeling	Product applications	Number of samples	Malayan box turtle DNA detection	Malayan box turtle contamination (%) in individual product
M	100% Tongkat ali powder	Stimulate libido, Promote semen quality, Muscle growth, Anti-inflammation, Blood circulation, Male enhancement and fertility	9	5/9	55.55 %
N	Radix gentinae, Radix bupleuri, Radix scutellariae, Fructus gardenia, Semen plantaginis, Radix angelicae sinensis, Radix rehmsnniae, Radix glycyrrhizae	Deafness, Hypochondriac pain, Irritability, Headache, Dizziness, Swollen sensation in ears/head, Swollen genitalia, Leucorrhea,	9	4/9	44.44 %
O	Radix gentinae, Radix bupleuri, , Rhizomzz alismatis, Caulis clematidis, Semen plantaginis, Radix angelicae sinensis, Radix, Radix glycyrrhizae	Hypochondriac pain, Irritability, Headache, Dizziness, Deafness, Swollen sensation in ears/head, Swollen genitalia, Leucorrhea, Acute icteric hepatitis, Urethritis	9	0/9	0.00%
P	Radix scutellariae, Fructus gardenia, Rhizomzz alismatis, Caulis clematidis, Semen plantaginis, Radix angelicae sinensis, Radix rehmsnniae, Radix glycyrrhizae	Liver, Gallbladder fire, Headache, Tinnitus, Hypochondriac pain, Irritability, Dizziness, Deafness, Swollen genitalia, leucorrhea	9	4/9	44.44 %
Q	Radix gentinae, Radix bupleuri, Radix scutellariae, Fructus gardenia, Rhizomzz alismatis, Caulis clematidis, Semen plantaginis, Radix angelicae sinensis, Radix rehmsnniae,	Migraine, Headaches, Acute pelvic inflammation , Swollen testes, Leucorrhea, Hyperthyroidism, Acute conjunctivitis, Acute liver fire	9	3/9	33.33 %

4.11 Optimization of SYBR Green Duplex Real-Time PCR System

A novel SYBR Green duplex real-time PCR system for MBT detection was developed by combining two sets of primers specific for MBT cytb (120 bp) and universal eukaryotic 18S rRNA (141 bp). Initially a singleplex PCR system was optimized and Ct values were recorded using DNA templates extracted from meat, skin, shell and bone tissues of MBT and cross tested against 20 non-target species (Table 4.8). Subsequently, the duplex system was established by primers for the internal control. However, no significant differences in Ct values were observed between the two systems. In order to eliminate any non-specific detection of SYBR Green PCR signal that might come from the formation of primer dimers and any other non-specific products (Kubista et al., 2006), melting curve analysis of the post-PCR samples was executed by plotting the variation in fluorescence as a function of time (*dF/dT*) against the melting (*Tm*) temperature of the reaction products. Since *Tm* depends on the length, base composition (GC/AT ration) and concentration of the target DNA sequences, the amplified PCR products for MBT (120 bp) and eukaryotic target (141 bp) were clearly differentiated through the specific *Tm* for each target. Two different peaks at 74.63 ± 0.22 and 81.40 ± 0.31 °C in a single melting curve were the obvious representation of MBT and eukaryotic positive control, respectively (Ririe et al., 1997; Varga & James, 2006) (Figure 4.16).

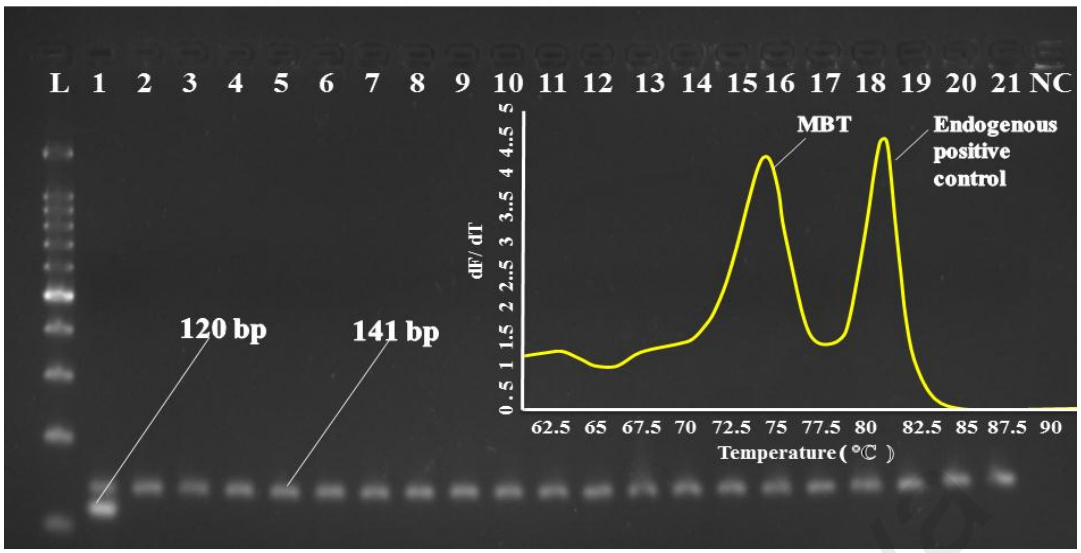


Figure 4.16: Specificity of Malayan box turtle (MBT) specific primers against 20 different species**.** Shown are *lane L*: ladder DNA; and *lanes 1–21*: PCR products from MBT target (120 bp) and eukaryotic endogenous control (141 bp). Malayan box turtle-specific product was amplified only from Malayan box turtle (*lane 1*), but endogenous control was amplified from all species (*lanes 1–21*): Malayan box turtle, pond slider turtle, chicken, sheep, goat, cow, water buffalo, deer, pig, duck, pigeon, dog, monkey, cat, rat, salmon fish, carp fish, cod fish, prawn, wheat and cucumber, respectively. The *inset* is the melting curve of the SYBR Green PCR for MBT and endogenous control.

4.12 Selectivity of the Real-time PCR Assay

Cross-reactivity of the designed primer were checked using the duplex PCR system wherein the MBT primers were challenged against the DNA templates of 20 different species including the Chinese edible frog (*Hoplobatrachus rugulosus* accession: NC_019615.1), chicken (*Gallus gallus* accession: KP269069.1), cow (*Bos taurus* accession: GU947021.1), goat (*Capra hircus* accession: KR059217.1), pig (*Sus scrofa domestica* accession: AP003428.1), pigeon (*Columba livia* accession: KP168712.1), sheep (*Ovis aries* accession: NC_001941.1), duck (*Anas platyrhychos* accession: EU755253.1), buffalo (*Bubalus bubalis* accession: NC_006295.1), giant river prawn (*Macrobrachium rosenbergii* accession: NC_006880.1), dog (*Canis lupus familiaris* accession: KF907307.1), cat (*Felis catus* accession: NC_001700.1), rat (*Rattus rattus* accession: NC_012374.1), monkey (*Macaca fascicularis* accession: NC_012670.1),

venison (*Odocoileus virginianus* accession: KM612279.1), Atlantic cod (*Gadus morhua* accession: NC_002081.1), salmon (*Salmo salar* accession: NC_001960.1), carp (Cyprinus carpio accession: KU050703.1), wheat (*Triticum aestivum* accession: NC_007579.1) and cucumber (*Cucumis sativus* accession: NC_016005.1); but no cross-reactivities were observed (Table 4.8). While the amplification signals (Ct values) of the MBT-specific PCR for the MBT-containing samples were 17.40±0.27–21.61±0.34, Ct values of the other species were ≥ 38 in a 40-cycle PCR reaction (Table 4.8 and Figure 4.16). On the other hand, the endogenous Ct values for all samples were found to be 18.02±0.20–24.30±0.23; this reflected the presence of good-quality DNA in all specimens but eliminated the possibilities of potential false-negative detection. The slight variation found for of Ct values (17–21) for the meat, skin, bone and shell tissues of the MBT could be attributed to heat, pressure, physical stresses, variable copies of mitochondrial DNA and presence of various inhibitors in different tissues. In some instances, negative controls yielded a positive signal at 38.5 cycles and, thus, it was inferred that any Ct ≥ 38 was non-specific for the MBT targets (Table 4.8). The specificity of the duplex assay was further confirmed by the melting curve wherein two distinctive peaks at 74.63 ± 0.22 and 81.40 ± 0.31 °C were the clear signatures for MBT (120 bp) and endogenous positive control (141 bp), respectively (Ririe et al., 1997; Varga & James, 2006) (Figure 4.16). Several factors such as dye types and concentrations, salt environment, primer dimers, unpredicted amplicons, inhibitors and variations in melting programs might result in false positive outcomes (Hennenfent & Herrmann, 2006; Varga & James, 2006). Thus the duplex PCR products were confirmed and detected in 2% agarose gel which clearly reflected MBT (120 bp) and universal eukaryotic product (141 bp), eliminating the doubts of any false negative detection (Figure 4.16).

