DEVELOPMENT OF SHORT AMPLICON-LENGTH PCR-RFLP ASSAY FOR THE DETECTION OF MACACA FASCICULARIS MEAT UNDER COMPLEX MATRICES

University of Malaya

NUR RAIFANA BINTI ABDUL RASHID

**INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2015

DEVELOPMENT OF SHORT AMPLICON-LENGTH PCR-RFLP ASSAY FOR THE DETECTION OF MACACA FASCICULARIS MEAT UNDER COMPLEX MATRICES

NUR RAIFANA BINTI ABDUL RASHID

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSHOPY

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

University of Malaya

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **NUR RAIFANA BT ABDUL RASHID**

Registration/Matric No**: HGA120006**

Name of Degree: **Master of Philosophy (M. Phil)**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

DEVELOPMENT OF SHORT AMPLICON-LENGTH PCR-RFLP ASSAY FOR THE DETECTION OF MACACA FASCICULARIS MEAT UNDER COMPLEX MATRICES.

Field of Study: **Biology and Biochemistry**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:24 August, 2015

Subscribed and solemnly declared before,

Witness's Signature Date:24August, 2015
Name:
Designation:

University of Malaya

ABSTRACT

The macaque (*Macaca fascicularis*) monkeys are the third-largest primate population which are abundant in tropical forests. Despite being the potential carrier of Simian Immunodeficiency, Ebola and Corona viruses as well as religious and wildlife restrictions, macaques have been widely hunted and consumed in many countries. However, in spite of being a potential adulterant of common meat, methods to detect monkey species in food are rarely documented. To fill up this research gap, here a monkey-specific polymerase chain reaction (PCR) assay targeting a short site (120bp) of mitochondrial d-loop gene was described since short-length targets are thermodynamically more stable than the longer ones under compromised states. The theoretical specificity of the primer pair was confirmed against 51 species, including 34 primates of which 13 species were from Macaque genera. The primers were fairly conserved for most of the Macaques but greatly polymorphic for other primates, demonstrating its universal signature for macaque detection. However, due to wildlife restriction, the practical specificity was tested only against 17 terrestrial and aquatic-species and no cross-species amplification was detected under raw, processed and admixed states. The sensitivity of the assay was 0.0001ng DNA under pure states and 0.1% monkey meat in binary meat mixtures. Finally, the assay was validated by digesting the PCR products with *Alu*I and *CViKI*-1 and distinctive restriction fingerprints for macaque identification were demonstrated both under raw meat and commercial meatball products. RFLP analysis further authenticated the originality of the PCR product and distinctive restriction patterns were found upon *Alu*I *and CViKI*-1 digestion. A micro-fluidic lab-on-a-chip automated electrophoretic system separated the fragments with high resolution. Definitely the assay would be useful to regulatory bodies for food and feeds along with wildlife protection agencies as a reliable authentication technique for the

University of Malaya

unambiguous tracing of monkey meat under various matrices including the processed food.

University of Malaya

ABSTRAK

Kera adalah spesis ketiga terbesar dalam populasi primat yang kebanyakannya terdapat di hutan tropika. Walaupun spesis kera tersenarai dalam Akta Perlindungan Hidupan Liar di Malaysia dan mempunyai potensi sebagai pembawa virus Immunodeficiency, Ebola dan virus Corona, namun aktiviti pemburuan species ini amat berleluasa baik di dalam negara mahupun luar negara. Setakat ini, kaedah untuk mengesan spesies monyet dalam makanan belum lagi didokumenkan walaupun penggunaan daging monyet untuk menjadi "bahan tambahan" sangat berpotensi tinggi. Untuk mengisi jurang kajian ini, di sini didirikan reaksi rantaian polymerase (PCR) khusus untuk spesis kera, menyasarkan 120 basa pasangan (bp) berasaskan mitokondria gen gelung-D memandangkan urutan (sekuen) pendek adalah termodinamik dan lebih stabil dalam keadaan ekstrim. Pasangan primer yang direka diuji spesifikasinya terhadap 51 spesies, termasuk 34 primat di mana 13 spesies adalah dari genus Macaque. Primer yang direka hampir sama dalam golongan sepsis kera tetapi sangat polimorfik untuk primat lain, menunjukkan universal yang berkesan untuk pengesanan DNA kera. Walaubagaimanapun, disebabkan oleh jumlah sampel hidupan liar yang terbatas, pengkhususan praktikal diuji hanya terhadap 17 daratan dan spesies akuatik dan pengesanan terhadap spesis lain adalah negatif dalam keadaan mentah, diproses dan campuran. Kepekaan ujian itu adalah 0.0001ng DNA dalam keadaan tulen dan 0.1% daging monyet dalam campuran binari daging. Akhir sekali, ujian ini disahkan pula dengan teknik pencernaan (RFLP) produk PCR menggunakan enzim *Alu*I dan *CViKI*-1 dan cap jari sekatan tersendiri bagi mengenal pasti kera telah menunjukkan kedua-dua di bawah daging mentah dan produk bakso komersial. Analisis RFLP lagi disahkan keaslian produk PCR dan corak sekatan tersendiri telah dijumpai pada pencernaan oleh *Alu*I dan *CViKI*-1 menggunakan kaedah elektroforetik automatik mikro bendalir makmal-di-atas-

University of Malaya

chip bagi memisahkan serpihan dengan resolusi tinggi. Sesungguhnya teknik ini adalah berguna untuk badan kawal selia makanan dan agensi perlindungan hidupan liar yang memerlukan teknik pengesahan untuk mengesan yang jelas daging monyet di bawah pelbagai matriks termasuk makanan yang diproses.

University of Malaya

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful. Alhamdulillah, all praises to Allah SWT, with His will and blessings, I managed to complete my dissertation and one step closer for my Master Degree.

First of all, I would like to express my heartfelt gratitude to my supervisors, Professor Dr. Sharifah Bee Abd Hamid and Dr. Md. Eaqub Ali for their utmost guidance throughout my research journey, for the encouragement and non-stop support from the beginning till the very end. Their willingness to sacrifice their precious time to monitor and guide me during the study period is highly appreciated. Special thank goes to Prof. Dr. Shuhaimi Mustafa, and Dr. Md. Mahfujur Rahman who shared their time and valuable knowledge for my academic research. I also would like to extend my deepest gratitude to Prof. Dr. Jennifer Ann Harikrishna for her help to use the facilities in CEBAR, IPPP, Universiti Malaya. I am very thankful to have Md. Abdur Razzak, Asing, Md Al Amin, Asing, and Azad as group members. For all the knowledge assistance, moral support, kindness and cooperation, thank you so much. Not to forget, my other research mates, Nurul Mujahidah, Fiqa, Ashraf, Dr. Faridah, Niza, Tika, Erfan, Nina, Iman and Rashid, thank you for making my adventurous Master journey more colourful, enjoyable and full of laughters.

I also would like to thank all NANOCAT staff and members for being there when I need help regarding my study. Most importantly, I would like express my greatest thank to my beloved parents and other family members for being such understand with my work and for the continuous support, precious prayers and unconditional love. At the end I would like express appreciation to my special friend, Muhammad Arif for always listen to me during my hard times, your advice and positive words are part of this success. Thank you so much.

University of Malaya

TABLE OF CONTENTS

CONTENT	PAGE
ORIGINAL LITERARY WORK DECLARATION……………………...	**iii**
ABSTRACT……………………………………………………………….	**iv**
ABSTRAK………………………………………………………………	**vi**
ACKNOWLEDGEMENT……………………………………………….	**viii**
TABLE OF CONTENTS………………………………………………	**ix**
LIST OF FIGURES…………………………………………………	**xii**
LIST OF TABLES…………………………………………………..	**xiv**
LIST OF SYMBOLS AND ABBREVIATIONS…………………………..	**x**
CHAPTER 1: INTRODUCTION……………………………………..	**1**
1.1 Project Rationale…………………………………………………	1
1.2 Research Gap…………………………………………………….	4
1.3 Study Objectives…………………………………………………	5
1.4 Scopes of Research………………………………………………	6
1.5 Thesis Organization……………………………………………...	6
CHAPTER 2: LITERATURE REVIEW…………………………………..	**8**
2.1 History of Food Authentication…………………………………….	8
2.2 Importance of Meat Identification Protocol………………………..	10
2.2.1 Consumers' Privilege………………………………………..	10
2.2.2 Religion………………..……………………………………	12
2.2.3 Culture and Lifestyle Factor………………………………	17
2.2.4 Wildlife Protection and Conservation………………….………	18

University of Malaya

2.3 *Macaca Fascicularis :* Background and Perspective…………… 21

2.4 Species Detection Methods……………………………………………… 24

2.4.1 Protein-Based Method……………………………………….. 24

2.4.2 DNA-Based Method ……………………………………... 25

2.4.2.1 Species-Specific PCR……………………………….. 29

2.4.2.2 Multiplex PCR………………………………………. 29

2.4.2.3 PCR-Restriction Fragment Length Polymorphisms (PCR-RFLP)………………………………………………… 29

2.4.2.4 Real-Time PCR (Quantification Assay)…………….. 31

2.4.2.5 PCR-Sequencing……………………………………. 32

CHAPTER 3: METHODOLOGY………………………………………. 33

3.1 Meat Samples Collection…………………………………………... 33

3.2 Design of Long-tailed Macaque Specific Primer 35

3.3 DNA Extraction……………………………………………………. 36

3.4 PCR Amplification………………………………………………… 37

3.5 Gel Electrophoresis………………………………………………… 38

3.5.1 Conventional Gel Electrophoresis…………………………... 38

3.5.2 Lab-on-a-Chip Microfluidic Automated Electrophoresis System……………………………………………………………….. 39

3.6 Limit of Detection ……………………………………..……….. 40

3.7 Target DNA Stability Test………………………………………….. 42

3.8 Enzymatic Digestion and RFLP Analysis…………………………. 43

CHAPTER 4: RESULTS…………………………………………………… 44

4.1 DNA Extraction……………………………………………………. 44

4.2 Specificity Test…………………………………………………….. 45

University of Malaya

4.2.1 Theoretical Analysis………………………………………… 45
4.2.2 Specificity Analysis by PCR……………………………….. 52
4.3 Assay Sensitivity…………………………………………………… 55
4.3.1 Sensitivity in Pure State…………………………………….. 55
4.3.2 Sensitivity under Binary Mixture Background……………… 56
4.3.3 Commercial Meat Product Analysis………………………… 58
4.4 DNA Stability Test………………………………………………… 59
4.5 PCR-RFLP Analysis……………………………………………….. 62
4.5.1 *In-Silico* Analysis……………………………………………. 62
4.5.2 PCR-RFLP Analysis in Pure State………………………….. 63
4.5.3 PCR-RFLP Analysis in Binary Mixture…………………….. 65
CHAPTER 5: DISCUSSION……………………………………………. 68
5.1 Samples Collection………………………………………………… 68
5.2 DNA Extraction……………………………………………………. 68
5.3 Specificity Test…………………………………………………….. 69
5.4 Limit of Detection………………………………………………….. 72
5.5 Target DNA Stability under Heat Treatment………………………. 74
5.6 RFLP Analysis……………………………………………………... 76
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS………... 79
6.1 Conclusions………………………………………………………… 79
6.2 Recommendations for Future Work……………………………… 81
REFERENCES………………………………………………………… 82
LIST OF PUBLICATIONS…………………………………………. 91
APPENDICES………………………………………………………… 96

University of Malaya

LIST OF FIGURES

FIGURES **PAGE**

Figure 3.1: Flow-Chart of Research Methodology………………………….. 34

Figure 3.2: Composition of 100 bp DNA ladder used in electrophoretic separation of PCR products………………………………………………… 39

Figure 3.3: Experion DNA chip 1K. The locations of the gel priming well (GS, highlighted) and alphanumeric priming codes are indicated………… 40

Figure 4.1: *In-silico* analysis of the monkey specific primers. Dendogram built from the 120 bp regions of d-loop gene sequences of monkey and other 17 land and aquatic species using Neighborhood-Joining method………… 49

Figure 4.2: Phylogenetic tree of 34 monkey species……………………… 50

Figure 4.3 3D plot showing the discrimination of long-tailed *M. facicularis* target in the primer binding sites of 51 species………………………………… 51

Figure 4.4: Specificity test gel image of macaque-specific primer pair against monkey and other 17 DNA animal species 52

Figure 4.5: Cross-specificity of monkey-specific primers against DNA of 17 different meat species by automated electrophoresis………………………. 53

Figure 4.6: Electropherogram of monkey-specific DNA (120bp) and 18S rRNA endogenous control (141 bp) PCR products of all non-target species ….54

Figure 4.7: Gel image of sensitivity test under pure state from 100 to 0.0001ng of total DNA extracted from monkey meat……………………… 55

Figure 4.8: Gel image (automated electrophoresis) of sensitivity analysis under pure from 100 to 0.00001 ng of total DNA extracted from monkey meat………………………………………………………………………. 56

Figure 4.9: Gel image of sensitivity test in monkey and beef admixed……… 57

Figure 4.10: Gel image of sensitivity test in monkey and goat admixed… 57

Figure 4.11: Monkey meat detection in commercial meatballs of chicken (Lanes 1-4) and beef (Lanes 5-8)…………………………………………… 58

Figure 4.12: Marker DNA stability test under boiling treatment………………59

University of Malaya

Figure 4.13: Marker DNA Stability Test under Microwave Treatment……. 60

Figure 4.14: Marker DNA Stability Test under Autoclaving Treatments…… 60

Figure 4.15: Restriction Maps of *Alu*I(Upper) And *CViKi*-1(Lower) on The 120 bp of Monkey D-Loop Gene…………………………………………… 62

Figure 4.16: Restriction Maps of *Alu*I (Upper) and *CViKi*-1 (Lower) on The 141 bp of 18s RNA Endogenous Control………………………………….. 62

Figure 4.17: Restriction Pattern Analysis of Monkey-Target (120 Bp) and Endogenous Control in Pure State………………………………………….. 63

Figure 4.18: Electropherogram Of Amplified Monkey DNA and E ndogenous Control after *Alu*IAnd *CViKI*-I Digestion 64

Figure 4.19: Specificity and Sensitivity Analysis in Binary Mixture of Monkey and Chevon………………………………………………………. 65

University of Malaya

LIST OF TABLES

TABLES	PAGE
Table 3.1: List of primer set used in the specificity test…………………….	35
Table 3.2: Parameters For PCR Assay Optimization……………………….	38
Table 3.3: Ingredients Used In Chicken, Beef And Monkey Meatball Preparation (Rohman et al., 2011)……………………………………….	42
Table 3.4: Restriction Enzyme Reaction Preparation……………………….	43
Table 4.1: Concentration of DNA Extracted From All Species in form of Raw, Heat-Treated and Meat Mixture (Binary and Commercial Product)…	46
Table 4.2: The mismatch comparison of the long-tailed macaque specific forward and reverse primers against 28 species …………………………...	47
Table 4.3: Pairwise distances of the monkey-specific 120 bp d-loop site against non-target tested species………………………………………….	48
Table 4.4 Analysis of beef and chicken meatball using *M.fascicularis* d-loop gene (120 bp)-based PCR assay	61
Table 4.5 Restriction sites of *Alu*1 on 120 bp monkey mt-D loop gene……	66
Table 4.6 Restriction sites of *CViKI*-1 on 120 bp monkey mt-dloop gene…	67

University of Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

$	: dollar
%	: percent
′	: prime
°C	: degree Celsius
μg	: microgram
μl	: microliter
μM	: micromolar
pg	: picogram
≥	: greater than or equal to
3D	: three dimensional
A	: adenine
A_{260}/A_{280}	: ratio of UV at absorbance 260 nm and 280 nm
ABI	: applied biosystem
ATP 6	: ATPase subunit 6
BLAST	: Basic Local Alignment Search Tool
bp	: base pairs
C	: cytosine
COI	: cytochrome c oxidase subunit I
Cq	: quantification cycle
CE	: capillary electrophoresis
Ct	: threshold cycle

University of Malaya

cyt b : cytochrome b

DBKL : Dewan Bandaraya Kuala Lumpur

dH_2O : distilled water

d-loop : displacement loop

DNA : deoxyribonucleic acid

dNTP : deoxyribonucleoside triphosphate

ds-DNA : double stranded- deoxyribonucleic acid

EC : European Commission

ELISA : enzyme-linked immunosorbant assay

E-nose : electronic nose

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

LOD : limit of detection

Ltd : limited

M.fascicularis *Macaca fasciularis*

MEGA5 : Molecular Evolutionary Genetics Analysis version 5

mg : milligram

$MgCl_2$: magnesium chloride

University of Malaya

Min : minute

ml : mililitre

mM : milimolar

Mt : mitochondrial

mtDNA : mitochondrial deoxyribonucleic acid

NCBI : National Center Of Biological Information

ND2 : 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

PCR : Polymerase Chain Reaction

PCR-RFLP : Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

psi : pounds per square inch

rpm : rotations per minute

rRNA : ribosomal ribonucleic acid

s : second

T : thiamine

Taq :*Thermus aquaticus*

Tm : melting temperature

UV : ultraviolet

w/w : weight/weight

University of Malaya

CHAPTER 1: INTRODUCTION

1.1 Project Rationale

Illegal trades of certain endangered populations are quite prevalent, threatening a multitude of species including primates, carnivores, ungulates and wild fowl in natural habitates (Fajardo et al., 2010). Although humans have hunted and eaten wild meat for millennia, consumption over the recent years has increased dramatically (Milner-Gulland and Bennett, 2003). The recent trend of meat preference shows higher interest in wild meat over the red meat due to its nutritional facts such as lower content total dietary and saturated fats (Hoffman & Wiklund, 2006). It has been a great appeal for exotic meat lover because of their exquisite tastes and healthier attributes in terms of lower fat (< 3%) and high protein contents (16-55%) (Hoffman & Cawthorn, 2012). In many part of Africa bush meat has been consumed and total consumption has been estimated to be 3.8 million tons of primate meat. For instance, it has been estimated that the exploitation of primates in the Tai region of Ivory Coast represents a market value of $124,031-136,688 per annum (Estrada, 2006). Meanwhile in Southeast Asia, Malaysia is one of the intensely hunting countries where approximately 108 million of bushmeat animals are killed for consumption in each year (Bennet,2002; Bennett et al,2000).