Table 4.8: Specificity and Ct values of the SYBR Green duplex real time PCR assay

Samples	Malayan box turtle specific PCR system	Eukaryotic (18SrRNA) positive control system
	Ct value and Standard deviation (±)	
Malayan box turtle raw meat	17.40 ± 0.27	18.20±0.23
Malayan box turtle sterilized meat	18.56± 0.34	19.01±0.23
Malayan box turtle raw skin	18.40± 0.29	18.30±0.40
Malayan box turtle raw shell	20.12± 0.29	19.20± 0.29
Malayan box turtle sterilized skin	21.61± 0.34	20.50±0.38
Malayan box turtle bone	20.20± 0.38	20.09±0.50
Frog meat	39.34±0.11	18.02±0.20
Chicken	38.70±0.23	19.30±0.34
Goat	40.00	21.03±0.24
Pig	39.02±0.23	18.40±0.38
Pigeon	40.00	20.04±0.28
Sheep	39.02±0.27	18.30±0.36
Venison	39.05±0.34	24.04±0.49
Rat	40.00	23.40±0.21
Cat	40.00	17.90±0.31
Dog	38.98±0.23	22.60±0.31
Duck	38.90±0.40	19.20±0.22
Cow	39.50±0.23	20.50±0.23
Monkey	39.50±0.21	19.20±0.23
Buffalo	38.92±0.32	18.30±0.21
Prawn	39.04±0.02	19.11±0.14
Salmon fish	39.50±0.23	24.30±0.23
Cod fish	39.02±0.42	19.42±0.22
Carp fish	40.00	23.20±0.15
Cucumber	39.97±0.31	22.41±0.31
Wheat	40.00	21.02±0.11

4.13 Limit of Detection (LOD) and Quantification (LOQ) and Efficiency of Real time PCR Assay

The LOD of an assay plays a critical role in the determination of marginal-level targets in adulterated foodstuffs. Thus, the LOD of this study was determined by two different indices. Firstly, the concentration of the pure meat DNA was measured at ≥ 100 ng μL^{-1} (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK) and then various

concentrations such as 100 (100%), 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 ng (0.00001%) were made by a 10-fold serial dilution using nuclease-free deionized distilled water (Figure 17(a)), as inaccuracies and inconsistencies had been observed in spectrophotometric readings when low concentrations (≤ 10 ng μL⁻1) were used. Ct (threshold cycle) responses of these series of diluted DNA were measured using the duplex real-time PCR system and signal (Ct) was detected with as low as 0.00001 ng DNA template at 38 ± 0.12 cycles (Figure 17(a)). This amplification signal was close to that of the negative control (≥ 38.50 ± 0.35 cycles) in the 40-cycle PCR system. Furthermore, several researchers concluded that the highest limit should be set at 38 cycles in the Applied Biosystems SYBR Green PCR Master Mix in order to eliminate the possibility of any false-positive detection that may come from the cross-reactivity or primer dimer formation (Druml et al., 2015a and b). Secondly, the validation was performed with mixed DNAs of MBT using two types of heat-treated (autoclaved) binary admixtures (Figure 4.17(b)) and six types of reference meat products (chicken and beef meatballs, burgers and frankfurters) (Figure 4.18), and clear signals were detected as low as 0.001% (w/w) MBT contamination. The amplification signals (Ct values) of reference meat products were in the range of 30.90 ± 0.20 to 33.32 ± 0.12 for 0.001% of MBT. The Ct values of the endogenous controls were in the range and 19.67 ± 0.32 to 21.17±0.65 of internal positive control (18S rRNA) in various specimens (Tables 4.10). There was no significant variation of Ct values of the endogenous positive control (18S rRNA) due to it (internal control) being responsible for all the meat DNA (target and non-target). The melting curves reflected target products from MBT and internal control at 74.63 ± 0.22 and 81.40 ± 0.31 °C, respectively. Since the Ct for the 0.001% MBT mix was 33.32 ± 0.12 or far below the LOD (≥ 38.5), the assay could be used to detect MBT materials in food items at levels much lower than 0.001% (Figures 4.17c and d and 4.18b-g). The limit of quantification(LOQ) was determined from the linear correlation of the logarithm of

DNA concentration and the Ct value obtained from binary admixtures (Table 4.9) and laboratory-made reference (Tables 4.9 and 4.10) meat products (Figures 4.17(b) and 4.18(a)). The contents of MBT and total DNA in unknown food samples were calculated from the standard curve for MBT (Cytb) and the reference eukaryotic (18S rRNA gene) control. The Ct values of the analyzed samples were normalized against the admixture standard curve and plotted against the logarithmic concentrations of the spiked MBT in binary admixtures and laboratory-made reference meat products. This resulted in a linear standard curve in the range of 10 to 0.001% (Figures 4.17(b) and 4.18(a)) with slopes that ranged between -3.36 and -3.52, which resulted in PCR efficiencies (E%) between 98.15 and 92.23% ; these were very close to the ideal value (100%) that comes from a slope of –3.32. The correlation coefficient (R^2) was between 0.9939 and 0.9994, indicating high linear relationship between the Ct value and the logarithm of the DNA concentration (Figures 4.17(a and b) and 4.18(a)). These results are in accordance with the European Network of GMO Laboratories (ENGL) guidelines (ENGL, 2008).

Finally, the lowest concentrations that were recovered from the admixtures and reference meat products were between 0.0005 and 0.003 ng, which arose from 0.001 % MBT spiked into the samples (Tables 4.8-4.10). While recoveries in reference meat products containing 10 to 1 % MBT meat were 98.19–124.97 %, they were 157.69–166.57 % for 0.001 % MBT contamination, suggesting some degrees of inaccuracies at lower concentrations (Tables 4.9 and 4.12). Earlier studies also reflect a higher percentage (144-178%) of recovery at lower (2% spiked samples) concentration (Druml, et al., 2015). It is difficult to find the same quantity DNA in all cells due to fivefold variation of DNA content per cell between tissues. (Floren et al., 2015). However, when real-time PCR-recovered values (y-axis) (Table 4.13) were plotted against the reference value (x axis), a calibration model was found with a very high correlation coefficient ($R^2 \geq 0.999$) (Figure 4.19). The deviation from the reference values at lower concentrations most probably

resulted from the unequal distributions of the trace amount of contaminated meat or higher error in the preparation of reference samples (Druml et al., 2015a and b). However, since most of the fraudulent mixing takes place at higher concentrations in order that companies may make extra profit, a little inaccuracy at lower level contamination does not practically hinder assay applicability since detection was performed at all concentrations (Moore et al., 2012). Tables 4.9 and 4.10 reflect a high content of MBT (10%) in reference meatball, burger, and frankfurter products, which does not show significant/cause a systematic error in MBT quantification. Furthermore, the repeatability of this assay was investigated with two different DNA-extraction kits, PCR master mixes and PCR machines along with a change of analyst on three independent days (Tables 4.8–4.12). The results indicated that these external variables such as operator, DNA extraction kit, PCR master mix and PCR instrument do not influence the final results; instead, all these indicate the assay reliability and robustness under various circumstances.

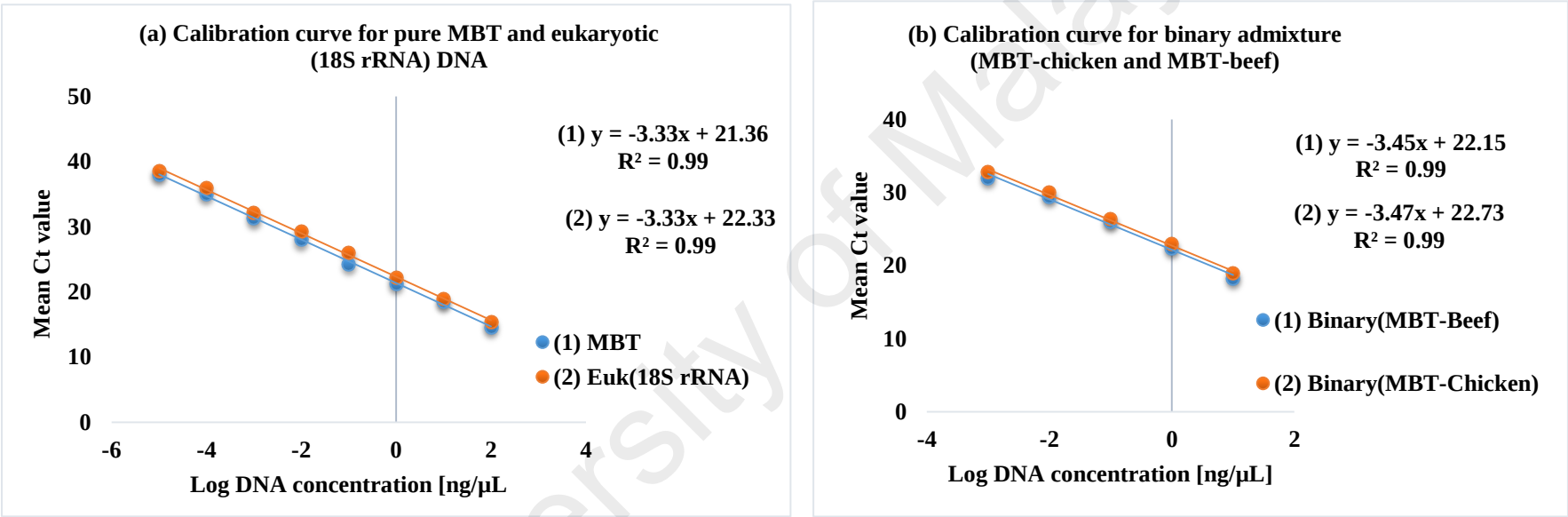


Figure 4.17: Standard (a) calibration curves for pure DNA(100 ng to 0.00001 ng) and (b) for binary admixtures DNA(10 ng to 0.001 ng) and melting (c and d) curves for binary admixtures**.** In the standard curve (b) shown are the recovery of MBT DNA from MBT- beef (1) and MBT – chicken (2) binary admixtures containing 10, 1, 0.1, 0.01 and 0.001% (w/w) MBT tissues with the balanced amount of beef and chicken, respectively. (c) and (d) are the melting curves for binary (1&2) admixture(MBT-beef and MBT-chicken) showing the distinctive peaks at 74.63 ± 0.22 °C and 81.40 ± 0.31 for MBT (120bp) and endogenous (141bp) targets.

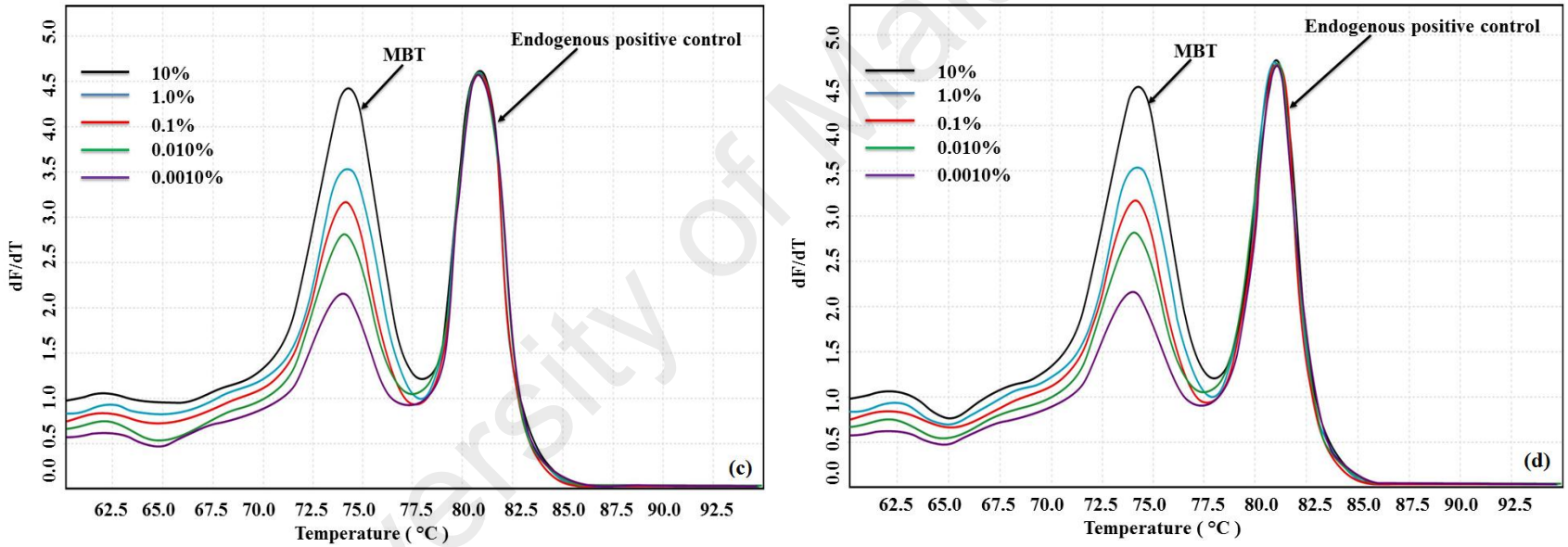
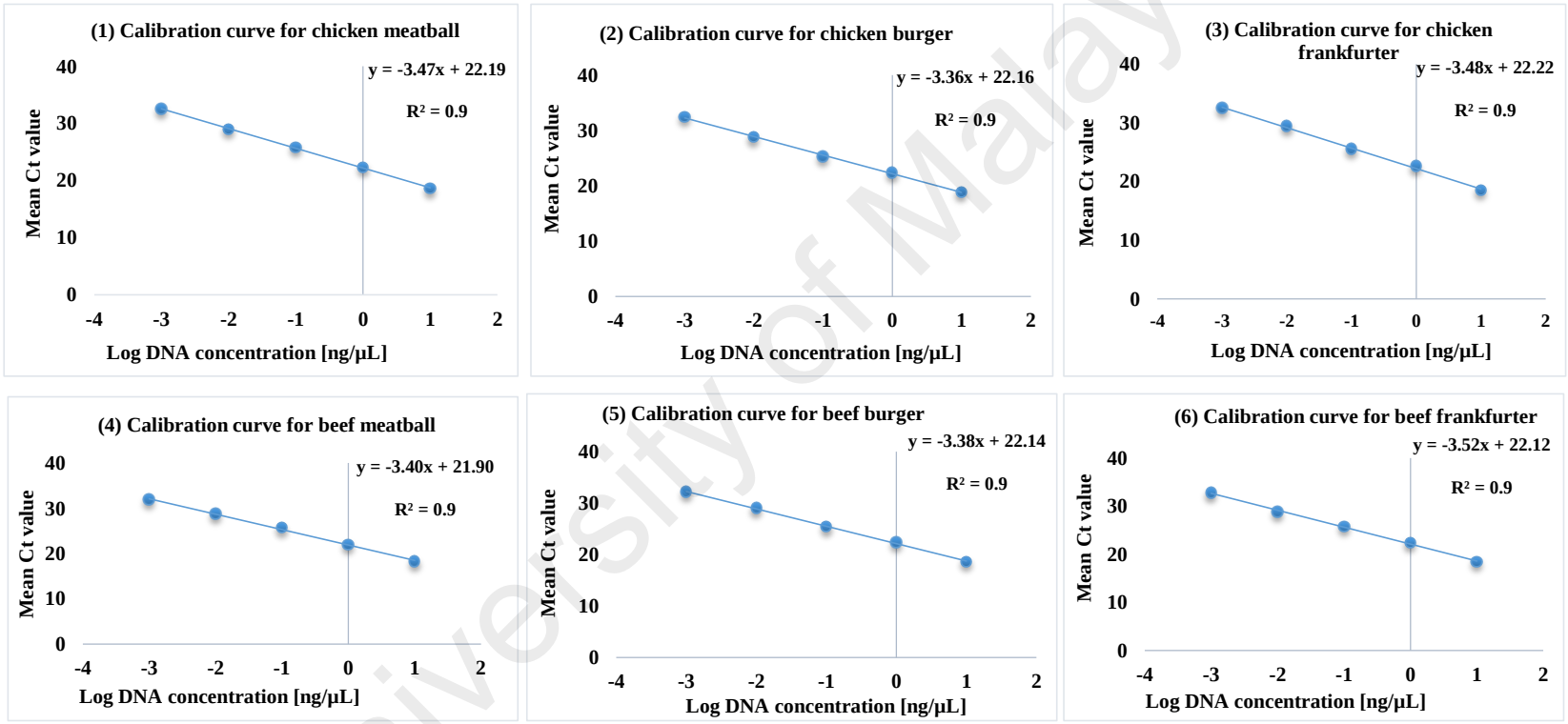