Long-tailed macaques (*Macaca fascicularis)* have been enlisted as the least concern species by IUCN (International Union for Conservation of Nature) Redlist (Ong & Richardson, 2008). However, the population of this species has greatly reduced as a consequences of enormous hunting both for consumption and research (Eudey, 2008). Over 5.5 million primate specimens had been traded legally or illegally between 1990 and 2004 according to the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) trade database (www.cites.org) (Rönn et al., 2009). This data is only a minor fraction of actual trades since illegal trades are often unreported and

University of Malaya

represent only less than 1% of the actual figure. The excessive harvesting of wild animals for meat and the concomitant declines in many species presents a major threat both to biodiversity and people's livelihoods (Hoffman & Cawthorn, 2012).

In 2010, an adulteration case of long-tailed monkey meat has been reported in Indonesia, where the meat was replaced for beef in meatball soup (Creagh, 2010). Recently, the Times of India reported the trades of monkey meat in certain regions of India is rampant for the export of monkey meat and brains to Africa, Japan, Korea, Taiwan, China and other countries through certain agencies (Drolia, 2014). In Malaysia, it could be frequently heard in folklores, that the aboriginal Malaysians consume monkey meat from the wild hunting. Eventhough, there is a lack of official documentation on this issue, it is believed monkey 72 species are certainly a potential candidate to be adulterated in food in certain areas where its population density is very high and could be easily hunted free of charge.

Bushmeat is also well known as a potential carrier of many emerging zoonotic diseases (Brown, 2004). For instances, the spread of Ebola infection is associated with consumption of bushmeat, mainly the chimpanzees found in western Africa (Georges-Courbot et al.,1997). Furthermore, trichinellosis has long been associated with consumption of undercooked meat from wild animals, such as bears, and now consumption of uncooked meat from deer and wild boar has recently been implicated with emergence of severe cases of Hepatitis E among the hunters in Japan (Chomel et al., 2007). Industrialized nations' new taste for exotic food has also been linked with various zoonotic pathogens or parasites, such as protozoa (Toxoplasma), trematodes (*Fasciola sp., Paragonimus spp.),* cestodes (*Taenia spp., Diphyllobothrium sp*.), and nematodes (*Trichinella spp., Anisakis sp., Parastrongylus spp*.) (Chomel et al., 2007). Despite

University of Malaya

representing a huge population, monkey cases are not well reported, leaving a clear room for the development of a reliable and convenient assay for the confirmed identification of *M. fascicularis* in food matrices and under compromised states.

For consumers protection and transparency maintenance in food business, a myriad of techniques including enzyme-linked immunosorbent assay (ELISA) (Ayaz et al., 2006),liquid chromatography (Chou et al., 2007), species-specific PCR (Kesmen et al., 2007), multiplex PCR (Matsunaga et al., 1999), randomly amplified polymorphic DNA (RAPD) (Arslan et al., 2006), PCR restriction fragment length polymorphism (RFLP), and real-time PCR (Dooley et al., 2004) and nanoparticle biosensors (Ali et al., 2011;2012c) have been proposed for the identification of meat species. In meat speciation, DNA-based techniques have been preferred over protein and lipid-based molecular identification schemes since DNA biomarkers, especially the short-length ones, are extremely stable even under harsh processing condition (heat, pressure and chemical additives) as well as compromised states such as natural decomposition or degraded specimens where protein-based markers are denatured or degraded and lipid-based biomarkers can be made rancid (Fajardo et al., 2010). DNA also preferred because of its universality in all cells, tissues and organs. Currently, PCR based DNA detection scheme is the method of choice since it can amplify multiple copies even from a single or few copies of target DNA, allowing the detection of very small amount of target biomaterials. Here a very short-amplicon-length (120bp) PCR assay was developed, targeting mitochondrial d-loop gene which is present in multiple copies and validated it by restriction fragment length polymorphism (RFLP) analysis for the authentic detection of macaque monkeys in raw, processed and admixed matrices.

University of Malaya

Species-specific PCR-RFLP assays are advantageous since they not only amplify-specific targets but also authenticate whether real-targets are detected through a post-PCR restriction digestion (Ali, Hashim, Mustafa, Che Man 2012). They have special interest in meat speciation because they exploit the sequence variations that exist within a defined region of target DNA, allowing differentiation of even closely related species by digestion of selected DNA fragments with appropriate restriction enzymes (Fajardo et al. 2008). Selection of shorter target is advantageous since it can survive under compromised states (natural decomposition or forceful breakdown of DNA) (Smith et al. 2002).

1.2 Research Gap

In meat speciation, DNA-based techniques are preferred over protein and lipid-based molecular identification schemes since DNA biomarkers, especially the short-length one, is extremely stable to harsh processing condition (heat, pressure and additives chemicals) and compromised states (natural decomposition) where most protein are denatured or degraded (Fajardo et al., 2010). Mitochondrial DNA (mtDNA) are especially suitable for meat speciation applications since they are found in multiple copies in each cells and their polymorphisms are adequate (Murugaiah et al., 2009). On the other hand, Polymerase Chain Reaction (PCR)-based detection schemes are amazing since they are simple, cost-effective and robust and can amplify marker DNA targets even from a single or few copies to detectable quantities (Ali et al., 2011). Species-specific PCR (Che Man et al., 2012; Haunshi et al., 2009; Karabasanavar et al., 2014; Mane et al.,2012) , multiplex PCR (Ali et al., 2015) (Dooley et al., 2004) randomly amplified polymorphic DNA (RAPD) (Arslan et al., 2006), PCR restriction fragment length polymorphism (RFLP) (Ali et al. 2012a), and real-time PCR (Ali et al., 2012b) are some of the significant reports for the identification of meat species.

University of Malaya

Species-specific PCR-RFLP is advantageous since it not only amplifies specific targets but also authenticate whether real-targets are detected through a post-PCR restriction digestion (Ali et al., 2012). It has special interest in meat speciation because 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). PCR-RFLP assays have been documented to distinguish between closest species such as cattle- buffalo and sheep-goat (Girish et al., 2005), swine and wildboar (Fajardo et al., 2008), various fish species (Wolf et al., 2000) and cattle and yak (Chen et al., 2010). However, no PCR-RFLP assay has been documented for macaque meat speciation. To fill up this research gap, a very short (120bp) target of d-loop gene were successfully amplified using macaque-specific primers and confirmed its authenticity by digesting with two different restriction-enzyme (*Alu*I and *CViKI*-1) and ensured distinctive restriction patterns by RFLP analysis using an automated electrophoresis system.

1.3 Study Objectives

The main purpose of this study was to develop a short-amplicon-length PCR-RFLP assay for the confirmed detection of *M. fascicularis*. The details of the objectives are outlined below:

a) To develop and characterize short-length DNA biomarkers for the detection of long-tailed macaque monkey meat under food matrices.
b) To optimize and validate the developed assay for the analysis of processed foods.

University of Malaya

1.4 Scopes of Research

The use of short DNA amplicons are gaining interest in food authenticity assessment. DNA sequence is advantageous thanks to its durability against harsh processing treatment, they still can be traced out even after they have been subjected to apply with high temperature and pressure (Ali et al., 2014).Though larger amplicons are detectable, amplicons with length less than 150 bp has been proved shown to give the highest sensitivity (the smaller the amplicon, the higher sensitivity) (Hird et at., 2006). This work has proposed the development of short amplicon assay based on sequences which are present in multiple copies in cell compared to other DNA to have a highly sensitive assay.

On the other hand, performance tests of the developed DNA primers targeting monkey mitochondrial d-loop gene was analysed by polymerase chain reaction (PCR) technique. However, the technique often reduces assay specificity, making the final results unreliable to come up with a solid conclusion (Hird et al., 2006). Plus, the verification of PCR product cannot be determined as the end-point PCR lack of information. Thus PCR-assay coupled with restriction fragment length polymorphism (RFLP) were used to identify authentic PCR-product if the amplicon contain appropriate restriction site (Aida et al., 2005, Murugaiah et al., 2009). This study proposed the development of PCR-RFLP assay with shorter amplicon containing markers that include an appropriate restriction site.

1.5 Thesis Organisation

There are five chapters in this thesis; Introduction, Literature Review, Materials and Methodology, Results and Discussion, and Conclusion. Introduction in Chapter 1 basically is a brief description of the whole study which includes the project rationale, research gap, scope of research and objectives of the study. Meanwhile, Chapter 2 covers

University of Malaya

a detailed literature review on the history of food authentication, importance of meat identification protocol, background of target species long-tailed macaque, and detection methods for species authentication. All materials and procedures done throughout the study are described in Chapter 3. Chapter 4 presents the results obtained including DNA extraction, specificity test, assay detection limit (sensitivity test) in various matrices as well as assay validation analysis by PCR-RFLP. In Chapter 5, the findings and the outcomes of the research are extensively discussed here. Lastly, the summary of the whole research is presented in Chapter 6, as well as the suggestions for future work.

University of Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 History of Food Authentication

Non-authentic foods as defined by Hargin (1996) are any unnatural foods, ingredients or derivatives purposefully manipulated by the manufacturers or suppliers to satisfy buyers' demands. He classified it in several ways: i) complete or partial omission or abstraction of valuable constituents; (ii) whole or partial substitution of a food component with an undeclared alternative (usually cheaper); (iii) concealment of damage or inferior foodstuffs; (iv) adulteration: addition of undeclared substances or material so as to increase product bulk or weight or make the product appear better value than its original contents. This issue is not new or isolated event since it was reported in 13th century A.D (Singh & Neelam, 2011). All food types are potentially susceptible to adulteration either with food originating from animals (meat product, fish, and dairy products) or plant (oil, cereals, nuts, soybean and alcoholic drinks). Authenticity issues that are often raised includes the origin of the food (geographical, sex, state of meat either wild or farmed), substitution of cheaper materials (less-value species, blood plasma, MRM, fats or plant protein), treatment (irradiation/thermal/frozen/thawed) and genetically modified organism (Hargin, 1996).

Historically, meat is not widely referred as being a major contributor to the list of products associated with adulteration, probably because it is generally sold fresh with easily recognisable appearance (Hargin, 1996). However, the growing world population has put the demand for proteins on meats. The global meat consumption has been estimated to be 41.2 kg per capita per year (Cawthorn, Steinman, & Hoffman, 2013) and it has been on increasing trends. The ongoing modernization and increased wages are enabling people to enjoy meat cuisines, keeping the consumption increasing. It is

University of Malaya

estimated that total meat intake by less-developed countries has crossed 107 million metric tons per year (Delgado, Rosegrant, Steinfeld, Ehui, & Courbois, 2001).

To keep up with this trend, food companies are vigorously competing to produce more meat supply including raw meat itself and various meat products. Minced meat, sausages, burger patty, meatball are the most common ones and are being widely consumed around the world regardless of the brands. Among the choices, consumers tend to buy the processed meat products since they are ready-made, time-saving, and could be consumed without much efforts. However, consumers nowadays becoming more alert on the increasing happenings of fraud labelling and adulteration (Stamouli et al., 2006). Consumers often rely on the information given on the labelling which reflects the quality attributes, nutritional information, surveillance assurance and thus give confidence to consumer on a particular product (Bernués, Olaizola, & Corcoran, 2003). The European Parliament and the Council of Agricultural Ministers agreed on a new regulation that obliged the EU beef industry to label the origin of beef and beef products being sold (EC Regulation 1760/2000). In this way, consumers benefit from EU-wide compulsory beef labelling rules. Apart from the compulsory label system, a voluntary scheme also permits other quality indications to appear on the labels of beef and beef products. Any undeclared species could be detected due to the concern of consumer's health as they might consist of fatal bacteria/virus presented in the unknown species (Bernués et al., 2003).

Despite having stringent labelling rules the authenticity of the halal meats and meat products have been seriously questioned in several countries (Lever & Miele, 2012). This is due to the growing meat adulteration issues around the world such as the recent horse meat controversy across Europe (Castle, 2013), multiple species substitute (rat and fox for mutton in China) (Kaiman, 2013) and mislabelling of pork or its derivatives in

University of Malaya

several countries (Aida et al.,2005). These incidences reflect fraud and/or incorrect labelling are a never-ending issue, and it has given researchers, regulators, manufacturers and distributors a brain-storming on what to detect, when to detect and how to detect species ingredients in foods to ensure transparency and fair trade in food business (Ali et al.,2014). Thus verification of food ingredient is a must not only to protect honest traders and manufacturers, but also to safeguard public health and customers' interest in terms of religious credence, personal choices and of course hard-earned wages (Ali et al.,2013) .

2.2 Importance of Meat Identification Protocol

2.2.1 Consumers' Privilege

Consumers must have the right to make a well-defined purchase decision by giving them product details before the purchase has been. Lifestyle, culture, religion, diet and health concerns are the major factors that influence purchase decision. The recent fraud labelling issues have made people critical to justify the product details given on the labels. Foods basically provide nutrients for body however any misleading substances such as allergens can results threatening reaction to sensitive person. Several groups of ingredients with the highest possibility of causing allergy could be found in the Codex Alimentarius FAO/World Health Organization (WHO) and the European Commission (Mafra et al., 2008). These include milk, eggs, fish, crustaceous, peanuts, soybean, walnuts, whey and other cereals with gluten.

Although meats are widely consumed all over the world, meat and meat products seemed to lack inspection in terms of meat origin, quality, cleanliness as well as the packaging which sometimes results in various public health crises (Sofos, 2008). The food-borne diseases caused by pathogenic microorganisms were among the top highlighted consequences related to meat adulteration. Report from United States

University of Malaya

Department of Agriculture Food Safety and Inspection Service (USDA/FSIS) showed, from 1998 to 2002, up to 18 million kg of fresh meat and processed meat products were recalled due to contamination of *E. coli, Salmonella sp. and L .monocytogenes* ,while fresh meat usually associated with gram negative bacteria *E.coli, Salmonella sp.,* ready-to-eat meats (manufactured, fermented and processed meats) are more likely to have high risk to contain gram positive *L. monocytogenes* (Sofos, 2008). Data from New South Wales (NSW) Food Authority website (http://www.foodauthority.nsw.gov.au/), shows the same result in which processed meats are associated with the said bacteria mainly caused by the activity of slicing and throughout the manufacturing procedure. According to the report on the website, there were 49 recalls on ready-to-eat meat products since 2004 in Australia due to the same reason. In addition, animal health issues such as bovine spongiform encephalopathy (BSE), avian influenza and swine influenza too, gives the overwhelming situation since cows, poultry and pig are the major meat resources play an important role for food and livelihoods in many countries.

Apart from that, the controversial horsemeat contamination in meat products that spread around U.K last year has caused a major anxious among consumer due to dangerous drugs (phenylbutazone, a painkiller and fevers cure) that horse meat might contain and might enter the food chain once it is consumed (McKie, 2013). Similar incident might happen due to pests and wild meats adulterated into common meats. Studies showed wild animals contained numerous fatal bacteria and parasites and caused various zoonoses, such as anthrax, brucellosis, tuberculosis, toxoplasmosis, trichinosis, and bovine spongiform encephalopathy (BSE) that may be transmitted from animals to human (Ramanzin et al., 2010). They are also associated with several diseases that affect humans such as monkeypox, which originated from African rodents; the SARS coronavirus, which has been associated with the international trade in small carnivores

University of Malaya

(Wyler, 2008). In addition, primates were reported to carry Simian Immunodeficiency Viruses (SIV) which caused HIV/AIDS through consuming primates as well as Ebola haemorrhagic fever in case of direct contact with infected great apes (Bennett, 2002).

2.2.2 Religion

Apart from the adverse impact on societal health, meat-adulterations have considerable impacts on religions, cultures, personal budgets and fair-trades (Ali et al., 2012a). Some of religions have outlined specific food consumption laws either due to ancient history, or as a respect for God or for the good reason behind the do's and don'ts do's of food intake (Meyer-Rochow,2009). Hindus are, for instance, prohibited to eat cow meat (sometimes it's milk derivatives) because they believe cow is one of the God's representative hence consider it as a sacred animal (Meyer-Rochow, 2009). Meanwhile, Judaism dietary laws is all about anything that "fit or proper" for consumption, with a clear forbidden of pig in their meal and restriction of milk and meat combination in the diet (Regenstein et al.,2003). Certain types of grains are also restricted on their Holiday of Passover. The kosher market is currently expanding especially in Europe while it has a strong footing in the United States. It has been estimated that about half of packaged goods in common American supermarket are certified as kosher (Regenstein et al.,2003).

Compare to Judaism, Christianity and Islam have not strongly promoted vegetarian diet. However, religion that originated in ancient India (Hinduism, Jainism and Buddhism) have strongly practiced vegetarianism in daily life. While vegetarianism is mandatory for everyone in Jainism, it is advocated by some influential scriptures and religion authorities of Hinduism and Buddhism (Davidson, 2003). Jain may never consume meat even meat from an animal that has died because they believe a natural death contains innumerable *nigodas* and must be absolutely avoided. Thus the

University of Malaya

vegetarianism arises from the necessity of purifying the soul of its attachments to and contamination from matter. The ultimate objective is the denial of the body and purification of the soul, as a necessary step to win the soul's release from matter (Davidson,2003).