Figure 17, continued

Table 4.9: Repeatability and recovery of MBT targets in binary admixture (MBT-beef and MBT-chicken) (w/w%)

Binary admixture(10-0.001)(w/w)%																														
	Spike level: 10%						Spike level: 1.0%						Spike level: 0.1%						Spike level: 0.01%						Spike level: 0.001%					
Meat Product Name	MBT Ct Value	Mean Ct Value	RSD(%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)
Binary(MBT-Beef)	18.56	19.58	4.43	101.3	1.32	5.56	23.11	22.08	4.17	112.4	12.4	1.05	27.34	26.83	2.65	132.5	32.5	0.044	29.45	29.39	2.32	148.7	48.7	0.008	30.24	30.74	3.92	158.1	58.1	0.003
	21.00						22.54						26.58						30.25						29.76					
	20.00						20.54						25.78						28.56						32.67					
	19.45						22.65						26.58						28.67						29.46					
	18.87						21.56						27.85						30.00						31.56					
Binary(MBT-Chicken)	18.34	19.93	4.85	99.34	-0.7	6.40	24.35	22.24	4.88	110.2	10.2	1.38	28.56	27.2	4.04	136	36.00	0.051	27.78	29.9	6.11	151.4	51.4	0.009	29.57	31.33	6.51	161.2	61.2	0.003
	20.14						22.16						27.00						30.00						34.65					
	21.34						21.75						26.34						29.54						32.76					
	20.15						21.46						25.75						28.95						29.56					
	19.68						21.48						28.34						33.24						30.11					



(a)

Figure 4.18: Standard (a) and melting (b-g) curves for chicken (b, c, d) and beef (e, f, g) meat products. In the standard curve (a) shown are the recovery of MBT DNA from chicken (1-3) and beef (4-6) meatball (1, 4), burger (2, 5) and frankfurter (3, 6) products containing 10, 1, 0.1, 0.01 and 0.001% (w/w) MBT tissues, respectively. (b)-(g) are the melting curves for (1)-(6).

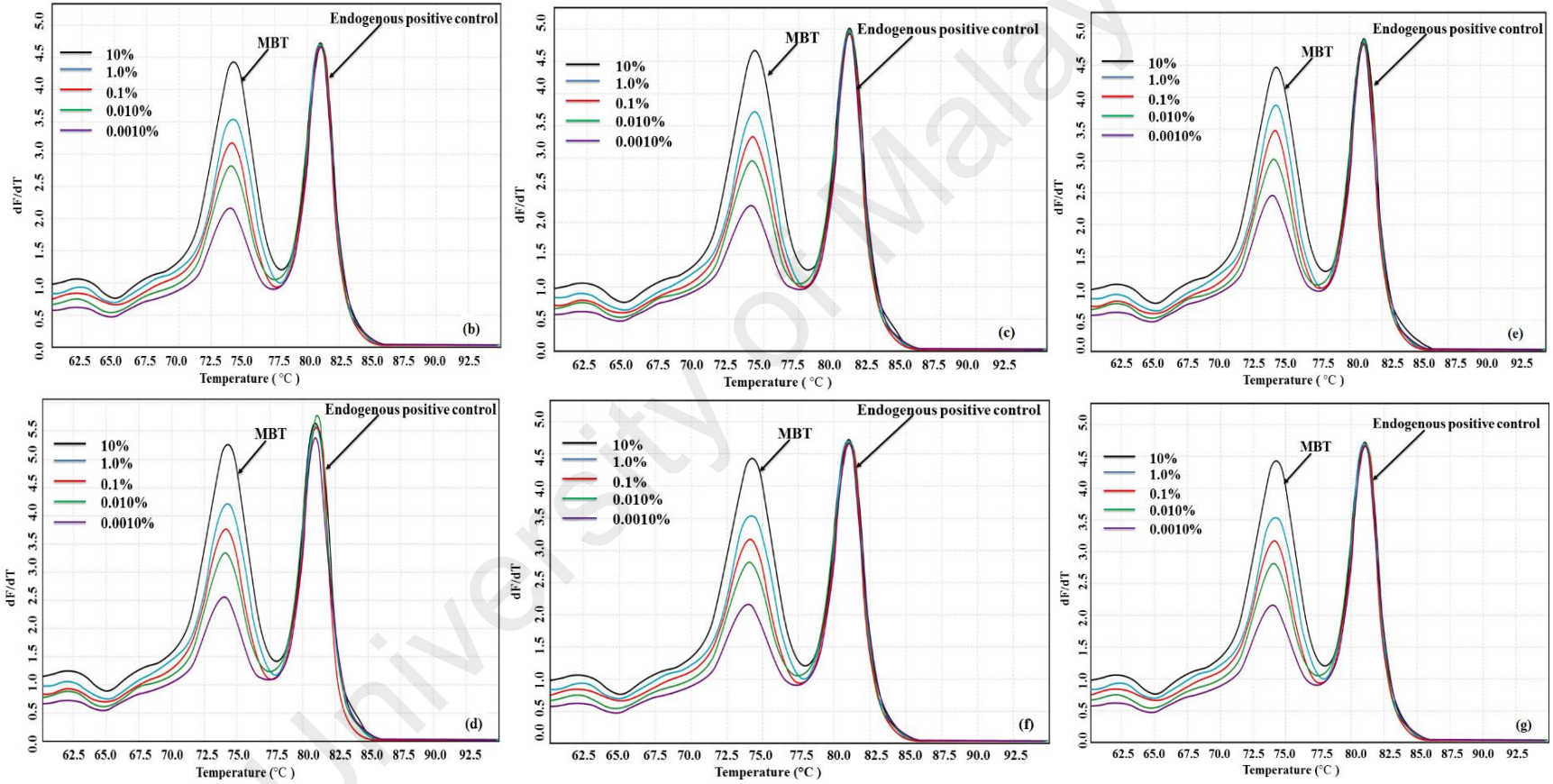


Figure 4.18, continued

Table 4.10: Repeatability and recovery of MBT targets in reference meat products using Yeastern genomic DNA extraction kit

Yeastern Genomic DNA Mini Kit																													
	Spike level: 10%						Spike level: 1.0%						Spike level: 0.1%						Spike level: 0.01%						Spike level: 0.001%					
Meat Product Name	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)
Chicken meatball	20.20	20.23	2.50	102.49	2.49	3.670	22.54	22.32	3.15	114.44	14.44	0.9200	24.76	25.18	2.67	127.13	27.13	0.1370	30.20	29.08	3.21	143.11	43.11	0.0100	29.54	30.90	3.89	157.69	57.69	0.0030
	20.13						22.14						24.11						28.56						30.10					
	21.12						21.14						25.67						27.81						32.00					
	19.56						23.30						26.00						30.12						32.65					
	20.12						22.50						25.34						28.70						30.21					
Chicken burger	19.30	19.67	4.83	98.19	-1.81	5.500	22.10	23.71	4.82	120.58	20.58	0.3450	28.00	27.45	2.66	136.75	36.75	0.0260	28.07	29.06	2.97	143.37	43.37	0.0088	34.22	33.32	2.32	161.31	61.31	0.0005
	20.03						24.43						26.32						29.76						32.15					
	18.67						25.12						27.02						28.14						34.14					
	19.00						22.65						27.52						30.25						33.00					
	21.34						24.26						28.40						29.10						33.11					
Chicken frankfurter	21.32	21.32	3.62	104.99	4.99	1.811	24.31	24.31	3.15	124.97	24.97	0.2510	28.32	28.32	4.49	136.47	36.47	0.0188	30.12	30.12	3.60	150.27	50.27	0.0053	30.76	31.62	3.82	159.46	59.46	0.0019
	20.00						23.00						28.78						31.36						29.67					
	22.15						25.14						26.64						28.36						32.65					
	21.11						24.10						30.40						31.12						32.76					
	22.03						25.00						27.46						29.64						32.26					

Note: RSD (Relative standard deviation), Recov (Recovery), Syst (System)

Table 4.10, continued

Yeastern Genomic DNA Mini Kit																													
	Spike level: 10%						Spike level: 1.0%						Spike level: 0.1%						Spike level: 0.01%						Spike level: 0.001%					
Meat Product Name	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)
Beef meatball	19.86	20.12	1.53	99.63	-0.37	3.347	25.03	24.80	0.81	120.87	20.87	0.1400	28.21	28.34	2.49	131.43	31.43	0.0100	31.26	30.11	4.99	144.78	44.78	0.0038	32.54	31.76	3.25	159.57	59.57	0.0013
	19.75						24.67						28.34						27.80						29.83					
	20.10						24.76						27.83						32.10						31.56					
	20.62						24.52						29.67						30.11						32.60					
	20.25						25.03						27.67						29.30						32.27					
Beef burger	22.00	21.34	3.12	102.64	2.64	1.721	23.04	23.07	2.63	111.19	11.19	0.5300	28.04	28.00	2.18	134.67	34.67	0.0184	30.67	30.67	1.71	150.64	50.64	0.0029	32.46	32.46	2.23	162.68	62.68	0.0080
	21.23						23.01						28.36						29.87						33.00					
	20.30						23.00						26.84						31.24						33.25					
	21.07						24.11						28.61						30.36						32.45					
	22.12						22.20						28.15						31.23						31.15					
Beef frankfurter	22.34	21.81	4.93	109.29	9.29	1.233	25.02	24.14	2.09	113.92	13.92	0.2640	29.12	28.56	3.68	139.37	39.37	0.0188	31.56	31.56	5.06	158.81	58.81	0.0021	33.45	33.23	1.79	166.57	66.57	0.0007
	21.00						24.17						28.00						32.34						34.16					
	23.70						23.45						27.00						30.25						33.00					
	21.00						24.06						28.56						29.56						33.20					
	21.03						24.00						30.13						34.10						32.34					

Note: RSD (Relative standard deviation), Recov (Recovery), Syst (System)

Table 4.11: Repeatability and recovery of MBT targets in reference meat products using NucleoSpin® extraction kit

NucleoSpin(R) DNA Extraction Kit																													
	Spike level: 10%						Spike level: 1.0%						Spike level: 0.1%						Spike level: 0.01%						Spike level: 0.001%					
Meat Product Name	MBT Ct Value	Mean Ct Value	RSD(%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)
Chicken meatball	20.15	20.64	4.63	101.79	1.79	2.800	22.10	22.65	4.75	116.09	16.09	0.7370	25.13	25.25	3.48	128.69	28.69	0.1310	29.45	29.03	2.07	145.16	45.16	0.0106	32.00	31.28	3.28	158.95	58.95	0.0024
	21.00						21.45						26.65						28.56						30.00					
	20.18						23.00						24.35						28.45						31.56					
	22.32						24.56						25.76						30.00						32.65					
	19.56						22.12						24.35						28.67						30.21					
Chicken burger	20.34	19.79	3.91	99.77	-0.23	5.073	23.17	24.14	3.87	121.83	21.83	0.2570	28.34	27.27	2.82	135.99	36.00	0.0302	28.07	28.62	3.33	144.36	44.36	0.0119	34.00	33.18	2.69	162.50	62.50	0.0005
	20.67						25.67						27.56						27.56						31.65					
	18.73						24.35						27.67						28.14						34.14					
	19.00						24.35						26.45						30.25						33.00					
	20.21						23.14						26.32						29.10						33.11					
Chicken frankfurter	21.23	20.35	4.20	106.97	6.97	3.440	24.43	24.11	3.59	123.96	32.96	0.2860	28.34	28.36	1.89	137.29	37.29	0.0172	30.45	30.37	3.32	150.82	50.82	0.0045	31.23	32.03	2.09	161.15	61.15	0.0015
	21.32						24.32						27.87						32.14						31.23					
	19.00						25.00						28.43						29.11						32.65					
	20.10						24.36						29.31						30.35						32.76					
	20.12						22.45						27.83						29.78						32.26					

Note: RSD (Relative standard deviation), Recov (Recovery), Syst (System)

Table 4.11, continued

NucleoSpin(R) DNA Extraction Kit																													
	Spike level: 10%						Spike level: 1.0%						Spike level: 0.1%						Spike level: 0.01%						Spike level: 0.001%					
Meat Product Name	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)	MBT Ct Value	Mean Ct Value	RSD (%)	Recov (%)	Syst error (%)	MBT DNA (ng/µL)
Beef meatball	19.67	20.22	3.50	100.15	0.15	3.119	25.35	24.91	2.01	121.39	21.39	0.1300	29.34	28.44	4.24	131.89	31.89	0.0120	30.45	30.20	3.29	145.20	45.20	0.0036	32.11	31.55	2.72	157.22	57.22	0.0015
	20.16						25.00						26.78						28.67						30.00					
	20.11						24.32						30.23						31.78						31.56					
	19.60						25.53						27.87						30.11						31.56					
	21.56						24.35						28.00						30.00						32.54					
Beef burger	24.34	21.82	7.39	102.60	2.60	1.243	24.11	23.63	3.94	110.15	10.15	0.3630	28.56	28.53	2.92	133.54	33.54	0.0128	30.34	30.72	3.19	148.13	48.13	0.0028	32.00	32.67	1.40	161.17	61.17	0.0007
	22.45						23.54						27.76						30.10						33.25					
	19.56						25.00						27.45						29.56						33.11					
	22.00						23.34						29.56						32.35						32.54					
	20.76						22.17						29.34						31.23						32.45					
Beef frankfurter	21.17	22.01	3.12	107.80	7.80	1.076	23.16	24.12	3.84	114.80	14.80	0.2700	28.56	28.66	2.31	139.10	39.10	0.0140	32.00	32.77	3.30	157.60	57.60	0.0009	35.00	33.35	2.92	165.10	65.10	0.0006
	22.00						24.35						29.53						32.34						33.56					
	21.43						25.61						28.56						31.43						33.00					
	22.34						23.10						27.56						34.00						33.20					
	23.11						24.36						29.11						34.10						32.00					