For Muslim, HALAL certified meats and meat products have been rapidly expanding and huge attention has been given to it to fulfil Muslim's dietary requirements and huge market demands (Hanzaee & Ramezani,2011). Since the Muslim population has crossed 1.8 billion, halal markets are getting challenging and Halal foods demand are rising every year. Global halal market nowadays appears to be one of the fastest growing business sectors in the world as they estimated to worth more than USD2.3 trillion (World Halal Forum, 2013). Focusing on the food sector, it contributed approximately USD700 billion to global halal industry in order to fulfil the requirement of nearly 1.8 billion Muslim population in the world (World Halal Forum, 2013). The rising of Halal food demand reflects that the consumers have put their trust to the authorities to serve the best for them which include the sources, cleanliness, and ingredients. The renowned term "Halal" is originated from an Arabic word to describe anything that is lawful by Shariah (Islamic Law). Therefore, Halal food is any food that is permissible to be consumed as described in the holy book (The Qur'an), the Hadith (the practices and sayings of Prophet Muhammad (peace be upon him) and the fiqah (the consensus Opinions of a group of Islamic Scholars). Generally, every food is Halal except what has been prohibited by the Islamic law as Haram (not allowed). The concept of Islamic dietary rules mainly stressed on consumption of only good things and to avoid anything that is harmful for body and health as demonstrated in Quran verses below:

"O People, eat from the land what is permitted & good & do not follow in the footsteps of Shaitan, for he is an open enemy to you"

University of Malaya

(*AlBaqarah, 2:168)*

"O ye who believe! Eat of the good things where with WE have provided you and render thanks to ALLAH if it is He whom you worship"

(Al-Baqarah 2:172)

Discussion on Muslim's meat intake and consumption were heavily discussed in Bonne & Verbeke (2008). According to them, Muslim migrants in America and Europe are heavy consumers of meats. Although the halal market in North America and the United Kingdom have been the subject of some studies, research on European Muslims food choice in general and meat consumption in particular is extremely common. Results of a household panel survey in the Netherlands (Foquz, 1998) shows that Muslims consume meats quite heavily. The average meat consumption per Dutch consumer was 35.6 kg in 1998. Turkish consumers, however, ate on average 61.3 kg meat per year and Moroccan consumers 57.1 kg per year. The total spending power of Muslims in the US was estimated at $12 billion in 1999, of which $3 billion went for meat and poultry (Bonne & Verbeke,2008) The global halal market for foods is estimated to be 1.5 billion consumers (Bonne & Verbeke,2008) which means that one in four consumers worldwide buys halal products. Nowadays, Muslims are making their presence to be felt socially and politically, putting the demand for halal-labelled food products on the rising spree. In France, for example, the first fast food restaurant, Burger King Muslim, opened in 2005 targeting young Muslims desiring halal convenience foods. It differentiates itself from other ethnic, halal restaurants by publicly confirming its Islamic identity and thereby it responds to the rise of a strong Islamic attitude among young Muslims expressed by consuming halal foods and wearing Islamic inspired clothing (Bonne & Verbeke, 2008).

University of Malaya

For Muslim, the great concern regarding diet is to totally avoid the consumption of pig meat in their meal. This prohibition was clearly stated several times in the holy Quran (Al-Baqarah 2:173, Al-Ma'idah, 5: 3, Al-'An`ām 6:145 ,An-Nahl 16:115)

"He only prohibits for you the eating of animals that die of themselves (without human interference), blood, the meat of ***pigs****, and animals dedicated to other than God. If one is forced (to eat these), without being malicious or deliberate, he incurs no sin. God is Forgiver, Most Merciful."* (Al-Baqarah 2:173)

"Forbidden unto you (for good) are carrion and blood and flesh of the ***swine,*** *and that over which is invoked the name of other than Allah, and the strangled, and the dead through beating, and the dead through ,falling from a height, and that which has been killed by (the goring of) horns, and the devoured of wild beasts, unless you have cleansed (by slaughtering) it in the proper, lawful way, while yet there is life in it, and that which has been immolated unto idols. And (forbidden is it) that ye swear by the divine arrows. This is an abomination."* (Al-Ma'idah, 5: 3)

"I do not find within that which was revealed to me [anything] forbidden to one who would eat it unless it be a dead animal or blood spilled out or the flesh of ***swine*** *- for indeed, it is impure - or it be [that slaughtered in] disobedience, dedicated to other than Allah . But whoever is forced [by necessity], neither desiring [it] nor transgressing [its limit], then indeed, your Lord is Forgiving and Merciful."(*Al-'An`ām 6:145)

"He has only forbidden to you dead animals, blood, ***the flesh of swine****, and that which has been dedicated to other than Allah. But whoever is forced [by necessity], neither desiring [it] nor transgressing [its limit] - then indeed, Allah is Forgiving and Merciful."*

University of Malaya

(An-Nahl 16:115)

Pig meat and its derivatives (e.g., gelatin made from bones, cartilage, tendons and skin of pigs) are strictly prohibited to be consumed by the Muslims. Often, there are significant reasons behind the prohibition in Islamic Law. For example, pig is believed to be unclean. It has been proven by numerous studies that it is associated with various health disadvantages following consumption. Pig poses resistant virulent bacteria that can results in food-borne disease (Zhou et al., 2012; Valentin-Weigandb et al.,2014), trichinosis roundworm infestation (Conlan et al., 2014) and well-known contain very high unhealthy fats (Enser, Hallett, Hewitt, Fursey, & Wood, 1996; Wood & Hughes, 2007). Due to the religious restriction, the substitution of porcine derivatives issue drew a great attention from both Muslims and Kosher practisers. Many food manufacturers all over the world choose pork as meat substitute obviously since they are cheap and abundantly available in farms. Beside the meat adulteration, pork derivatives include pig fat (lard), mechanically recovered meats (MRM), porcine gelatine and porcine blood plasma were used in the meat processing industry (Nakyinsige, CheMan, & Sazili, 2011).

Muslims are not merely forbidden consuming meat of pig or its derivatives, there are descriptive outline regarding the criteria of food that are permissible or not listed in the Holy Quran. All verses cited above also mention the animal must be slaughter properly with the name of Allah, not to consume the dead animal, either naturally or killed by other animal, and blood. According to the Shaykh (Mufti) Muhammad Ibn Adam (Darul Iftaa), one of the main criteria of prohibited animals are those animals that attack with their fangs among predatory animals, such as lions, wolves, dogs, cats, bears, monkeys, elephants. Also impermissible are those that attack with their claws, their nails that is, of birds such as the falcon, the eagle and others of that type. Unlike pig and domesticated donkey which is impermissible as clearly stated in the Quran and Sunnah,

University of Malaya

monkey is included in the prohibited list because they are scavengers that hunt with sharp teeth/ fangs. Besides that, a question and answer session (through email) has been carried out with local Selangor’s Mufti and according to him, all food and drinks are permissible to consume as long as there are no prohibition of it stated in Quran or Sunnah. However, according to Mazhab Shafie in this case (monkey meat), is categorized under Haram species based on a Hadith narrated by Abu Tha'laba al-Khushani which reported that Allah's Messenger (ﷺ) is prohibited from the eating of all fanged beasts of prey (Sahih Muslim). Thus, Selangor’s Mufti concluded that monkey is prohibited to consume.

2.2.3 Culture and Lifestyle Factor

Culture and lifestyle also play an important role in species identification. Lifestyle such as practicing vegetarian are one good example to describe how food involved purchase decision making. Though there are several types of vegetarians, the main idea is abstaining to have meat in from the diet (Ikeda, 1999). People are also concerned about their health they become really concern about details ingredient whether the food has high content fat, unsafe additive, preservative, or colouring (Ikeda, 1999). In Western culture, health professionals are trained to view food as a source of nutrients, which provide energy, regulate body processes, and furnish essential compounds needed for growth and maintenance of the human body (Ikeda, 1999). The assumption is that people will purposely choose foods that contribute to their long-term physical wellbeing by reducing their risk of chronic disease (Ikeda, 1999). Chinese practices Yin & Yang philosophy , considered yang, or ‘hot’, while others are considered yin, or ‘cold’. If an illness is considered yang, then the patient needs to consume yin foods, and vice versa. The categorization of foods as hot or cold is not necessarily consistent between or within cultures. The best way health professionals can deal with the inconsistency is to ask if the

University of Malaya

patient is avoiding or favouring any particular foods when treating their condition (Ikeda, 1999).

2.2.4 Wildlife Protection and Conservation

The consumers not only expect their foods to be safe, healthy, and tasty, but also expect the products of which his food is made to be produced and transformed in concordance with good cultivating practices, and with the greatest respect for the environment and animals welfare (Pascal and Mahe, 2001). However, the recent trend of meat preference is quite worrying especially to those who are concern about healthy diet regime, they refuse or trying to reduce red meat intake due to its total dietary fat and saturated fat content (Hoffman & Wiklund, 2006). Therefore, wild/game meat now gradually is becoming their top choice. Wild meats have been a great appeal for exotic meal lover because of their exquisite tastes and healthier attributes because of their contents of low fats (< 3%) and high proteins (16-55%) (Hoffman & Cawthorn, 2012). South Africa, Australia, Europe and America are the major producers of game meats such as deer, kangaroo and ostrich and hence these animals are reared in farms for meats (Hoffman & Wiklund, 2006). However, insufficient domestic supply and overprices of red meats in some developing countries might push consumers to hunt their own animal proteins from natural habitats such as forest and bushes (Hoffman & Cawthorn, 2012). But the main concern is the way these wild animals are obtained either with permits or illegally caught since they are live wild and easily caught without the supervision of authority bodies. Though most of the country will export them legally for consumption and research study, there are no doubt that illegal poacher or personal hunters make profit buy trading in the black market. In Africa, wild meat trade for consumption purpose is huge and this including species such as primates, ungulates and wild fowl (Hoffman &

University of Malaya

Wiklund, 2006). This issue, apart from the meat species adulteration, are one of the main concerns related to the importance of species identification procedure.

According to Interpol, wildlife trafficking is the second largest form of black market commerce, behind drug smuggling and just ahead of illegal arms trade. This wildlife market that regulated by the UN 1975 Convention on the International Trade in Endangered Species involves number of countries worth an estimated $159 billion, involves the annual trade in more than 350 million animals and plants (Warchol, 2004). Globally, trade in illegal wildlife estimated to be worth at least $5 billion and potentially in excess of $20 billion annually and reasons behind the current demand for illegal wildlife and related products appear to vary according to regions and cultures (Wyler & Sheikh, 2008). Throphies, souvenier, body accessories and sold as pet are the end product of the purchased of wild animal in Europe. However, most of the demand (Asian, African, European) mainly due to human consumption; either as a protein source or as traditional medicine (Engler, Parry-Jones, & Europe, 2007; Lin, 2005; Wyler & Sheikh, 2008). It has been believed that exotic meats could lift up the internal energy, give longer life and increase juvenility and youth hood (Hoffman & Cawthorn, 2012). It includes the trade in live exotic and endangered animals (mammals, birds and reptiles) sold to private collectors, pet shops, animal brokers, game farms, biomedical labs, circuses, and even exotic meat dealers (Burgener, 2002; Speart, 1993).

Total wild meat harvest for consumption across central Africa is estimated to be between 1 million and 3.4 million tonnes per annum (Wilkie & Carpenter 1999; Fa & Peres 2001), and in Brazilian Amazon is estimated to be between 67,000 to 164,000 tonnes per year (Robinson & Redford, 1991). A variety of different wildlife species inevitably remain a cheap source of protein for many population groups, particularly in

University of Malaya

the developing world, and as such contribute substantially to food security in these regions (Wilkie and Carpenter, 1999; Fa et al., 2001). The market trend in favour of game meats has been reflected by the increase from 600,000 head of game in 1964 to 18.6 million in 2007, with a result that 80% of game animals are being kept on private land. The capacity of most countries to import food to fulfil their protein requirements is often limited due to the global scarcity in meat supply, the associated foreign exchange burden, and the low disposable incomes of many of their citizens (Hoffman & Cawthorn, 2012). Although humans have hunted wildlife for over 100,000 years, consumption has increased considerably over the past few decades (Milner-Gulland and Bennett, 2003). In Africa, large mammals, primates, antelope, frogs, snakes, rodents, bats, and even insects and termites often sold on the road side or at local markets to supply a much needed source of cash revenue. The primary motivation to engage in such outlawed practices appears to be an economic gain. It is important to mention, however, that in many developing countries the income derived from illegal wildlife poaching and trading is often vital for sustaining the livelihoods, and even traditions and culture of impoverished peoples. From an extensive review on the magnitude of bush meat exploitation and consumption it is clear that this contributes between 20% and 90% of the animal protein eaten in many regions of Africa (Fajardo et al., 2010) (Hoffman,2012). Nevertheless, with the escalating demand for animal protein and the high prices associated with such products, it has been inevitable that the inhabitants of many regions of the world have become increasingly reliant on the harvesting of local wildlife species for subsistence (Hoffman & Cawthorn, 2012)

Meanwhile in Southeast Asia, wildlife has being manipulated from this biodiversity hotspot at more than six times the sustainable rate and possibly the largest in the world (Bennett, 2002; Lin, 2005). The data reveal the export of just over 35 million

University of Malaya

CITES-listed animals from Southeast Asian countries in a ten-year period from 1998 to 2007 (Nijman, 2010). In East Malaysia, more than 100 million of wild animal been hunted every year and 23500 tons were shot for consumption annually (Bennett, et al., 2000; Bennett, 2002). Malaysia is one of the major exporters for group animal including butterflies, seahorses, fish reptiles, corals and mammals, and the European Union and Japan have been the most significant importers of wild-caught animals from Southeast Asia in the last decade (Nijman, 2010).It has been believed that exotic meats could lift up the internal energy, give longer life and increase juvenility and youth hood (Hoffman & Cawthorn, 2012). When traded, these resources can further provide cash revenue where few alternative sources of income exist. In addition, wild animals can also serve as important contributors to national economies through tourism and the sale of wild animal product. The food taboo on consumption of exotic wild animal meat also make sense as they served as tonic food and prolong human's live. And for this reason, meat from wild animal has risen in demand over the supply and consumers are willing to pay for whatever the price threaten the wildlife population (Fajardo et al., 2010).

2.3 *Macaca fascicularis*: Background and the Perspective.

Macaques (genus *Macaca*) are an Old World Monkey which falls in the family Cercopithecids with the only in genus in cercopithecine (Md-Zain et al., 2010). The genus comprises up to 20 species (Abegg & Thierry, 2002) and one of its species, *Macaca fascicularis* is the third most successful non-human primate species. This species which has several name such as long-tailed macaque, crab-eating monkey as well known as cynomolgus macaque in medical research, can be found abundantly in mainland and island of Southeast Asia (Fooden, 1995) including Malaysia, Singapore, Indonesia, Brunei, Cambodia, Laos, Philippines, Thailand, Vietnam, Myanmar, Bangladesh and India and many smaller islands associated with these land masses (Brandon-Jones et al.,

University of Malaya

2004). The total population of *M.fascicularis* around the world is not well known, however, in the late 1980s, estimated the entire natural population of the species was approximately 5 million individuals (Fooden , 1995) and the most recent population was estimated about 3 million long-tailed macaques (Fooden,2006; Fuentes, 2011).

In Malaysia, this is the most common monkey species and can be found easily nationwide especially in urban areas (PERHILITAN, 2006). Macaque research study in Malaysia focusing more on the behaviour, ecology, taxanomy and phylogenetic study of the species (Abdul-Latiff et al., 2014; Hambali, Ismail, Zulkifli, Md-Zain, & Amir, 2012; Md-Zain et al., 2010).

Long-tailed macaque has been enlisted as the least concern species by IUCN (International Union for Conservation of Nature) Redlist (Ong & Richardson, 2008). However, their population is decreasing due deforestation and enormous hunting for consumption and research (Eudey, 2008). According to the CITES trade database (www.cites.org), more than 5.5 million primate specimens were traded legally and illegally from 1990 to 2004 and *M. fascicularis* is the most widely-traded species of mammal listed on the CITES. The most recent reported that Vietnam Cambodia, Laos, Indonesia, Philippines, and Myanmar, in ascending order, are now are the main exporter of this species. Long-tailed macaque is imported mainly by USA to be used as animal model in biomedical research (Street, Kyes, Grant, & Ferguson, 2007).

The exact number of illegal trade is undefined; but it is believed that about 1% of it represents the illegal trade Macaque. However, it was suggested that this is a mere percentage and therefore the total number of specimens traded are underestimated, since illegal trades usually remain not documented (Rönn et al., 2009). The greatest factors in primate population declines are habitat destruction, hunting and disease (Walsh et al., 2003). The estimated yearly hunt or export genera macaque is approximately 270,000 individuals throughout the world (Nijman, 2010).

University of Malaya

Eventhough most of the exported macaques were used for laboratory research in United States, however macaque trafficking is widespread in the Indochinese region where wild-caught macaque were exported for captive breeding but were sold for consumption (Eudey, 2008). Primates are an important source for food protein, particularly in parts of Africa, Asia and the Amazon region of South America. There is speculation that they are also being sold illegally to countries like China, South Korea, Taiwan, and Japan for consumption in exotic dinner (Murali, 2013). In addition, primates are also hunted for skins and body parts, as ingredients in traditional medicine, as pets and for exhibition. Exotic animals include frog, turtle, snakes, wild boar, monkey and tiger usually are consumed by people who practice traditional medicine and believe this kind of food are good for their health (Milner-Gulland, 2003).

In addition, a case found on monkey meats been added in meatball soup sold by Indonesian (Trowbridge, 2010). The report mentioned the reason of using monkey meat remain the same, because of the lower price or no commercial price compared to beef or lamb. There is a shortage of well documented reports about monkey meat adulteration in conventional meats in Malaysia. This might be due to the lack of attention paid to this area. However, monkey species are certainly a potential candidate to be adulterated in certain areas where its population density is very high and could be easily hunted without any offered prices or under the overlook of regulatory bodies.

The excessive harvesting of wild animals for meat and the concomitant declines in many species presents a major threat both to biodiversity and people's livelihoods (Hoffman & Cawthorn, 2012). Because of the great concern on species threats, illegal trades, meat fraudulence, and consumers' health, there is a need of meat speciation techniques, especially for wild meats such as macaque meats. Thus the development of a

University of Malaya

reliable and convenient assay for the identification of *M. fascicularis* is extremely desirable.

2.4 Species Detection Methods

The ever-increasing meat fraudulent issues give researchers a task and challenge to determine the ideal detection technique of various animal species. The current detection methods for food authenticity are enormous, however for species identification mainly focussed on DNA and protein analysis (Ballin et al.,2009). Protein-based methods include immunological assay (ELISA), electrophoretical and chromatoghraphy (GCMS, HPLC, LC/MS/MS). Initially, many tests were directed towards the identification of protein fractions in foods, including isoelectric focussing and ELISAs (Bottero & Dalmasso, 2011). However, these techniques gradually less favaourable mainly due to low specificity and unsuitability in the case of complex matrices subjected to processing, such as chilling, salting, seasoning and, most importantly, heat, which induces marked structural modification of proteins (Bottero & Dalmasso, 2011).