Note: RSD (Relative standard deviation), Recov (Recovery), Syst(System)

Table 4.12: PCR efficiency and limit of detection (LOD) and quantification (LOQ) of MBT specific SYBR Green PCR for the admixed and reference meat products of chicken and beef origins

Samples	Efficiency(%)	LOD (%)	LOQ (ng/µL)	Mean LOQ (ng/µL)
Binary (MBT-Chicken)	94.08	0.001	0.0031	0.003
Binary (MBT-Beef)	94.50	0.001	0.003	
Chicken Meatball	94.08	0.001	0.003	0.0021
Beef Meatball	96.78	0.001	0.0013	
Chicken Burger	98.15	0.001	0.0005	0.0042
Beef Burger	97.60	0.001	0.008	
Chicken Frankfurters	93.77	0.001	0.0019	0.0013
Beef Frankfurters	92.23	0.001	0.0007	

Table 4.13: Average recovery value of the real time PCR using 150 reference meat products samples

Yeastern Genomic DNA Mini Kit															
Product name	Spike level 10%			Spike level 1%			Spike level 0.1%			Spike level 0.01%			Spike level 0.001%		
	Recovery (%)	Mean(%)	RSD(%)	Recovery(%)	Mean(%)	RSD(%)	Recovery(%)	Mean(%)	RSD(%)	Recovery(%	Mean(%)	RSD(%)	Recovery(%)	Mean(%)	RSD(%)
Chicken meatball	102.49	102.87	3.51	114.44	117.66	4.08	127.13	134.30	2.98	143.11	148.50	3.72	157.69	161.21	1.77
Chicken burger	98.19			120.58			136.75			143.37			161.31		
Chicken frankfurter	104.99			124.97			136.47			150.27			159.46		
Beef meatball	99.63			120.87			131.43			144.78			159.57		
Beef burger	102.64			111.19			134.67			150.64			162.68		
Beef frankfurter	109.29			113.92			139.37			158.81			166.57		

NucleoSpin(R) DNA Extraction Kit															
Product name	Spike level 10%			Spike level 1%			Spike level0.1%			Spike level 0.01%			Spike level0.001%		
	Recovery (%)	Mean	RSD(%)	Recovery(%)	Mean	RSD(%)	Recovery(%)	Mean	RSD(%)		Mean	RSD(%)	Recovery(%)	Mean	RSD(%)
Chicken meatball	101.79	103.18	3.03	116.09	118.04	4.05	128.69	134.42	2.59	145.16	148.54	3.10	158.95	161.01	3.77
Chicken burger	99.77			121.83			135.99			144.36			162.50		
Chicken frankfurter	106.97			123.96			137.29			150.82			161.15		
Beef meatball	100.15			121.39			131.89			145.20			157.22		
Beef burger	102.60			110.15			133.54			148.13			161.17		
Beef frankfurter	107.80			114.80			139.10			157.60			165.10		

RSD (Relative standard deviation)

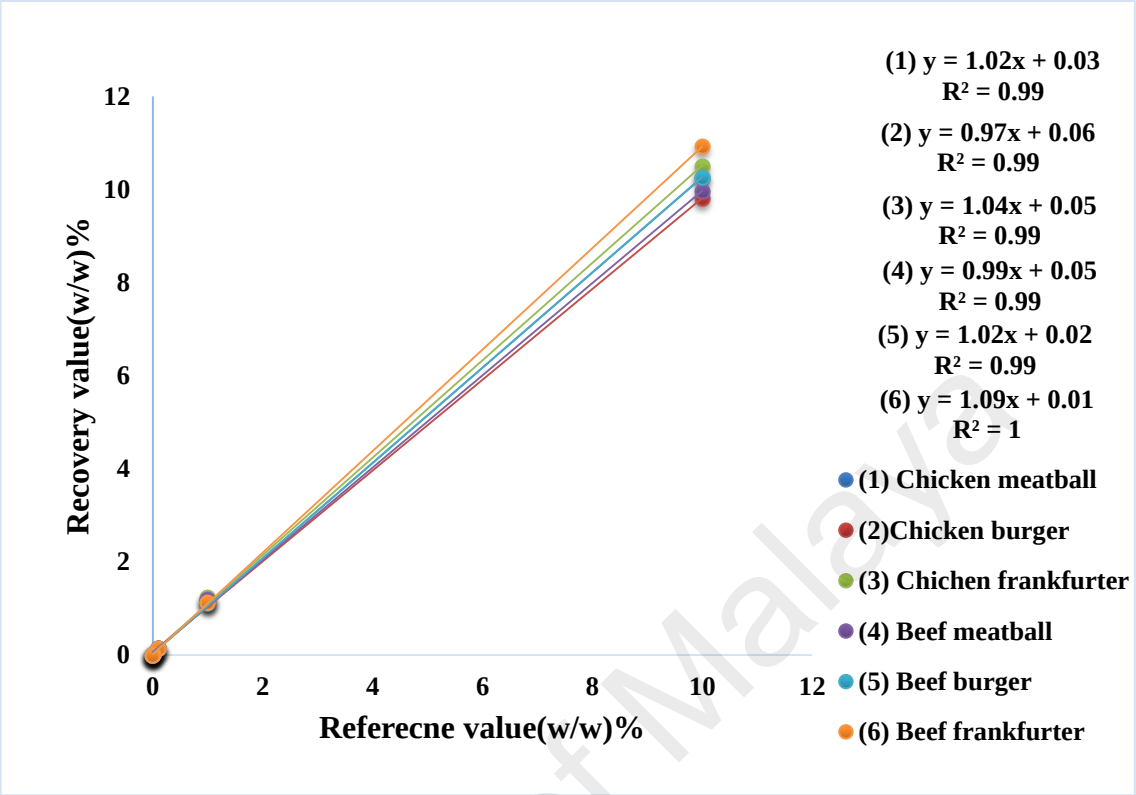


Figure 4.19: Relationship between the reference and recovery value (%). Relationship between the reference and recovery value (%). The MBT tissues were determined by SYBR Green duplex PCR from various meat products (meatballs, burgers, frankfurters from chicken beef, respectively) and plotted against the reference values.

4.14 Meat Product Analysis

In order to validate the real-time technique for screening meat adulteration in food products, deliberately adulterated reference meat products (chicken and beef meatballs, frankfurters and burgers) were prepared (Ali et al., 2012; Razzak et al., 2015) having 10, 1, 0.1, 0.01 and 0.001% (Table 4.6) contribution from ground MBT meat. All meat products were autoclaved at 120 °C under 45 psi pressure for 2.5 h on three different days to address the stability issues when denaturated (Tables 4.8-4.10; Figures 4.18 (b)–(g)) since this thermal treatment was previously used in food forensic investigations to benchmark target biomarker stability (Arslan et al., 2006; Haunshi et al., 2009; Ilhak & Arslan, 2007). The Ct and *Tm* values were in the range of 19.67 ± 0.42 to 33.32 ± 0.32 (Tables 4.9) and 74.63–74.65 ± 0.22°C for MBT and 81.40-81.76 ± 0.30 °C for internal

control (Figures 4.18 (b)–(g)), respectively, for all the reference meat products, demonstrating that the fluctuations in assay outcomes were insignificant in response to changes in the extraction kit or other variables. Furthermore, 33.32 ± 0.32 Ct for 0.001% MBT in meatball, burger and frankfurter products showed that this novel technique could be used to trace MBT meat in the food chain to detect even less than 0.001% (w/w) adulteration. Finally, a total of 189 samples including eight Halal branded meatballs (A–H), seven burgers (I–O) and six frankfurters (P–U) from each of chicken and beef origins were screened with the duplex SYBR Green real time PCR assay (Table 4.6), but none of the commercial meat products yielded a positive amplification signal for MBT, reflecting the respect for Halal products in Malaysia. On the other hand, the Ct and *Tm* values for the eukaryotic controls were 19.20 ± 0.32 to 23.42 ± 0.25 and 81.40 ± 0.31 to 81.50 ± 0.28 °C, demonstrating the presence of amplifiable DNA in all specimens (Fajardo et al., 2008). Thus, the experimental as well as the theoretical specificity, stability and sensitivity of this novel assay show it as a reliable and rapid technique for the authentication of MBT materials in the food chain. Malaysia has shown a strong commitment towards the development of Halal hub industries and as a competitive partner in the global Halal food business. Therefore, the absence of MBT materials in the Malaysian Halal food chain was not a surprise (Ali et al., 2012).

4.15 Quantitative Screening of Traditional Chinese Medicines

The widespread trade in various TCMs, which are claimed to provide multi-cure and natural remedies to common ailments, has become a disastrous threat to the existence of several wild species, including MBT. Although most of them are claimed to be plant products, several highly endangered species, such as rhinos, crocodiles, turtles, tigers and elephants, which are enlisted in the CITES Appendix I and II, have continued to be killed to supply the raw materials of these medicines which have a huge market in Southeast Asia and Chinese communities around the world (Lee et al., 2014). In many cases,

enforcement of regulation becomes impossible because the endangered animals are sold in forms, such as powder and jellies made from ground bones, shells and skins, which are difficult to detect by customs officials (Alves & Rosa, 2005; Lee et al., 2014). Herbal jelly powder is a jelly-like Chinese medicine traditionally sold and consumed as 'guilinggao' or 'turtle jelly' or 'Chinese medicine dessert' among the vast Chinese communities around the world. It is popular natural desert with Wuzhou origins and its typically made with secrete recipes that have been passed from generation to generation. According to the Chinese medicines, herbal jelly is thought to be good for the skin, promoting a healthier complexion upon repeated consumption and it able to clean toxins from the body. Other positive effects include the improvement in circulation, muscle growth, kidney and liver functions, relief of itching and dermatitis (Chen et al., 2009b; Dharmananda, 2005). Herbal jelly is made from powdered plastron, which is the bottom shell of the turtle *Cuora trifasciata*, which is commonly known as 'three-lined box turtle' or 'golden coin turtle', along with a variety of herbal products such as China roots, Smilax glabra, known to contain natural remedies for many diseases (CARR, 1991; Chen et al., 2009b; Dharmananda, 2005). Despite commercial farming across China, the golden coin turtle is extremely expensive (Haitao, et al., 2008), encouraging the use of more commonly available turtle species in guilinggao or herbal jelly powder (Chen et al., 2009b; da Nóbrega et al., 2008; Dharmananda, 2005). Considering this, we attempted to screen MBT materials in 120 traditional Chinese jelly powder of eight different brands sold in various Chinese medicines shops across Malaysia (Table 4.14). About 23.33 % of the 120 tested products were found to be MBT-positive, reflecting the widespread consumption and uses of MBT materials in these medicines, but the information was not included in the product labels (Table 4.14). The MBT presence was reflected through the Ct values and melting curves at 26.37 ± 0.32 to 31.67 ± 0.42 (Table 4.14) and 74.63 to 74.71.65 ± 0.22 °C, respectively. Due to the unavailability of the medicinal formulas or

documents containing sufficient information, dummy medicinal products could not be made in the laboratory for medicinal product screening validation. However, the amount of the contaminating MBT target (0.00157 to 0.0612 ng/ µL corresponding to 26.37 ± 0.32 to 31.67 ± 0.42 Ct values) in the tested samples was calculated by plotting the Ct values in the pure DNA standard curve (pure MBT DNA) and using equations (1) to (3) and was found in the range of 25.33 ± 0.50% to 38.23 ± 0.34% (Table 4.14), when only the total DNA of the source materials was considered and other materials like polysaccharides and non-DNA biomaterials were not measured (Table 4.14).

Since herbal products are regarded as low risk and natural sources for the cure for many diseases, traditional medicines are not under stringent regulation. In this regard, the herbal products studied here clearly reflect that such declarations are not correct at all times; the grave concern is that MBT ingredients were not declared in the labels and that most of these preparations were claimed to be plant products and hence there are no religious obligations since plant products are permitted in all religions. Recently, an Australian study found that high rates of adulteration (92%), substitution and mislabeling are widespread in herbal TCMs; the undeclared ingredients were either illegal or potentially hazardous to consumers (Alves et al., 2013; Coghlan et al., 2015; Ernst & Coon, 2001). Thus the 23.33% herbal jelly powder samples tested in our laboratory provided a 100% matching of DNA materials with *Cuora amboinensis* species, reflecting a clear breach of wildlife conservation law in the preparation and selling of traditional Chinese herbal products (Adeola, 1992; Alves & Rosa, 2005; Angeletti et al., 1992). This is also contrary to the regulation of the United States (USFDA), UK (MHRA) and Australia (TGA) (Bennett et al., 2002; Robinson & Bennett, 2000, 2002), which demand the mandatory declaration of product ingredients. Published reports show that wild and domestic animals and their by-products, such as hooves, skins, bones, feathers and tusks, are used in the preparation of curative, protective and preventive medicines (Anyinam,

1995), causing over-hunting and massive threats to wildlife (Bennett et al., 2002). Moreover, some organs and animal by-products, such as bones and bile, can be a source of Salmonella infection that causes chronic diarrhoea and endotoxic shock. In this context, the possibility of transmitting infections or ailments from animal preparations should be seriously considered (Still, 2003).

Table 4.14: Analysis of traditional Chinese herbal jelly powder using MBT specific PCR assay

Code of medicinal items	Information of the products labeling	Product applications	Number of samples	Number of detection samples	Contamination (%) of Malayan box turtle	Mean Ct value & Concentration[ng/µL]
A	Chinese herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	3/15	25.33-28.37	26.37 & 0.0612; 27.12 & 0.037; 28.14 & 0.018
B	Chinese herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	4/15	25.48-27.70	27.54 & 0.0272; 29.15 & 0.009; 30.00 & 0.005; 31.67 & 0.00157
C	Herbal jelly powder	Nocturnal enuresis, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Anti-inflammation, Appetizer	15	4/15	35.50-37.20	27.46 & 0.028; 29.31 & 0.008; 29.75 & 0.006; 31.17 & 0.0022
D	Herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Anti-inflammation, Appetizer	15	5/15	36.32-38.23	26.38 & 0.0613; 28.45 & 0.0145; 28.67 & 0.0125; 28.74 & 0.012; 29.24 & 0.0084

Code of medicinal items	Information of the products labeling	Product applications	Number of samples	Number of detection samples	Contamination (%) of Malayan box turtle	Mean Ct value & Concentration[ng/µL]
E	Guilinggao powder (Chinese herbal jelly powder)	Pimples, Blood Circulation, Male fertility, Strength of knees, Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	3/15	30.45-36.72	28.14 & 0.0180; 28.76 & 0.0117; 30.15 & 0.0044
F	Chinese herbal jelly powder (guilinggao)	Gall bladder, Hepatitis, Herpes, Shingles, Hyperthyroidism, Migraines and Jaundice, Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	6/15	35.56-37.60	26.37 & 0.0612; 27.46 & 0.028; 29.31 & 0.008; 30.00 & 0.005; 30.56 & 0.0033; 31.43 & 0.0037
G	Guilinggao powder(herbal jelly powder)	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	3/15	31.23-33.56	27.54& 0.0272; 29.75 & 0.006; 30.67 & 0.0031
H	Chinese herbal jelly powder	Nocturnal enuresis, Anti-inflammation, Dessert soup, Muscle growth, Relieving itching, Reducing acne and kidney restoration, Blood circulation, Appetizer	15	0/15	0.00	0.00-0.00