On the other hand, DNA-based methods includes DNA hybridization, PCR-based techniques (species-specific PCR, multiplex PCR, real-time PCR, PCR-RFLP, DNA sequencing), microarray technique, DNA barcoding, and nanobiosensor. Detection based on DNA are more interesting because of the characteristic DNA itself; well-informative, exceptionally stable, and its abundance. Other methods such as spectroscopy technique (UV, NIR, Raman, and U-Vis) and electric nose are also reliable.

2.4.1 Protein-based Methods

Even though the exploitation of protein for species analysis (especially processed foods) are less preferred due to thermal intolerance of some proteins, yet this approach is well-developed, particularly Enzyme-linked Immunosorbent Assay (ELISA), electrophoretic techniques such as Polyacramide Gel Electrophoresis (PAGE) Sodium

University of Malaya

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Isoelectric Focusing (IF), and chromatography techniques namely Gas Chromatography Mass Spectrometry (GCMS) , Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS). There are several protocol of involved immunological assay but the most common ones is ELISA. Basically, ELISA is a specific test between antibody and antigen/protein reaction on a solid surface. It is specific, sensitive, more rapid yet less costlier than genetic methods for routine analysis of large sample numbers (Asensio, González, García, & Martín, 2008). Numbers of commercial immunoassay kits for detection of raw and heat-treated sample were already available in market since decades (Meyer & Candrian, 1996), and even specifically for bovine detection (Ballin, 2010). (Chen & Hsieh, 2000) were the first ones to develop an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody to a porcine thermal stable muscle protein (skeletal muscle) for detection of pork in cooked meat products with the sensitivity of the assay was 0.5% (w/w) in meat mixtures. Immunological techniques such as ELISA have the disadvantage of being relatively insensitive to differences between closely related species (Nakyinsige, CheMan, & Sazili, 2011) and often hindered by cross-reactions occurring among closely related species (Fajardo et al., 2010). It also requires production of high titer antisera with specific antibodies for each meat species. The development process is time-consuming and the resultant assays detect only one target at a time.

On the other hand, electrophoretic procedure (polyacrylamide gels, concentration-gradient gels and pH gradient gels, 2-dimension gel electrophoresis) also applicable where protein were separated within gel assisted by electric power, and the output come as characteristic band patterns gel (Meyer & Andrian, 1996). Among all, the recent improvised two-dimensional (2-DE) is currently the top choice due to its ability to authenticate the freshness and discriminate either wild or farmed fish in both raw and processed product (Montowska & Pospiech, 2010). Lastly, Chou et al. (2007) was able

University of Malaya

to qualitatively detect a variety of meats, including pork using liquid chromatography methods that focus on protein profiles. Aristoy and Toldra (2004) used the examination of dipeptides, carnosine, anserine and belanine to qualitatively identify pork. However, the method was only applicable when different species were not mixed.

2.4.2 DNA-based Methods

The DNA molecule is extremely useful tool for molecular species identification mainly because of its exceptional stability, abundance, and richness of information (Pereira, Carneiro, & Amorim, 2008). Stability of DNA is the key of the preference especially to detect processed food products. Meat products undergone extreme routine process where high temperature and pressure were applied and this affect the biological content of the meat tissue. Unlike protein which easily denature with thermal, double stranded DNA are highly stable with stress condition, meaning good quality DNA could be extracted from various type of foods (fresh, frozen food products, processed, degraded and mixed form) (Lenstra, 2003) for evaluation. In addition, DNA can be found in multiple copies in cell organism or fluids with nucleated cells (or non-nucleated cells with plastids and/or mitochondria) make it suitable for detection though with little concentration/amount of sample. DNA also provide more information than proteins due to the degeneracy of the genetic code and the presence of large non-coding stretches (Pereira et al., 2008). Along with those favourable criteria of DNA, Polymerase Chain Reaction (PCR) is the essential technique to utilise DNA as a target of detection by amplifying from a single DNA into multiple copies. In order to develop species detection assay, it is essential to establish a specific biomarker of the desired species. Both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have been used in numerous researches according to the specification and the usefulness in the study.

University of Malaya

Mitochondrial DNA or mtDNA is another type of DNA that can be found in every nucleated cell. The mtDNA genome size is relatively small compared to nuclear genes with approximately 16,500 bp and relatively uniform in size among vertebrate and invertebrate animals. MtDNA is circular and includes for 12sRNA, 16SRNA, Cytb, CytC , ND5, and a D-loop region). However, mtDNA are favourable targets for species detection purpose due to rapid evolution and higher mutation fixation rate; about 5 to 10 times higher than nuclear DNA, mostly maternally inherited thus easier to study (Walker, Smith, & Smith, 1987). MtDNA generally evolves much faster than nuclear DNA and thus enables even closely related species to be differentiated and identified suitable biomarkers to identify even the closely related species (Teletchea et al., 2005). MtDNA can be found in almost all of the cells and due to multiple copy number (>1000x copies of nuclear DNA), it can survive extreme food processing conditions, high temperature and pressure, and environmental stresses, allowing target detection in compromised samples (Ali et al., 2012d; Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Mane, Mendiratta, & Tiwari, 2012). Different regions in mtDNA evolve at different rates, providing a range selection of regions to be chosen as a target, depending on the purpose of study (Mohamad, 2013).

2.4.2.1 Species-Specific PCR

Most of work related to DNA analysis has focussed on using PCR to amplify the specific areas of DNA of interest (Reid, O'Donnell, & Downey, 2006). The principle of PCR is that specific lengths of DNA can be copied multiple times to provide a sufficient amount of that area of DNA to be analysed using a variety of methods with electrophoretic techniques (conventional gel or automated electrophoresis) or in real time PCR, sometimes referred to as qPCR (quantitative PCR) being the most frequently used (Ballin, 2010). Species-specific PCR is so-called because a specific target gene of a species is

University of Malaya

selected and primer was design exclusively based on the sequence information of the gene, as a result the product size of the amplicon is predictable after the DNA amplification. This is usually followed by cross-amplification of the specific primer with the non-selective species DNA in order to confirm the specificity of the primer. Previous work that applied this method include detection of bovine DNA (Calvo, Rodellar, Zaragoza, & Osta, 2002), water buffalo and goat DNA (Karabasanavar et al., 2011a; Karabasanavar, Singh, Umapathi, Kumar, & Shebannavar, 2011b), sheep DNA (Martín et al., 2007), pork, horse, cat, and dog DNA (Ilhak & Arslan, 2007). The specificity of the PCR assay is not only dependent on the specific nucleotide sequence of the species, but also on the source of gene. Intra- and interspecies sequence variability and copy number of the nuclear and mitochondrial DNA can influence the performance of the PCR reaction in detection and quantification of a particular target (Mohamad, Sheikha, Mustafa, & Mokhtar, 2013). Among mtDNA genes, cytb is the most frequent target gene used in species identification (Abdulmawjood, Schönenbrücher, & Bülte, 2003; Aida, Che Man, Wong, Raha, & Son, 2005; Rahman et al., 2014; Wolf, Burgener, Hübner, & Lüthy, 2000) because often used for phylogenetic studies and as reference gene in species-specific PCR. It contains both variable and conserved regions that are sufficient to resolve divergence at a population level and give high sensitivity (1pg) (Mohamad et al., 2013). Besides that, the study on 12S rRNA (Girish et al., 2005; Martín et al., 2007), d-loop (Kumar, Singh, Singh, & Karabasanavar, 2011; Mane, Mendiratta, & Tiwari, 2009), subunits of NADH dehydrogenase 2,5 and ATPase 6, 8 (Kesmen, Sahin, & Yetim, 2007) were also provided detection limit from range 2% to 0.01% in mixture which offer a highly sensitive species detection system.

University of Malaya

2.4.2.2 Multiplex PCR

While species-specific PCR only target for a single species, multiplex PCR, in contrast, does simultaneous amplification of multiple DNA targets in a single reaction vessel (Ali et al.,2014).This technique has gained huge attention among researchers due to its outstanding multi-target detecting technique in a single assay platform, which is time and cost saving. However, the establishment of multiplex PCR is more complex and difficult than conventional PCR. It requires complicated primers design for multiple species and tough primer specificity and reaction optimization (melting, annealing, and elongation temperatures) (Ali et al., 2014; Matsunaga et al., 1999). Multiplex PCR also apparently is less sensitive compared to simplex PCR. Matsunaga (1999) developed multiplex assay of goat, chicken, cattle, sheep, pig and horse, while Di Pinto et al. (2005) targeted horse and pig , both with limit of detection 0.25 ng. Nejad et al. (2014) detected poultry, camel, donkey, goat, cattle a low as 0.05 ng. As comparison, most of the simplex PCR showed higher assay sensitivity 0.01 ng of pork, horse and donkey (Kesmen et al., 2007) and 0.0001ng of detection of pork and goat, all in pure state (Ali, Hashim, Mustafa, & Che Man, 2012a; Karabasanavar et al., 2011b).

2.4.2.3 PCR- Restriction Fragment Length Polymorphisms (RFLP)

PCR-restriction fragment length polymorphism (RFLP) is another potential and promising technique in determining the species of the meat. The method involves the digestion of PCR products by a restriction enzyme that cleaves at a species-specific restriction site. PCR–RFLP is a good option to further confirm the PCR product as compared DNA sequencing since it is time saving, and does not need expensive analytical tools (Haider et al., 2012). Right away after PCR protocol, amplicons usually subjected to incubation with two or more restriction enzymes and empirical observations made as to their utility for species differentiation (Lockley & Bardsley, 2000). Mane et al. (2012)

University of Malaya

digested the PCR product of buffalo (d-loop) gene with *Bam*HI to further confirm the primer specificity after no positive result obtained when cross-amplified the primer with other 5 animal species. PCR–RFLP also received special interest for meat speciation because 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). Previous reports on PCR-RFLP have focused their interest on mostly with the aim to distinguish between closely related species, as done by Chikuni et al. (1990). In his study, the satellite marker which was supposed to bind only with sheep DNA was seen amplify both sheep and goat DNA due to high homology of both species. Later, it was found that the two species are distinguished by four different restriction site. Other similar works include differentiation of cattle and buffalo and sheep and goat (Girish et al., 2005), swine and wild boar (Fajardo et al., 2008), various fish species (Wolf et al., 2000) and cattle and yak (Chen et al., 2010). This method is cost-effective in large scale food traceability program, especially for traceability analysis to be undertaken by developing countries, compared to direct sequencing (Murugaiah et al., 2009).

However, some criteria need to be met when handling the RFLP technique. It is important to use two or more types of restriction endonuclease and requires large amplicon size in order to be cut and analyse in the conventional gel electrophoresis. Since the movement of DNA is dependent on the concentration of the agarose gel, it is impossible to trace small DNA fragment, especially for size lower than 50 bp. Alternatively, DNA analysis of fragments as low as 15bp sized can be visualised using the current technology of automated electrophoresis system. Ali et al. (2012a) analysed their small size of restriction digested product (49,33 and 27 bp) by using a chip- based capillary electrophoresis incorporated in Agilent 2100 Bioanalyzer. Other similar automated instrument that allows detection of small fragments are QIAxcel capillary

University of Malaya

electrophoresis system (Qiagen) and Biorad Experion. Except for the high cost of the machine, this advanced tools is favourable as it is rapid, automated and offer better reproducibility, give high in resolution with less reagents and samples (≈1µl), and need no post-PCR hazardous chemicals (i.e the usage of Ethidium Bromide for gel electrophoresis).

2.4.2.4 Real-time PCR (Quantification Assay)

Meanwhile species-specific PCR only generate qualitative information, real-time PCR is a both qualitative and quantitative detection, which the latter is highly advantageous where the accumulation of amplified PCR product can be monitors time-by-time with the aid of fluorescent labelled dye. The ability to monitor the progress of DNA amplification in real time depends on the chemistries (between fluorescent probes and DNA-attached-dye) and instrumentation used (Pereira et al., 2008). Several types of probes exist but the most common is based on the use of a TaqMan fluorogenic probe (Rodríguez et al., 2004). The probe, labelled with both a reporter and a quencher dye, binds to a target DNA between the flanking primers. During PCR amplification the 5'–3' exonuclease activity of the *Taq* DNA polymerase cleaves the probe hybridized to the template, releasing the 5' reporter from the quenching effects of the 3' quencher. Fluorescence emission is measured during the reaction, and is directly proportional to the amount of specific PCR products (Rodríguez et al., 2004). This technique has been employed to detect beef, pork, lamb, chicken and turkey meats and the target product was short amplicons (<150 bp) of regions of the cytochrome b gene with limit of detection of 0.1% in pure and 0.5%in mixture (Dooley, Paine, Garrett, & Brown, 2004). Besides , this method also has successfully identified 16S rRNA of turkey, beef, pork and sheep and cyt-b gene of meat in complex food products, with the range of detection 0.02 pg and 0.80 pg and at 1% in mixture (Cammà, Di Domenico, & Monaco, 2012).. Real-time PCR is

University of Malaya

an automated process which eliminates the end-point analysis, increased sensitivity by eliminating non-target DNA and relatively fast genotyping method (Pereira et al., 2008). However the drawbacks of this method is that it only allows the amplification of short amplicons PCR (maximum 150bp) (Lenstra, 2010), and there are 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).

2.4.2.5 PCR-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 online-available DNA sequence database. Five types of tuna species from cooked and canned tuna were sequenced and identified from the amplification of universal primer of cytochrome b (Unseld, Beyermann, Brandt, & Hiesel, 1995). Employing the same gene target (cytb) and the same technique, goose species in commercial goose salami (Colombo, Marchisio, Pizzini, & Cantoni, 2002) and protected game animal Chamois (Colombo, Cardia, Renon, & Cantoni, 2004) were successfully detected and the sequencing result showed perfect matching (100% homology for chamois) with the published database . Other work includes sequencing of 28 different mammals (mostly wild animals) were amplified and sequenced targeting partial 12S rRNA and the partial 16S rRNA sequences (100bp), as a result indicates high divergence factor and clearly distinguished among all 28 species , with the exceptions of the closely related pig and wild boar, the different seals and the deers (Karlsson & Holmlund, 2007). However, the method may present constraints in cooked or processed samples with degraded DNA and it is further restricted in the analysis of mixed-species meats as the heterogeneous amalgam of sequences from different species hinders result interpretation (Fajardo et al., 2010).

University of Malaya

CHAPTER 3: METHODOLOGY

This chapter is aimed to provide the materials and detail procedures that reflect the objectives of this study. This chapter has been divided into several sub-sections; sample collection, assay specificity test, sensitivity test, biomarker stability test, screening of commercial meat product and lastly enzymatic digestion and RFLP analysis. A flowchart of the experimental and analytical steps is presented in **Figure 3.1.**

3.1 Meat Sample Collection

All raw meat samples (chicken, beef, buffalo, goat, lamb, duck, pork, venison, carp, cod and salmon) were purchased three different outlets in triplicates on three different days from various markets in Selangor (Pasar Borong Jalan Othman, Petaling Jaya and Pasar Borong Selangor, Serdang) as well as AEON Supermarkets in Kuala Lumpur, Malaysia. Other species, such as quail, pigeon and turtle, were bought from Pudu Wet Market, Kuala Lumpur. Three different individuals of cat and dog meat samples were collected after being euthanized by the authorized personnel of Dewan Bandaraya Kuala Lumpur, Malaysia. Meanwhile, the target species, macaque meat samples from three individuals of *Macaca fascicularis* were provided for study purpose by the Department of Wildlife and National Parks (DWNP) Peninsular Malaysia (Cheras, Kuala Lumpur). For commercial meatballs, a total of 4 different brands of beef (Figo, Resipi Nenek, Marina, Ayamas) and chicken meatball (Ayamas, A1, Ayam Dinding and Farm Best) were bought in different stores in Mid Valley Megamall, Kuala Lumpur. All samples were stored at -20 °C until further use to prevent enzymatic degradation of DNA.

University of Malaya

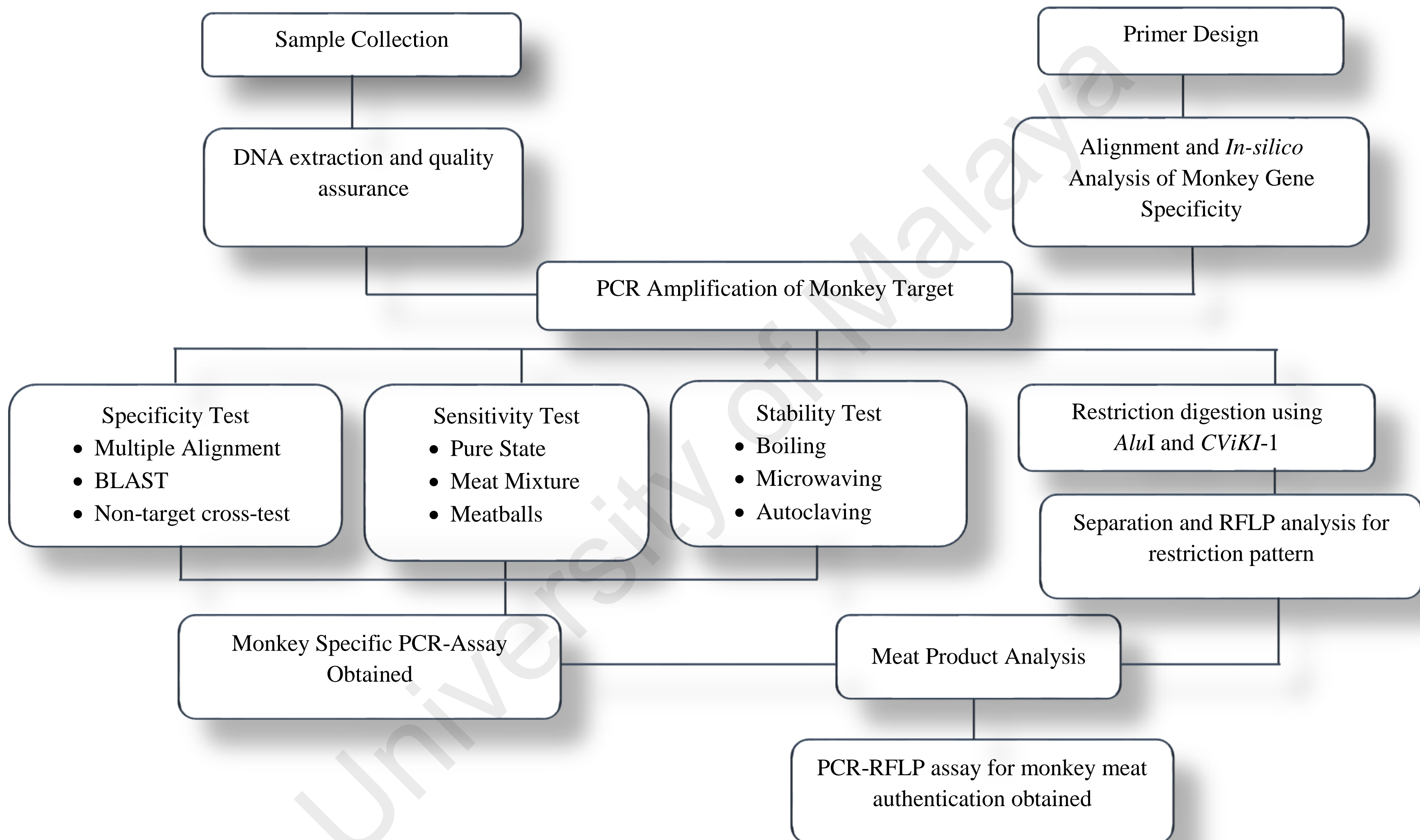


Figure 3.1: Flow-Chart of Research Methodology

University of Malaya

3.2 Design of Long-tailed Macaque Specific Primer

Macaca fascicularis mt-d-loop gene sequence (FJ906803.1) was retrieved from National Centre of Biotechnology Information (NCBI) and primers were designed *in-silico* using the online-available software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). A pair of primers targeting a 120 bp fragment of the d-loop gene were selected (Table 3.1). Meanwhile the endogenous control primer (Eukaryotic 18S rRNA) was selected based from Rojas et al. (2010).