CHAPTER 5: CONCLUSION AND RECOMMENDATION FOR FUTURE WORK

5.1 Conclusion

The Malayan box turtle *(Cuora amboinensis)* is one of 18 native freshwater turtle and tortoise species in Malaysia. The highest rate of exploitation of this species has been reported in East Asian countries for use in sex stimulants and invigorating traditional Chinese medicines, resulting in a significant decline of its population and putting it at risk of extinction. Moreover, its slow reproductive cycle due to late maturity as well as the production of few eggs has placed this species on the verge of disappearance, causing enormous potential harm to biodiversity. Therefore, it is feared that continuous, high-volume exploitation combined with its vulnerable life history characteristics might lead to a serious population decline, making the MBT extinct, at least locally. Since, 2005, the Malaysian government banned the hunting of this species for sale in domestic or international markets, but it clandestine trade under the labels of permissible meat is greatly suspected. Thus, there is a need for a reliable tracing method for MBT identification and quantification before this vulnerable species disappears from its natural habitats.

Stability, sensitivity, robustness and precision under various food-processing conditions are the benchmarks of an assay to be acceptable for authentication studies. Moreover, short-length PCR targets perform better over longer ones. Consequently, two different PCR targets (120 bp, which was developed in this study, and 165 bp, which was shortest in length among the published reports) were subjected to extensive boiling (100 °C for 60, 90, 120 and 150 min), harsh autoclaving (120 °C for 60, 90, 120 and 150 min at 45 psi) and microwaving treatments (600, 650 and 700 W for 30 min). It was found that the 120 bp target survived and, hence, was amplified under all treatment conditions. But the 165 bp target failed to withstand boiling at 100 °C for 150 min, autoclaving at

120 °C for 150 min, and microwaving stress at 700 W for 30 min. Furthermore, the sensitivity of the newly developed target (120 bp) (0.0001 ng at pure state and 0.01% at complex background) was higher than that of the published target (165 bp) (0.001 ng at pure). This superior target stability and sensitivity under extreme treatments of boiling, autoclaving and microwave cooking suggests that this newly developed assay would be suitable for any forensic identification of Malayan box turtle species, even in severely degraded specimen and better than those of previously reported assays. The assay performed outstanding specificity when cross-challenged against 20 commercially important species. Additionally, theoretical analysis reflected very close matching with the nine critically endangered *Cuora* species, but there was a huge genetic distance from the other reptile species such as tortoise and turtles that belong to non-*Cuora* genera, crocodiles, snakes and lizards. Thus the designed assay has the potential to be used as a universal probe for the detection of vulnerable *Cuora* genus.

The originality of the PCR products was confirmed firstly, by sequencing and secondly by RFLP analysis. For sequencing, PCR products were cloned in a plasmid vector (PJTI1.2) and using the TOPO^{TM} TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). DH5α-T1 *Escherichia coli* (Invitrogen) were used and incubated at 37 °C on agar plate containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for transformation and multiplication. Forty colonies were selected from each agar plate, and plasmid DNA were purified using the QIAprep Spin Miniprep Kit (Qiagen Gmbh, Hilden, Germany). Cloned DNAs were analyzed by sequencing with universal LpJET1.2 forward and reverse primers using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Perkin-Elmer/Cetus, Norwalk, Connecticut, USA). Chromatogram sequencing files were inspected with Chromas 2.2 (Technelysium) (http://technelysium.com.au/wp/), and similarity analysis of the obtained DNA sequence was carried out using ClustalW software and online based BLAST software in NCBI.

The study of PCR-RFLP assay for the detection of MBT under complex food matrices increased the confirmation and validity in assay authentication with eliminating the ambiguities**.** Furthermore, the use of an endogenous control (141 bp) effectively eliminated the chances of a false negative detection, and the false positive identification was similarly avoided through the use of a negative control. Additionally, the lower limit of detection (0.01% MBT meat and 0.0001 ng DNA) has made the assay suitable for the detection of marginal levels of adulteration in popular food products such as meatballs, burgers and frankfurters.

Furthermore, the study emulated the real forms of adulteration practices by making binary and ternary admixtures with various percentages of the target meat in a wide range of food products. Commercially available meat products and Chinese traditional medicine were screened to optimize and establish the validity of the assay for the analysis of marketed food, and medicine and the distinctive *Bfa1*-restriction profiles further authenticated the origin of the amplified products.

One of the biggest challenges was the extraction of DNA from the Chinese Herbal Jelly powder. Herbal jelly powder is made with multiple herbal components with turtle plastron and shell materials. There was no optimized method for the extracting DNA from the herbal jelly powder. For the first time, this study has optimized and validated DNA extraction protocol by extracting DNA from 120 herbal jelly powder.

Nevertheless, **t**he duplex SYBR Green real-time PCR system for the detection and quantification of Malayan box turtle (MBT) materials in food chain is a significant contribution to forensic science and conservation biology. Distinctive Ct values and melting curves for MBT (120bp) and eukaryotic control (141bp) even at lower concentrations (0.00001 ng pure DNA or 0.001% (w/w) MBT meat) have made it trustworthy for the unambiguous tracing of MBT materials in food chain or any forensic

or archaeological investigations. Furthermore, a high correlation coefficient (R^2=0.999) between the recovered and reference values for 10-0.001% MBT adulteration was a strong piece of evidence that this automated real-time PCR assay has sufficient merit to be used for the confirmed MBT detection in any forensic studies.

To validate the assay, 273 commercial traditional medicines and 183 commercial food samples were analysed and 66% of the 273 TCM tested samples were found to be MBT positive but no commercial food were contaminated, although positive results were found in lab made reference meat products. The proven success of the assay to identify authentic MBT target DNA from various food matrices and traditional Chinese medicine strongly suggested that the assay could be used by the regulatory bodies, archaeologists and wildlife protection agencies to prevent or reduce the illegal trades of MBT materials in all possible routes and safeguard this endangered species in natural habitats, preserving the biodiversity and ecological balances.

5.2 Recommendations for Future Work

The developed food and TCM authentication assay described in this study is a promising tool to detect and quantify specific DNA targets from contaminated and highly degraded samples. Detection of specific DNA targets is very useful technique to distinguish permissible and non-permissible animal materials in food and medicinal products and to safe guard human health, religious views and to secure fare trade as well as to prevent the illegal wildlife trade. Although the designed primers were speculated to be a universal marker for all 12 *Cuora* species, we could not cross-challenged the primers against the DNA of all *Cuora* species because of the unavailability of the samples from all species. This was due to geographical distance and sample unavailability in one country, regional embargo and legal and regulatory issues for the transportation of samples from other countries. So it would be ideal if samples could be collected from all

Cuora species and a confirmatory test performed to prove universality of the developed primers. The developed method was applied and successfully detected MBT in food and traditional Chinese medicine specimens; the reliability of the assay could be further enhanced if it could be tested for cosmetics and other health care products.

Human food and medicinal products are composed of multiple species. So the development of multiple species' targets and detecting them in a multiplex PCR platform might ensure better customer satisfaction since it could identify whether other elements are present or not. So future work can explore the application of multiple species' targets which can detect and quantify different species' DNA from complex pools of food matrices. A multiplex PCR assay platform can be designed by incorporating specific primer pairs from different potential Haram (not allowed) meat species such as pork, cat, rat, horse etc. in a single assay run. Such an assay technique definitely could save time and deliver more information to the consumers. Additionally, target biomarker could be integrated with the new technology such as Single Molecule Detection Technique for the determination of single DNA molecules of the target species in forensic samples analysis (https://www.thebalance.com/what-s-required-for-superior-pcr-results-375480).

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