Table 3.1 List of primer set used in the specificity test

Primer Name	Primer Sequence (5'-3')	Length (bp)
Monkey Forward Primer	TGA AAT CAA TAT CCC GCA CA	20
Monkey Reverse Primer	CTG GTT GTT ATG GCC CTG AG	20
Eukaryotic 18S rRNA Forward Primer*	GGT AGT GAC GAA AAA TAA CAA TAC AGG AC	29
Eukaryotic 18SrRNA Reverse Primer*	ATA CGC TAT TGG AGC TGG AAT TAC C	25

*The primer pair of Eukaryotic 18S rRNA followed Rojas et al. (2010).

The specificity of the primers was tested in 3 different ways. Firstly by using online Basic Local Alignment Tool (BLAST) against non-redundant nucleic acid sequences in NCBI data base (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). Secondly, the primers were multiple aligned with 17 other species (cow (*Bos taurus*) :AB003801.1, sheep (*Ovis aries*) : KJ954145.1, goat (*Capra hircus):* KM360063.1, chicken (*Gallus*

University of Malaya

gallus): KM096864.1, buffalo (*Bubalus bubalis):* NC_006295.1 , pig (*Sus scrofa)*: AF034253.1, deer (*Cervus nippon*): AB211429.1, duck (*Anas platyrhynchos)*: EU009397.1, salmon (*Oncorhynchus gorbuscha*): NC_010959.1, carp (*Cyprinus carpio*) KF856965.1, cod (*Gadus chalcogrammus*): NC_004449.1, turtle *(Cuora amboinensis*) : NC_014769.1, rat (*Rattus norvegicus*) : KM577634.1, cat (*Felis catus*): NC_001700.1, dog (*Canis lupus*) : NC_008092.1, quail (*Coturnix japonica*): AP003195.2, and pigeon (*Columba livia*) : NC_013978.1) using ClustalW sequence alignment program (http://www.genome.jp/tools/clustalw/) to identify conserved sequence regions and total mismatch between the target and non-target species. Finally, the specificity was confirmed by PCR via cross-amplifying reactions with the DNA templates of all non-target species. The primers were synthesized and supplied by the First Base Laboratories Sdn. Bhd., Selangor, Malaysia.

3.3 DNA Extraction

Total DNA was extracted from 30 mg of each meat sample (raw, admixed and commercial meatballs) using a Genomic DNA Mini Kit for Animal Tissues (Yeastern Biotech Co. Ltd, Taipei) following the manufacturer's instructions without any modification. The DNA extraction was divided into five main steps i.e tissue dissociation, lysis, DNA Binding, washing, and DNA elution. About 30 mg of animal tissue was ground inside the 1.5 ml micro-centrifuge tube using the provided micro-pestle, before it was added with 200 µl of GT Buffer for homogenizing. Later, 20 µl of Proteinase K was added to the mixture, vigorously mixed, and incubated in 60 °C of shaking water bath for half an hour. The mixture then was added with 200 µl of GBT Buffer, and incubated in water bath with the same conditions until the clear lysate appeared. In DNA binding step, a 200 µl of absolute ethanol was added and mixed, centrifuged and the pellet in column used for washing step by using W1 buffer. After centrifuging, the column again was

University of Malaya

washed with buffer and subsequently centrifuged twice. The last step of DNA extraction is to elute DNA using Elution buffer provided in the kit. The pre-heated elution buffer was added onto the pellet inside the column, left for three minutes and centrifuged for the yield. The concentration and purity were checked by UV-VIS spectrophotometer (Biochrom Libra S70, Biochrom Ltd., UK) taking absorbance at 260-280 nm. The extracted DNA was kept in -20 °C freezer until used.

3.4 PCR Amplification

Target DNA was amplified in a 250 µl PCR tubes in 20 µl reaction mixture containing 4 µl of 5x Green GoTaq Flexi Buffer, 2.2 µl of 25 mM of MgCl, 0.4 µl of 0.2mM of each DNTP, 0.4 µM of each primers and 0.5 unit *Taq* polymerase and 10 ng of total DNA extracted from each sample. We also included 0.4 µM of Eukaryotic 18S rRNA primers as the internal control (Ali, Hashim, Mustafa, & Che Man, 2012a; Rojas et al., 2010) (Table 3.2). Meanwhile, the negative control (nuclease-free water) was used to eliminate contamination. All primers set were purchased from First Base Laboratories Sdn. Bhd., Selangor, Malaysia meanwhile PCR reagents from Promega Corporation (Madison, USA). PCR reaction was performed in Veriti 96-Well Thermal Cycler (Applied Biosystems Inc., CA, USA), following initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and the final extension at 72 °C for 5 min. PCR products were kept at -20 °C for further analysis.

University of Malaya

Table 3.2: Parameters for PCR Assay Optimization.

PCR Reagents	Initial Concentration	Final Concentration	Final Volume (Total Volume: 50µl)	Final Volume (Total Volume: 20µl)
Buffer	5X	1X	10 µl	4.0 µl
MgCl2	25mM	2.75	5.5 µl	2.2
Forward Primer	10mM	0.4mM	2.0 µl	0.8 µl
Reverse Primer	10mM	0.4mM	2.0 µl	0.8 µl
dNTPs mix	10mM each	0.2mM	1.0 µl	0.4 µl
Taq Polymerase	5U/µl	1.25U	0.25 µl	0.1 µl
DNA	10ng/µl	10ng	5 µl	2 µl

3.5 Gel Electrophoresis

3.5.1 Conventional Gel Electrophoresis

In order to examine the amplified DNA, PCR products were analysed by gel electrophoresis. Firstly, a 2 % (w/v) gel was prepared by mixing 3 g of agarose in 150 ml of 1 X ultra-pure grade Tris-borate-EDTA (TBE) buffer by heating in a microwave oven until totally dissolve. Next, 3-5 µl Fluorosafe DNA stain was added and mixed well into the molten gel. The molten gel mixture was cast in a tray containing the well comb for 20 min until set. The gel later was placed inside the gel tank filled with 1× TBE buffer. Wells were loaded with 5 µl of PCR product and 100bp DNA ladder (Promega, USA) (**Figure 3.2**) accordingly. Agarose, TBE Buffer and FluoroSafe DNA stain all purchased from 1st BASE. Gel electrophoresis was set to run at 120 volts for about 90 min. Lastly, the gel was visualized in a gel-imager (AlphaImager HP; Alpha Innotech Corp., Santa Clara, CA, USA).

University of Malaya

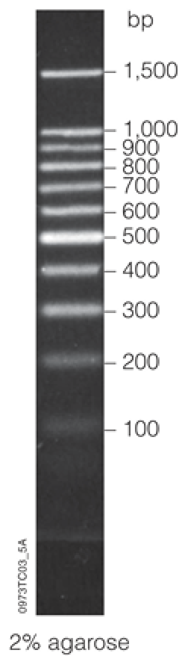


Figure 3.2: **Composition of 100 bp DNA ladder used in electrophoretic separation of PCR products.**

3.5.2 Lab-on-a-Chip Microfluidic Automated Electrophoresis System

The Experion system employs LabChip microfluidic technology to automate electrophoresis and analysis by integrating separation, detection, and data analysis within a single platform. Using much smaller sample and reagent quantities than standard analysis methods, the Experion automated electrophoresis system can be used both upstream and downstream of a number of nucleic acid and protein applications. In this work, Experion 1K DNA was used for nucleic acid analysis. The procedure started with preparation of Gel-Stain (GS) by adding 12.5 µl DNA stain to a tube of 250 µl DNA 1K gel Vortex the GS for 10 sec, and was spun down briefly in a microcentrifuge. The GS then was filtered by transferring it into spin filter and centrifuged for 15 min at 2,400 × g. All reagents provided such as gel-stain, DNA ladder, and buffer was spun down and equilibrated at room temperature for at least 10 min every time before used. For sample analysis, first of all, the chip was primed by pipetting 9 µl of GS into the gel priming well (labeled GS on the chip) and the priming station was set at C3 (option button on the

University of Malaya

station). After priming, another three GS wells were filled with the same amount. The rest of the wells were loaded with 5 µl of buffer each before loaded with 1µl of DNA ladder and 1µl sample (0.5-50ng/µl) in their particular (**Figure 3.3**).

Figure 3.3: Experion DNA chip 1K. The locations of the gel priming well (GS, highlighted) and alphanumeric priming codes are indicated.

The occupied chip was vortexed using the provided station for 1 min, and was inspected for the absence of any bubbles and contaminant such as dust. Lastly, the analysis was performed by placing the primed, loaded, and vortexed chip on the chip platform, and DNA 1K was selected in Experion software toolbar before start the process.

3.6 Limit of Detection under Pure State and Meat Admixtures.

The sensitivity was tested by dilution of DNA extracted from pure and admixed meats. Limit of detection (LOD) was determined by amplification of 10-fold serially diluted (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng DNA) monkey DNA. For binary mixture, two types of admixed were prepared; monkey-beef and monkey-goat mixtures in a total of 100g specimen by spiking monkey meats at a proportion of 10%, 5%, 1%, and 0.1%. The meats were first minced and then homogenised with a blender. To avoid

University of Malaya

contamination, each mixture was prepared separately using different material and different blender containers on three different days.

To adapt the real adulteration of commercial meatball products, three types of dummy meatball (pure beef, pure chicken, and monkey meatballs) were prepared following to recipe from Rohman et al. (2011) (**Table 3.3**). Pure meatballs were prepared with balanced amount of minced meat with breadcrumbs, cooking salt, spices and other ingredients. To obtain monkey meat contaminated meatballs, 10%, 5%, 1%, and 0.1%, of monkey meat were added to the total of 100 g of chicken and beef meat in the formulation. The mixture was homogenously mixed and well blended before it was given into ball shape and boiled for approximately 15 to 30 minutes so it cooked and suitable to eat (Ali et al., 2012a). An eukaryotic endogenous control (141 bp site of 18S rRNA) was used in every test to determine the quality of DNA in all meatballs and eliminate the probability of false negative detection.

University of Malaya

Table 3.3 : Ingredients used in chicken, beef and monkey meatball preparation (Rohman et al., 2011).

Ingredients	Chicken Meatball	Beef Meatball	Monkey Meatball
Minced meat	100g*	100g*	100g
Breadcrumbs	7.5 g	7.5g	7.5g
Chopped onions	5 g	5g	5g
Chopped ginger		1.5g	1.5g
Cumin powder		1.25g	1.25g
Garlic powder		1.25g	1.25g
Black pepper	0.14g		0.14g
Milk	0.01g		0.01g
Butter	3.28g		3.28g
Tomato paste		2.5g	2.5g
Salt	0.05g	0.05g	0.05g

* 1%, 0.5%, 0.2%, 0.1% and 0.01% of dog meat were mixed with a balanced amount of chicken and beef meat to make 100 g specimen of each meatball meat.

3.7 Target DNA Stability Test

Three types of food processing and cooking treatments were applied to the meat samples to test the stability of d-loop gene DNA target in processed meats. To mimic the normal way of cooking, meats were boiled at 60 °C, 80 °C and 100 °C for 30 min, microwave cooked at 300, 500 and 700 Watt for 30 min using commercial home microwave. Meanwhile, to simulate steaming and canning processes, meat samples were autoclaved at 121 °C under 14.5 psi for 30, 60 and 150 min.

University of Malaya

3.8 Enzymatic Digestion and RFLP Analysis

The verification of the 120 bp monkey d-loop gene sequence was confirmed by PCR-RFLP technique. First of all, 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). From the analysis, *Alu*I and *CViKI*-1 were verified whether the enzymes that met these criteria. Both enzymes were purchased from New England Biolabs, UK.

Restriction enzyme reaction was prepared as in **Table 3.4**. PCR products were digested with *Alu*I and *CViKI*-1 restriction endonucleases in 25 µl reaction mixture in separate tubes containing 1 µg of unpurified PCR product, 5U of enzyme, 1x digestion buffer and adjusted amount of sterilized distilled water. Digestion was carried out at 37 °C in a shaking water bath for 45 min. After 45 min, *Alu*I digestion was stopped by heating the mixture at 65 °C for 20 min. However, no enzymatic inactivation steps were required for *CViKI*-1 enzymes. For RFLP analysis, 1 µl digested product was applied to a microfluidic-lab-on-a- chip using 1k DNA a kit and was separated by Experion Automated Electrophoresis System (Bio-Rad, C.A, US).

Table 3.4: Restriction enzyme reaction preparation.

Components	*Alu*I (10000U/ml)	*CViKI*-1 (5000U/ml)
DNA (20ng/µl)	15ul	10ul
Steriled dH2O	14	23
Buffer (10x)	5	5
Restriction Enzyme	1ul	2

University of Malaya

CHAPTER 4: RESULTS

In this chapter, all the results from each sub procedure will be presented. The data including in the form of text, figures and tables will briefly presented and discussed.

4.1 DNA Extraction

Initially, DNA was extracted using a Genomic DNA Mini Kit for Animal Tissue DNA. This DNA isolation kit, however, not only extracts the whole genomic library, but also was designed for the purification of total DNA including mitochondrial DNA and viral DNA from a variety of animal tissues or cells. The kit came together with the micropestle which helps to homogenize tissue sample to shorten the time spent for cell lysis. As described in the protocol, this extraction method used proteinase K and chaotropic salt, guanidine hydrochloride to lyse cells and degrade protein. DNA in chaotropic salts binds to the glass fiber matrix of column and later on washed with elution buffer which actually is TE buffer, which contain low salt concentration. The DNA extracted from the raw meat sample showed good quality and less contamination found was produced at A260/A280 ratio of 1.70 to 2.00 (Table 4.1). In addition, the concentration also satisfactory when only about 30mg of meat tissue was needed to yield 100 to 200 ng/ul of DNA. In short, the extracted DNA isolates were fine but for commercial meat product (meatball) gave poor DNA yield and low in purity due to the additional ingredients of meatball. This shortcoming has been overcome by adding more meat sample (50-100mg), and repeating the elution step in order to increase the DNA recovery.

University of Malaya

4.2 Specificity Test

4.2.1 Theoretical Analysis

The specificity of monkey specific primer was first determined theoretically before it tested in real run. The primer set that generated from primer software (primer3plus) have been set to have special criteria; only short amplicon (not more than 150bp), to contain 40-80% of GC content, and melting temperature between 55-60 °C. We multiple aligned the 120 bp amplicon of monkey sequence with the rest non-target to find the similarity and mismatch. We found that the least mismatch was between monkey and rat and highest with cod fish (**Table 4.2**). From the mismatch result, we derived pairwise distance (**Table 4.3**), phylogenetic tree (**Figure 4.1)** and 3D plot (**Figure 4.3**) to show the clearer relationship between all the species.

University of Malaya

Table 4.1: Concentration of DNA extracted from all species in form of raw, heat-treated and meat mixture (binary and commercial product).

Type of Sample	Name	Average Concentration (ng/μl)	Purity (A_{260}/A_{280})
Raw meat	Monkey	112.3	1.850
	Cow	114.1	2.001
	Sheep	127.3	1.700
	Goat	161.0	1.765
	Chicken	138.2	1.822
	Buffalo	149.4	1.816
	Deer	130.8	1.691
	Pig	121.9	1.732
	Duck	154.7	1.880
	Salmon	286.3	1.791
	Carp	99.0	1.801
	Cod	156.8	1.893
	Rat	144.2	1.976
	Cat	98.9	1.685
	Dog	89.4	1.716
	Turtle	134.0	1.765
	Quail	210.4	1.759
	Pigeon	562.9	1.712
Heat Treated	Boiled	420.3	1.736
	Microwaved	665.4	1.704
	Autoclaved	102.8	1.828
Binary Meat Mixture (Raw)	Monkey+Beef	83.6	1.894
	Monkey+Goat	68.1	1.761
Commercial Meat Product (Beef Meatball)	Figo	58.5	1.550
	Resipi Nenek	48.8	1.667
	Marina	60.3	1.745
	Ayamas	98.1	1.832
Commercial Meat Product (Beef Meatball)	Ayamas	120.1	1.844
	A1	105.8	1.594
	Ayam Dinding	211.5	1.742
	Farm Best	99.3	1.886

University of Malaya

Table 4.2 The mismatch comparison of the long-tailed macaque specific forward and reverse primers against 28 species

	Forward Primer																				Mismatch
M. fascicularis	T	G	A	A	A	T	C	A	A	T	A	T	C	C	C	G	C	A	C	A	
M. nemestrina	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. arctoides	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. assamensis	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	1
M. tonkeana	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	1
M. silenus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. sylvanus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. thibetana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. fuscata	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
M. nigra	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	1
M. radiata	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	2
Cow	C	.	G	T	.	.	.	C	T	G	.	C	.	G	T	.	.	.	A	.	10
Sheep	C	.	G	T	.	.	T	C	T	G	.	C	.	G	T	.	.	.	A	.	11
Goat	C	.	G	T	.	.	T	C	T	G	.	C	.	G	T	.	.	.	A	.	11
Chicken	C	.	G	T	.	.	.	C	T	A	.	C	.	G	T	.	.	G	A	.	11
Buffalo	C	.	G	T	.	.	.	C	T	G	.	C	.	G	T	.	.	.	A	.	10
Deer	C	.	G	T	.	.	.	C	T	G	.	C	.	G	T	.	.	.	A	.	10
Duck	C	.	G	T	.	.	.	C	T	A	.	C	.	G	T	.	.	.	A	.	10
Salmon	C	.	G	T	.	.	T	T	T	G	.	C	.	G	T	.	.	G	A	.	12
Tortoise	C	.	G	T	.	.	.	C	T	A	.	C	.	G	T	.	.	.	A	.	10
Pig	C	.	G	T	.	.	T	C	T	G	.	C	.	G	T	.	.	.	A	.	11
Rat	C	.	G	T	.	.	.	C	C	G	.	C	.	G	T	.	.	.	A	.	10
Cat	C	.	G	T	.	.	.	C	T	G	.	C	.	G	T	.	.	.	A	.	10
Dog	C	.	G	T	.	.	.	C	T	G	.	C	.	G	T	.	.	.	A	.	10
Carp	C	.	G	T	.	.	T	T	T	G	.	C	.	G	T	.	.	.	A	.	11
Cod	C	.	G	T	.	.	T	T	T	G	.	C	.	G	T	.	.	G	A	.	12
Quail	C	.	G	T	.	.	.	C	T	A	.	C	.	G	T	.	.	.	A	.	10
Pigeon	C	C	.	T	.	.	.	C	.	C	.	.	A	A	T	C	.	T	.	.	10

	Reverse Primer																				Mismatch
M. fascicularis	C	T	C	A	G	G	G	C	C	A	T	A	A	C	A	A	C	C	A	G	
M. nemestrina	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. arctoides	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. assamensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. tonkeana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. silenus	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	T	.	.	A	4
M. sylvanus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	1
M. thibetana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. fuscata	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	2
M. nigra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
M. radiata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21
Cow	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	C	.	13
Sheep	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	C	.	.	C	.	13
Goat	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	C	.	.	C	.	13
Chicken	.	.	A	T	.	A	A	A	T	T	A	G	T	A	T	T	.	.	C	.	14
Buffalo	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	C	.	13
Deer	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	C	.	13
Duck	.	A	G	T	.	A	A	A	T	T	G	.	T	.	T	C	.	.	C	.	13
Salmon	.	A	A	T	.	A	A	A	T	T	G	.	T	.	T	G	.	.	C	.	13
Tortoise	.	A	G	T	.	A	A	A	T	T	G	.	T	.	T	T	.	.	T	.	13
Pig	.	A	G	T	.	A	A	A	T	T	A	.	C	.	T	T	.	.	C	.	13
Rat	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	.	.	12
Cat	.	C	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	C	.	13
Dog	.	A	G	T	.	A	A	A	T	T	G	.	C	.	T	T	.	.	C	.	13
Carp	.	A	G	T	.	A	A	A	T	T	G	.	T	.	T	.	.	.	C	.	12
Cod	.	A	A	T	.	A	A	A	T	T	G	.	C	.	T	C	.	.	C	.	13
Quail	.	A	G	T	.	A	A	A	T	T	G	.	T	.	T	C	.	.	T	.	13
Pigeon	.	A	G	.	.	.	A	.	T	.	G	.	.	.	T	T	A	A	T	.	10

Table 4.3 Pairwise distances of the *Macaca fascicularis* specific primer sites of D-loop gene against corresponding sites of 32 different tested species and most closely related species.

	120 bp Amplicon	M. fascicularis	M. arctoides	M. assamensis	M.nemestrina	M.tonkeana	M.silenus	M. sylvanus	M. thibetana	M. mulatta	M.fuscata	M.nigra	M.radiata	T. obscurus	T. cristatus	T. pileatus	T. germaini	P. melalophos	P. hosei	P.frontata	P. rubicunda	P. chrysomelas	P. potenziani	P.potenziani siberu	P.thomasi	P. comata	Nasalis larvatus	Rhinopithecus avunculus	Semnopithecus entellus	Colobus guereza	Procolobus badius	Pygathrix nemaeus	Pygathrix roxellana	Homo sapiens
120 bp Amplicon																																		
M. fascicularis	0.000																																
M.arctoides	0.000	0.000																															
M. assamensis	0.013	0.013	0.013																														
M.nemestrina	0.000	0.000	0.000	0.013																													
M.tonkeana	0.039	0.039	0.039	0.026	0.039																												
M.silenus	0.039	0.039	0.039	0.052	0.039	0.052																											
M. sylvanus	0.013	0.013	0.013	0.025	0.013	0.052	0.026																										
M. thibetana	0.025	0.025	0.025	0.038	0.025	0.065	0.038	0.038																									
M. mulatta	0.025	0.025	0.025	0.038	0.025	0.038	0.038	0.038	0.052																								
M.fuscata	0.000	0.000	0.000	0.013	0.000	0.039	0.039	0.013	0.025	0.025																							
M.nigra	0.026	0.026	0.026	0.039	0.026	0.066	0.066	0.039	0.052	0.052	0.026																						
M.radiata	0.093	0.093	0.093	0.079	0.093	0.079	0.108	0.079	0.093	0.093	0.093	0.106																					
T. obscurus	0.167	0.167	0.167	0.183	0.167	0.201	0.201	0.183	0.201	0.167	0.167	0.182	0.232																				
T. cristatus	0.167	0.167	0.167	0.183	0.167	0.201	0.201	0.183	0.201	0.167	0.167	0.182	0.232	0.013																			
T. pileatus	0.184	0.184	0.184	0.201	0.184	0.219	0.219	0.201	0.219	0.184	0.184	0.200	0.250	0.013	0.026																		
T. germaini	0.167	0.167	0.167	0.183	0.167	0.201	0.201	0.183	0.201	0.167	0.167	0.182	0.232	0.013	0.000	0.026																	
P. melalophos	0.199	0.199	0.199	0.215	0.199	0.234	0.234	0.215	0.234	0.215	0.199	0.215	0.282	0.126	0.142	0.142	0.142																
P. hosei	0.217	0.217	0.217	0.235	0.217	0.254	0.254	0.235	0.219	0.217	0.217	0.234	0.250	0.110	0.125	0.125	0.125	0.039															
P.frontata	0.200	0.200	0.200	0.217	0.200	0.236	0.236	0.217	0.236	0.200	0.200	0.217	0.267	0.095	0.110	0.110	0.110	0.026	0.013														
P. rubicunda	0.201	0.201	0.201	0.219	0.201	0.237	0.237	0.219	0.237	0.201	0.201	0.218	0.269	0.096	0.111	0.111	0.111	0.026	0.038	0.025													
P. chrysomelas	0.217	0.217	0.217	0.235	0.217	0.254	0.254	0.235	0.219	0.217	0.217	0.234	0.250	0.110	0.125	0.125	0.125	0.039	0.000	0.013	0.038												
P. potenziani	0.201	0.201	0.201	0.219	0.201	0.237	0.237	0.219	0.237	0.201	0.201	0.218	0.269	0.096	0.111	0.111	0.111	0.026	0.038	0.025	0.000	0.038											
P.p. siberu	0.217	0.217	0.217	0.235	0.217	0.254	0.254	0.235	0.254	0.217	0.217	0.234	0.285	0.110	0.125	0.125	0.125	0.039	0.025	0.013	0.038	0.025	0.038										
P.thomasi	0.219	0.219	0.219	0.236	0.219	0.255	0.255	0.236	0.255	0.219	0.219	0.235	0.287	0.110	0.125	0.125	0.125	0.013	0.025	0.013	0.013	0.025	0.013	0.025									
P. comata	0.200	0.200	0.200	0.217	0.200	0.236	0.236	0.217	0.236	0.200	0.200	0.217	0.267	0.095	0.110	0.110	0.110	0.026	0.013	0.000	0.025	0.013	0.025	0.013	0.013								
Nasalis larvatus	0.132	0.132	0.132	0.147	0.132	0.164	0.164	0.147	0.164	0.132	0.132	0.167	0.234	0.067	0.067	0.081	0.067	0.178	0.159	0.144	0.144	0.159	0.144	0.144	0.160	0.144							
R.avunculus	0.197	0.197	0.197	0.214	0.197	0.232	0.232	0.214	0.232	0.197	0.197	0.213	0.262	0.053	0.053	0.067	0.053	0.125	0.108	0.094	0.095	0.108	0.095	0.108	0.109	0.094	0.110						
S.entellus	0.160	0.160	0.160	0.176	0.160	0.192	0.192	0.176	0.192	0.160	0.160	0.195	0.262	0.081	0.081	0.096	0.081	0.142	0.125	0.110	0.111	0.125	0.111	0.096	0.126	0.110	0.066	0.080					
Colobus guereza	0.166	0.166	0.166	0.182	0.166	0.200	0.200	0.182	0.200	0.166	0.166	0.181	0.230	0.025	0.039	0.039	0.039	0.097	0.081	0.067	0.095	0.081	0.095	0.081	0.081	0.067	0.067	0.081	0.081				
Procolobus badius	0.130	0.130	0.130	0.145	0.130	0.162	0.162	0.145	0.162	0.115	0.130	0.164	0.214	0.039	0.053	0.053	0.053	0.126	0.127	0.112	0.112	0.127	0.112	0.127	0.127	0.112	0.053	0.097	0.067	0.039			
Pygathrix nemaeus	0.175	0.175	0.175	0.190	0.175	0.208	0.176	0.159	0.176	0.175	0.175	0.194	0.194	0.110	0.110	0.125	0.110	0.190	0.141	0.156	0.156	0.141	0.156	0.141	0.172	0.156	0.094	0.123	0.078	0.109	0.109		
Pygathrix roxellana	0.232	0.232	0.232	0.250	0.232	0.269	0.269	0.250	0.269	0.232	0.232	0.249	0.300	0.110	0.110	0.110	0.110	0.172	0.154	0.139	0.140	0.154	0.140	0.124	0.154	0.139	0.126	0.066	0.081	0.110	0.128	0.124	
Homo sapiens	2.228	2.228	2.228	2.228	2.228	2.133	2.187	2.278	2.175	2.133	2.228	2.087	1.998	1.943	1.850	1.943	1.850	3.015	2.732	2.776	2.638	2.732	2.638	2.732	2.687	2.776	2.587	2.587	2.685	1.998	2.447	2.587	2.228	0.000

University of Malaya

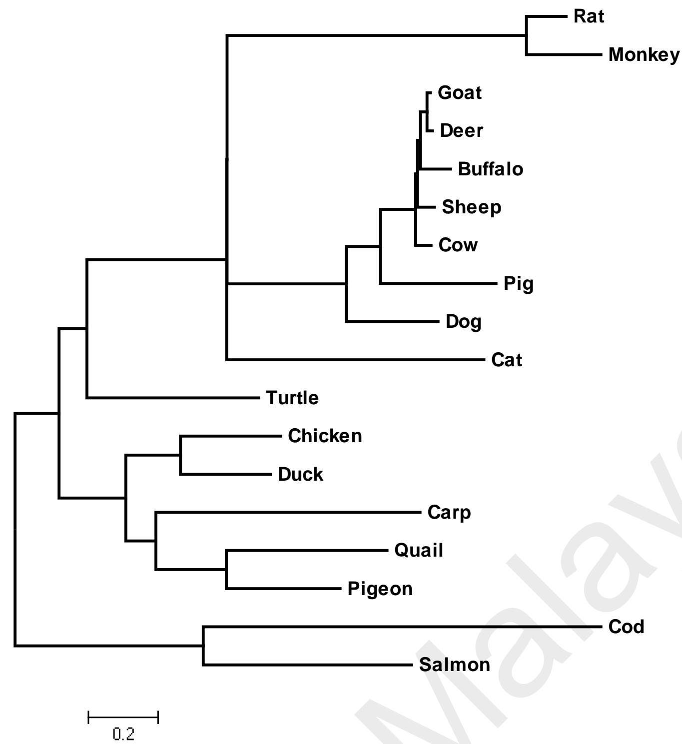


Figure 4.1: *In-silico* analysis of the monkey specific primers. Dendogram built from the 120 bp regions of d-loop gene sequences of monkey and other 17 land and aquatic species using Neighborhood-Joining method.

University of Malaya

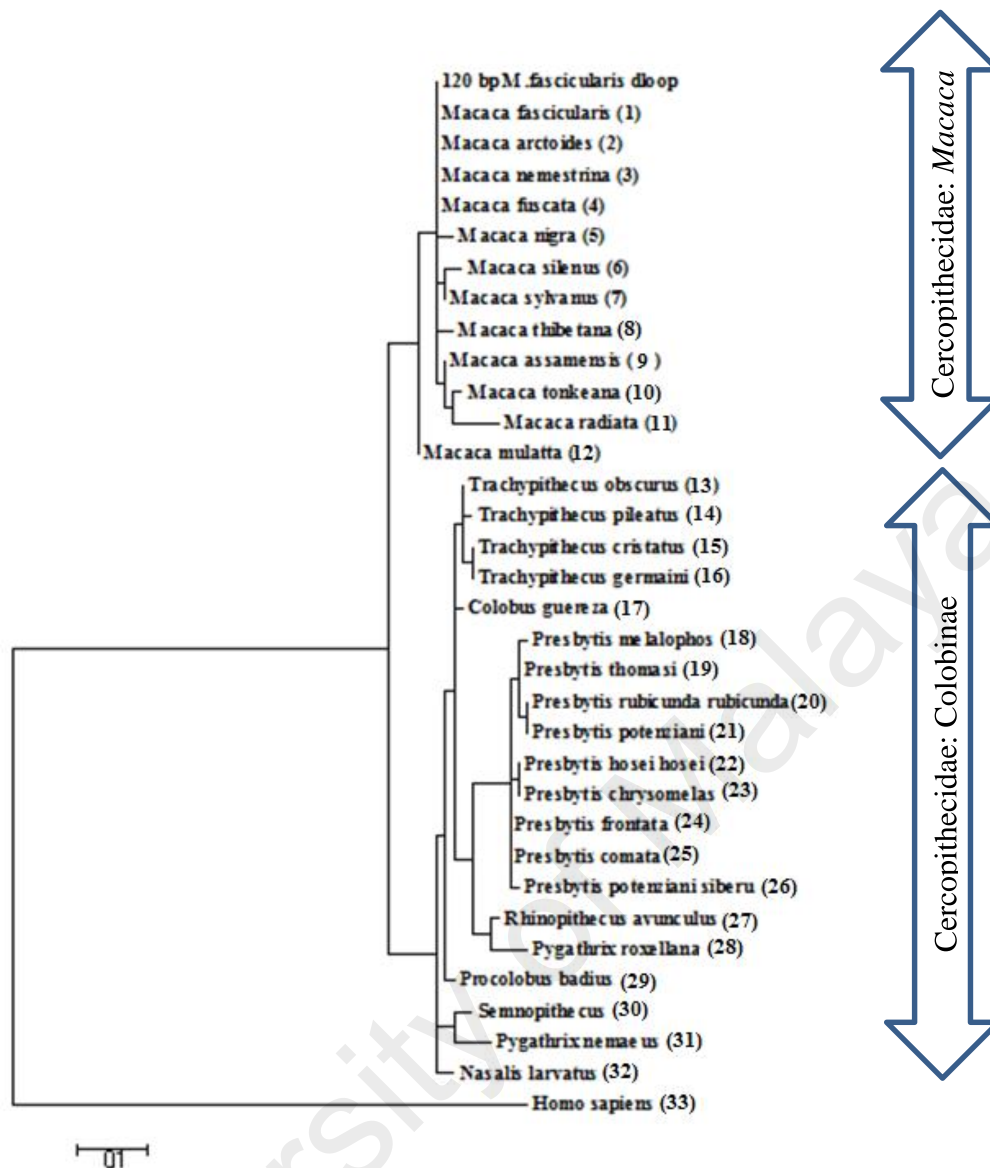
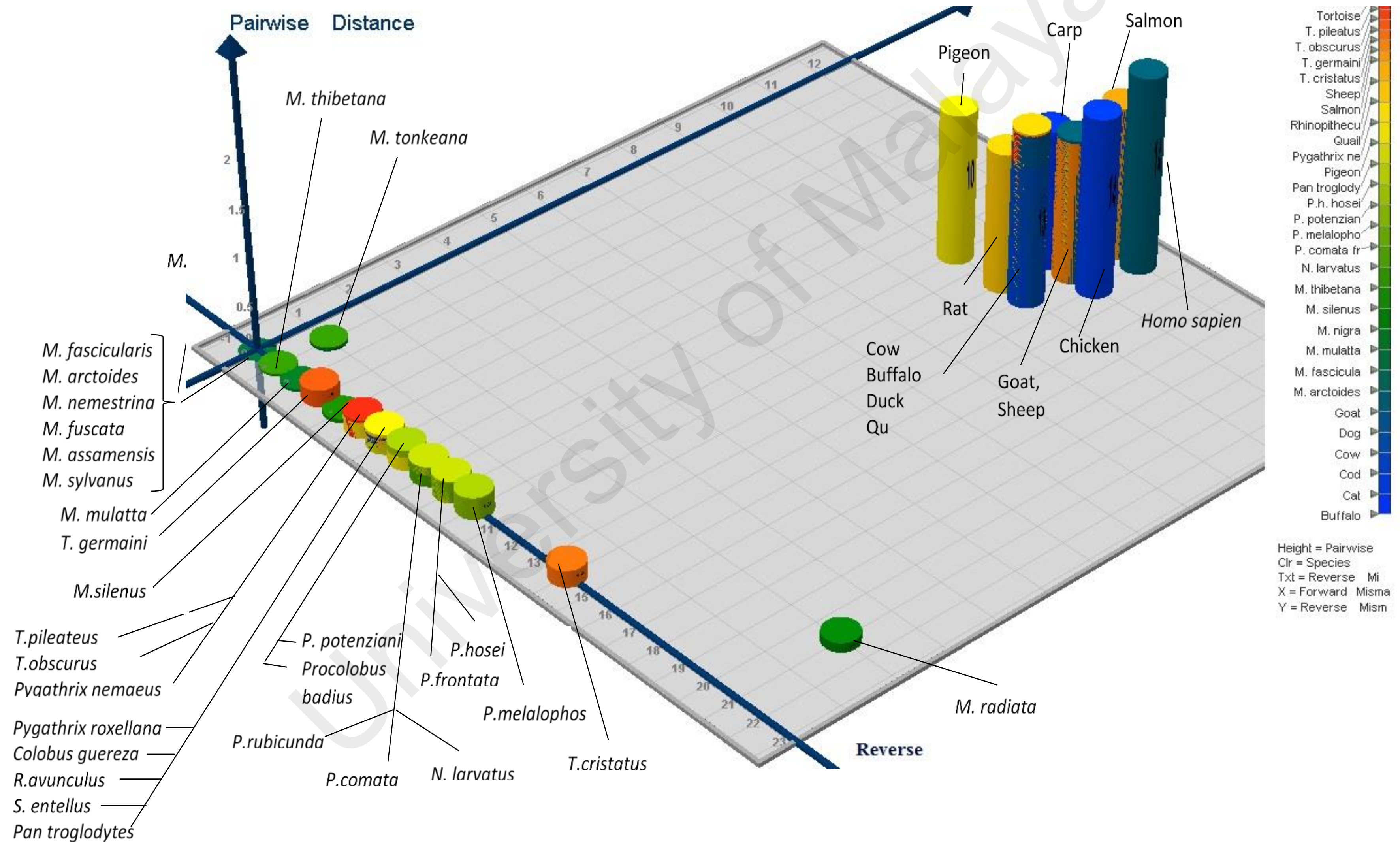


Figure 4.2: Phylogenetic tree of 12 Macaca (Genera *Cercopithecinae*) (1-12), 20 Colobines species (Genera *Presbytis,Trachypithecus, Semnopithecus, Rhinopithecus, Nasalis, Pygathrix, Colobus* and *Procolobus*) (13-32) *and Homo sapiens* (33) and 3D plot showing the discrimination of long-tailed *Macaque facicularis* target in the primer binding sites of 51 species .

University of Malaya

Figure 4.3 3D plot showing the discrimination of long-tailed *Macaca facicularis* target in the primer binding sites of 51 species.



University of Malaya

4.2.2 Specificity Analysis by PCR

Several optimization of PCR reaction have been carried out, and we found that the PCR condition with initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and the final extension at 72 °C for 5 min gave the best gel band, with high specificity to monkey DNA only while the other species produced no DNA amplification (**Figure 4.4**). Initially we cross-tested with only non-target DNA as negative control, but later endogenous control primer was used as positive control (**Figure 4.5**).

Figure 4.4: Specificity test. Gel-image of macaque-specific primer pair against monkey and other 17 DNA animal species. Lane L: 100 bp Ladder; Lane 1-18: 120bp PCR products from DNA template extracted from monkey, chicken, beef, chevon, lamb, buffalo, venison, duck, pork, quail, pigeon, salmon, carp, cod, turtle, dog, cat, and rat meats, respectively. Lane 19: Negative Control.

University of Malaya

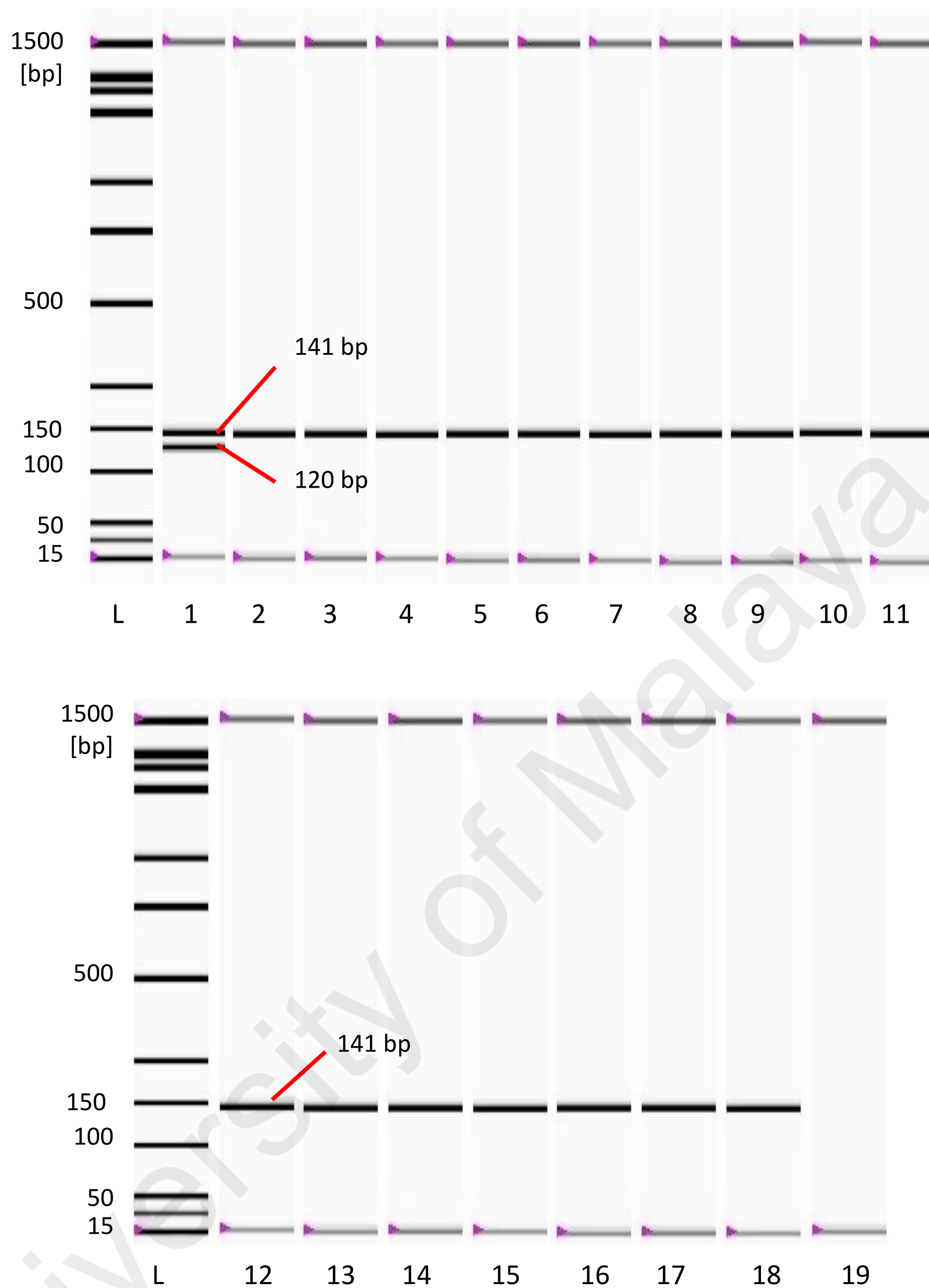
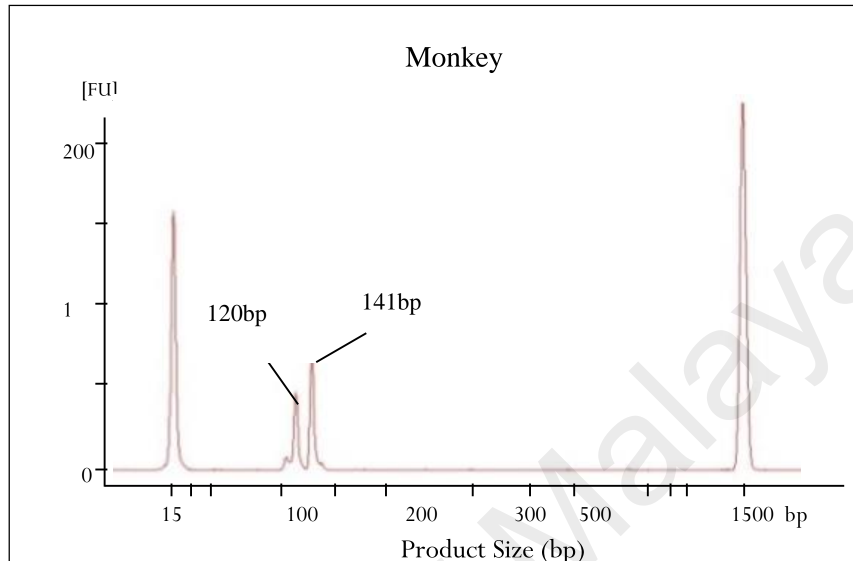


Figure 4.5 : **Cross-specificity of monkey-specific primers against DNA of 17 different meat species by automated electrophoresis**. Gel image shown; Lane L: 100 bp Ladder; Lane 1: monkey specific target (120bp) and endogenous control (141 bp); Lanes 2-18: endogenous control (141 bp) for cow, sheep, goat, chicken, buffalo, pig, deer, duck, salmon, carp, cod, turtle, rat, cat, dog, quail, and pigeon, respectively and Lane 19: negative control. A total of 10 ng DNA was used as template for each species.

University of Malaya

Electropherogram of monkey shows two peaks; 141 bp indicates the amplified endogenous control while 120 bp peak is represent monkey DNA detected by the system. The 15 bp and 1500 bp both represent lower and higher marker (**Figure 4.6**).



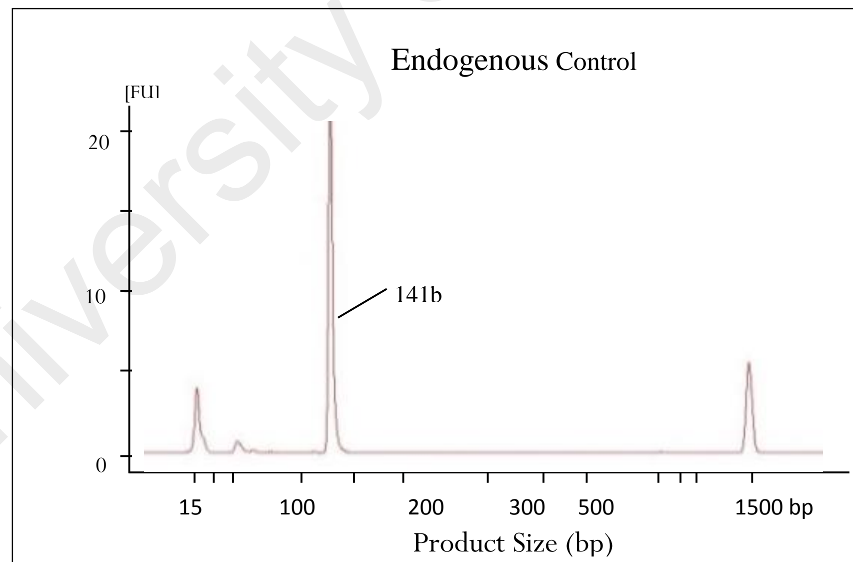


Figure 4.6: Electropherogram of monkey-specific DNA (120bp) and 18S rRNA endogenous control (141 bp) PCR products of all non-target species.

University of Malaya

4.3 Assay Sensitivity

4.3.1 Sensitivity in Pure State

Sensitivity of the assay was determined by two steps; firstly in pure state (DNA dilution) and secondly, by preparing binary meat mixture. In pure state, we determined the detection limit of the assay in pure state as low as 0.0001 ng for conventional gel electrophoresis (**Figure 4.7**) and up to 0.00001 ng for automated system (**Figure 4.8**).

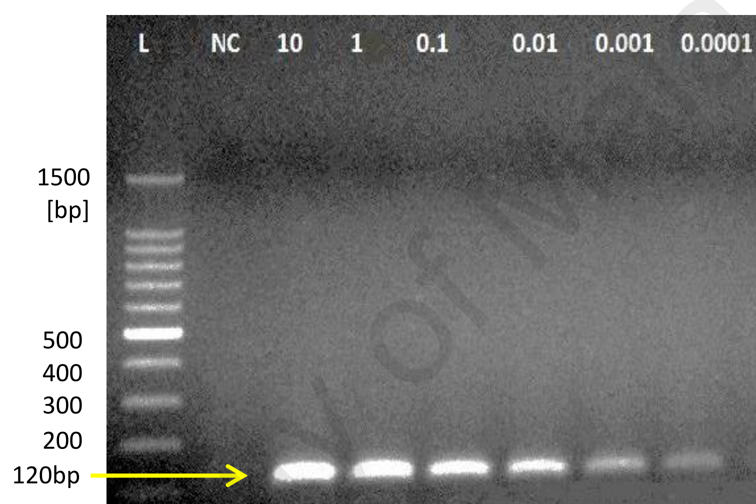


Figure 4.7: Gel image of sensitivity test under pure state from 100 to 0.0001ng of total DNA extracted from monkey meat. Lane L: DNA ladder, Lane NC: Negative Control.

University of Malaya

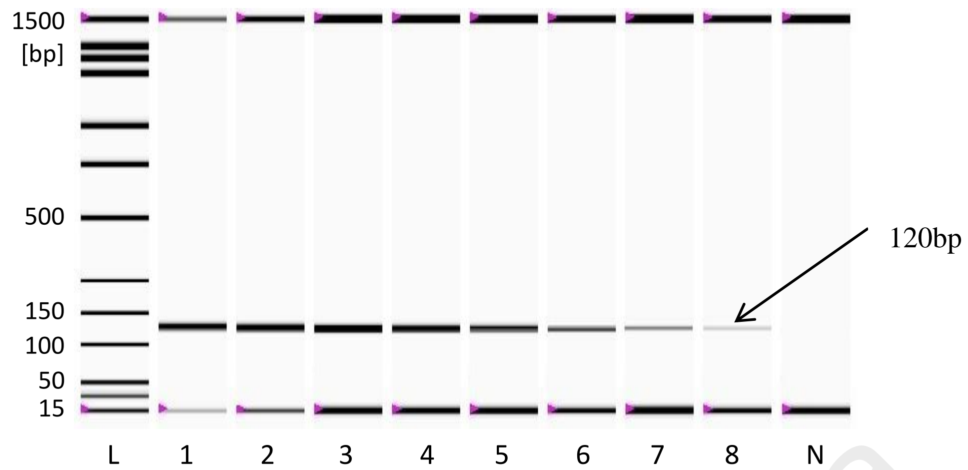


Figure 4.8: Gel image (automated electrophoresis) of sensitivity analysis under pure from 100 to 0.00001 ng of total DNA extracted from monkey meat. Lane L: DNA ladder, Lane N: Negative Control.

University of Malaya

4.3.2 Sensitivity under binary mixture background

For binary mixture, two types of admixed were prepared in a total of 100 g specimen by spiking monkey meats in beef and goat meat (separately) at a proportion of 10%, 5%, 1%, and 0.1%. We found that the limit of detection of both were 0.1%, with DNA concentration of 0.001ng (**Figure 4.9** and **Figure 4.10**).

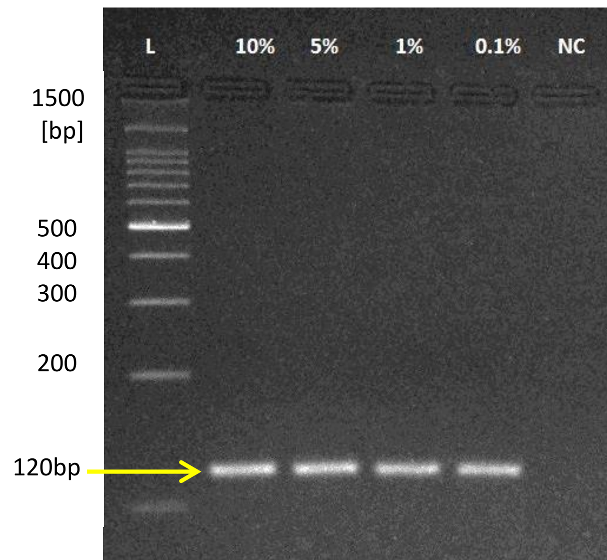


Figure 4.9: Gel image of sensitivity test in monkey and beef admixed. Shown are Lane L: DNA Ladder; Lane 1-4: 10%, 5%, 1% and 0.1% spiked monkey DNA. Lane NC: Negative control.

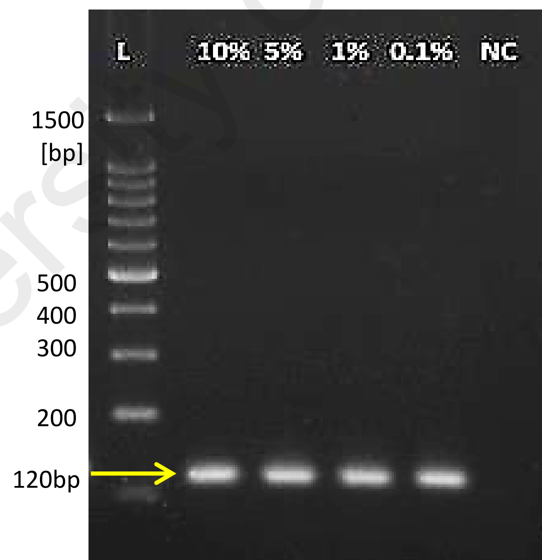


Figure 4.10: Gel image of sensitivity test in monkey and goat admixed. Shown are Lane L: DNA Ladder; Lane 1-4: 10%, 5%, 1% and 0.1% spiked monkey DNA. Lane NC: Negative control.

University of Malaya

4.3.3 Commercial Meat Product Analysis

A total of 4 different brands for each commercial chicken (Ayamas, A1, Ayam Dinding, and Farm Best) and beef meatball (Marina, Figo, Resipi Nenek, and Ayamas) were cross- tested with monkey specific primer in triplicates. To adapt the real adulteration of commercial meatball products, dummy meatballs were prepared by spiking 0.1% monkey meats following Ali et al. (2012). The composition of the dummy meatball products is given in table 3.8. An eukaryotic endogenous control (141 bp site of 18S rRNA) was used in every test to determine the quality of DNA in all meatballs and eliminate the probability of false negative detection. Result shown in **Figure 4.11** reflects no monkey DNA detected from all 8 type of commercial meatball. An eukaryotic endogenous control (141 bp site of 18S rRNA) was used in every test to determine the quality of DNA in all meatballs and eliminate the probability of false negative detection.

University of Malaya

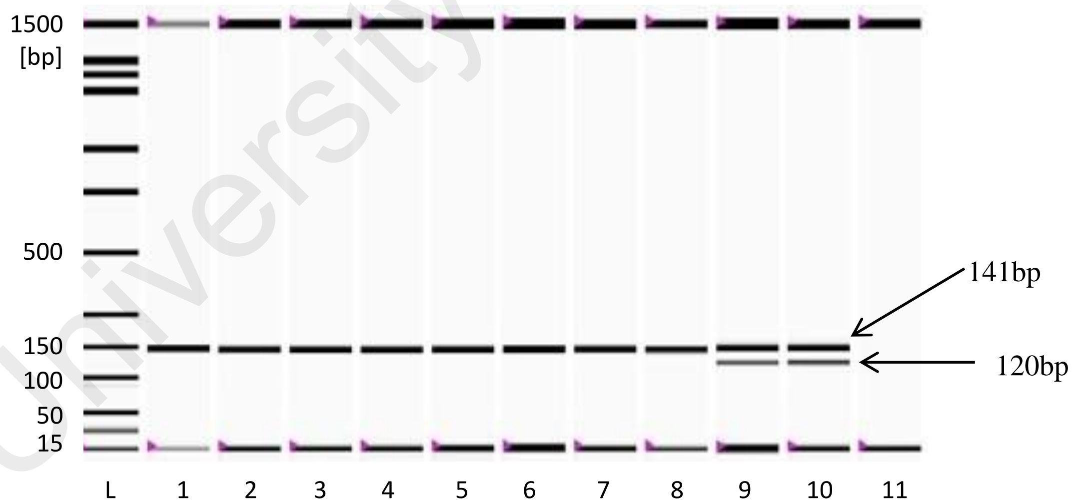


Figure 4.11 Monkey meat detection in commercial meatballs of chicken (Lanes 1-4) and beef (Lanes 5-8). Adulterated model meatballs from chicken (lane 9) and beef (lane 10) with 0.1% (w/w) monkey meat. Lane 11: Negative control.

4.4 DNA stability test

The stability of DNA in severe condition was studied by thermally exposing the monkey meat species in three ways; boiling, microwave cooking, and autoclaving. Boiling treatment was set for 30 min each at 60, 80, and 100°C. The microwave treatment was set half an hour at 300, 500 and 700W using a domestic microwave (ME711K, Samsung, Korea). On the other hand, autoclaving process was set at 120°C, 14.5 psi with different duration (30 min, 90 min and 150 min). DNA was extracted from all cooked meats, and concentration was checked and amplified by PCR. We also determined the sensitivity of the assay for DNA extracted from heat-treated meats (**Figure 4.12-14**).

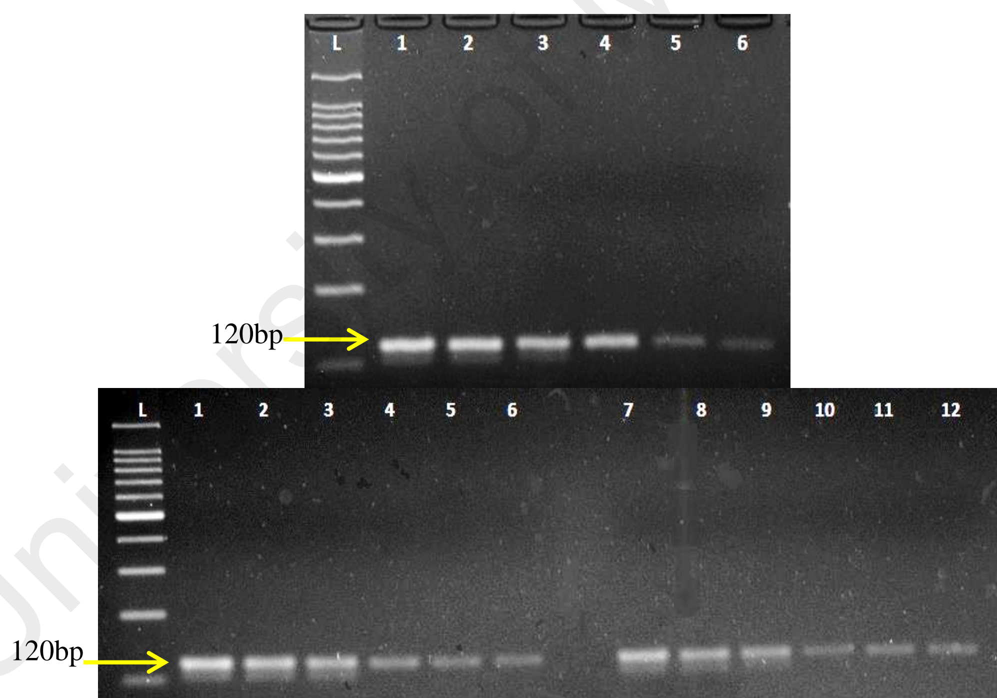


Figure 4.12: Marker DNA stability test under boiling treatment. Lanes 1-6; 7-12 and 13-18 represent boiling at 60; 80 and 100 °C, respectively, for 30 min. The six set of lanes demonstrate PCR products from 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng template DNA extracted from post-treated meats.

University of Malaya

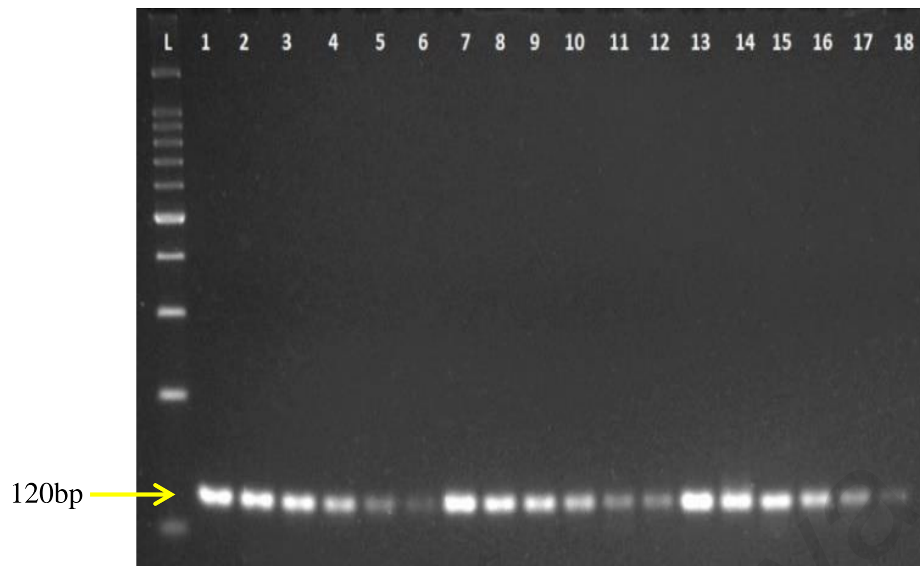


Figure 4.13: Marker DNA stability test under microwave treatment. Lanes 1-6; 7-12, and 13-18 represent microwave cooking at 300; 500 and 700 W, respectively, for 30 min. The six set of lanes demonstrate PCR products from 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng template DNA extracted from post-treated meats.

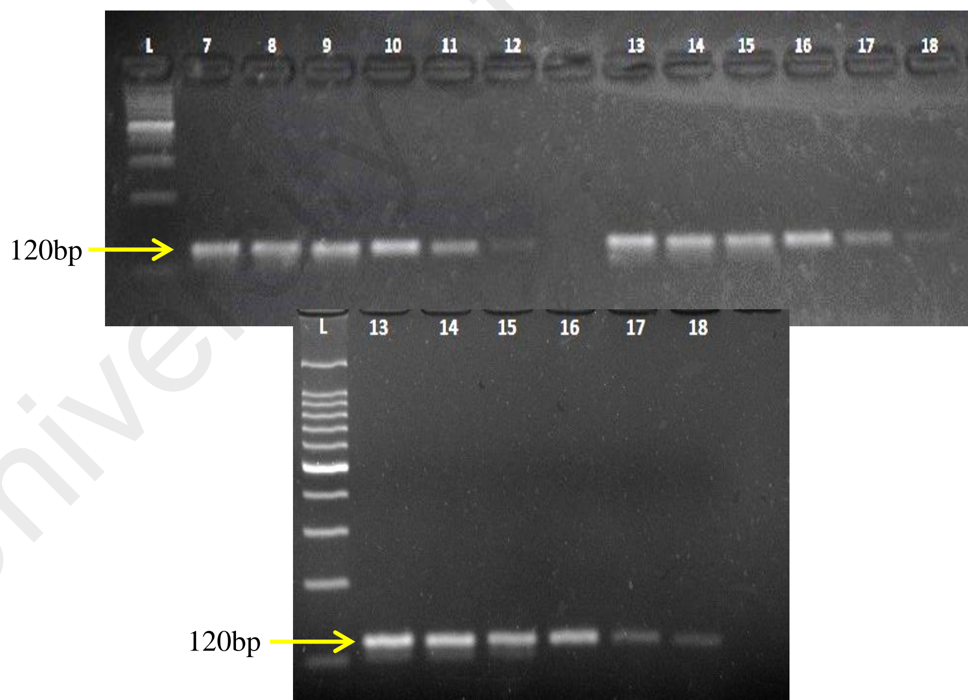


Figure 4.14: Marker DNA stability test under autoclaving treatments. Lanes 1-6; 7-12 and 13-18 represent autoclaving at 121 °C for 30, 90 and 150 min, respectively. The six set of lanes in every treatment demonstrate PCR products from 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng template DNA extracted from post-treated meats.

Table 4.4 Analysis of beef and chicken meatball using *M.fascicularis* d-loop gene (120 bp)-based PCR assay

Days	Raw Meat					Heat-Treated Meat					Detection Possibility (%)
	Pure Monkey Meat	Monkey-Beef Mixture	Monkey-Chevon Mixture	Commercial Chicken Meatball	Commercial Beef Meatball	Boiled Monkey Meat	Microwaved Monkey Meat	Autoclaved Monkey Meat	Commercial Chicken Meatball	Commercial Beef Meatball	
1	3	3	3	0/3	0/3	3	3	3	0/3	0/3	100
2	3	3	3	0/3	0/3	3	3	3	0/3	0/3	100
3	3	3	3	0/3	0/3	3	3	3	0/3	0/3	100

* Numerator denotes feline positive samples and denominator reflects total number of analyzed samples

University of Malaya

4.5 PCR-RFLP Analysis

4.5.1 *In-silico* Analysis

The verification of the 120 bp monkey d-loop gene sequence was confirmed by PCR-RFLP technique. Theoretically, *Alu*I and *CViKI*-1 enzyme cuts at two and four sites respectively (**Figure 4.15** and **4.16**). **Table 4.5 and 4.6** show restriction sites of *Alu*1 and *CViKI*-1 , respectively, on 120 bp monkey mt-dloop gene sequence.

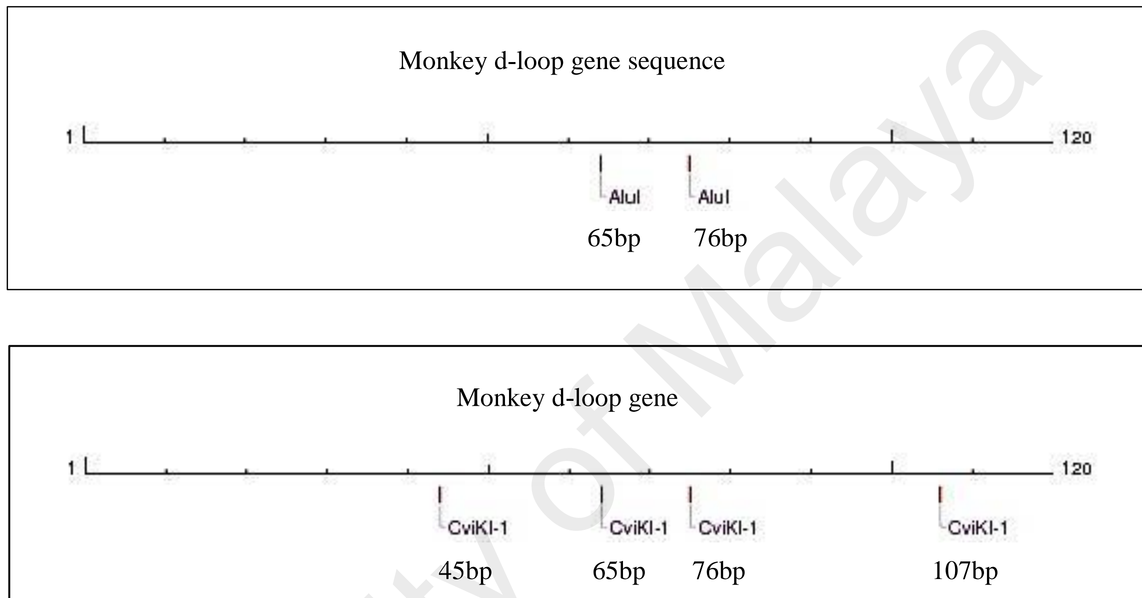


Figure 4.15: Restriction maps of *Alu*I (upper) and *CViKI-1* (lower) on the 120 bp of monkey d-loop gene.

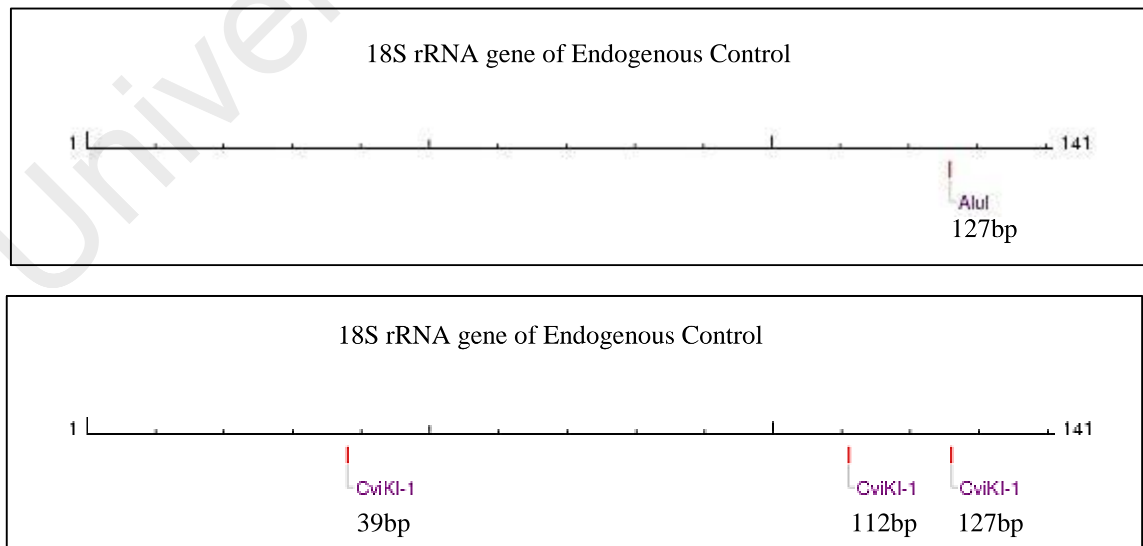


Figure 4.16: Restriction maps of *Alu*I (upper) and *CViKI*-1 (lower) on the 141 bp of 18S rRNA endogenous control.

University of Malaya

4.5.2 PCR-RFLP Analysis in Pure State.

Enzymatic digestion was initially performed on PCR product of monkey, cow and goat (**Figure 4.17** and **4.18**). **Figure 4.17** demonstrates restriction pattern analysis of monkey-target (120 bp) and endogenous control in pure state. *Alu*I digestion of the monkey PCR product give 65 and 44 bp (Lane 1) while *CViKI*-1 give (73, 45, 31 and 20 bp) in Lane 7. On the other hand, endogenous control (141 bp) produced two *Alu*I in lane 2 and 3 (127 and 14bp) and four *CViKI*-1 fragments (73, 39 and 15bp) in lane 8 and 9.

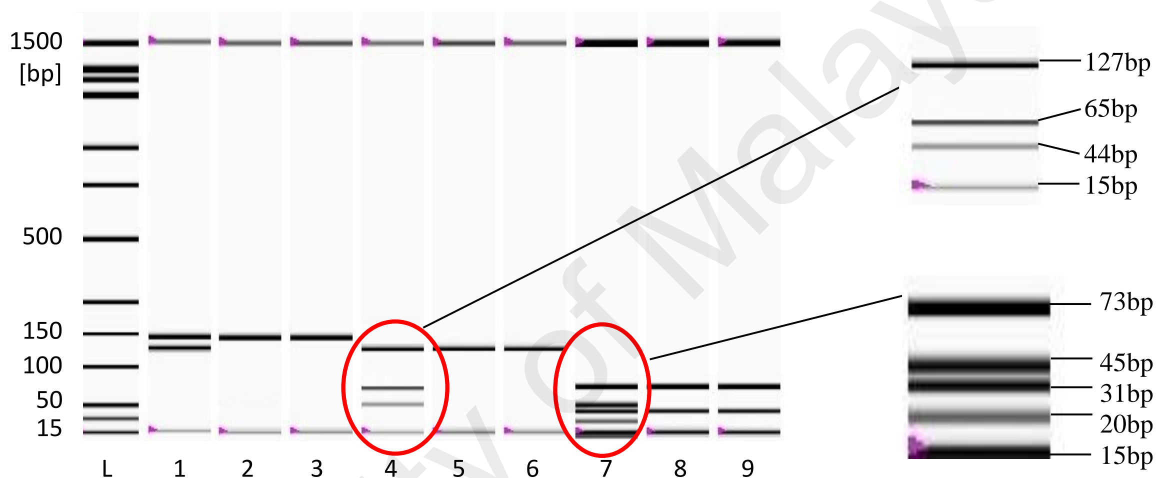
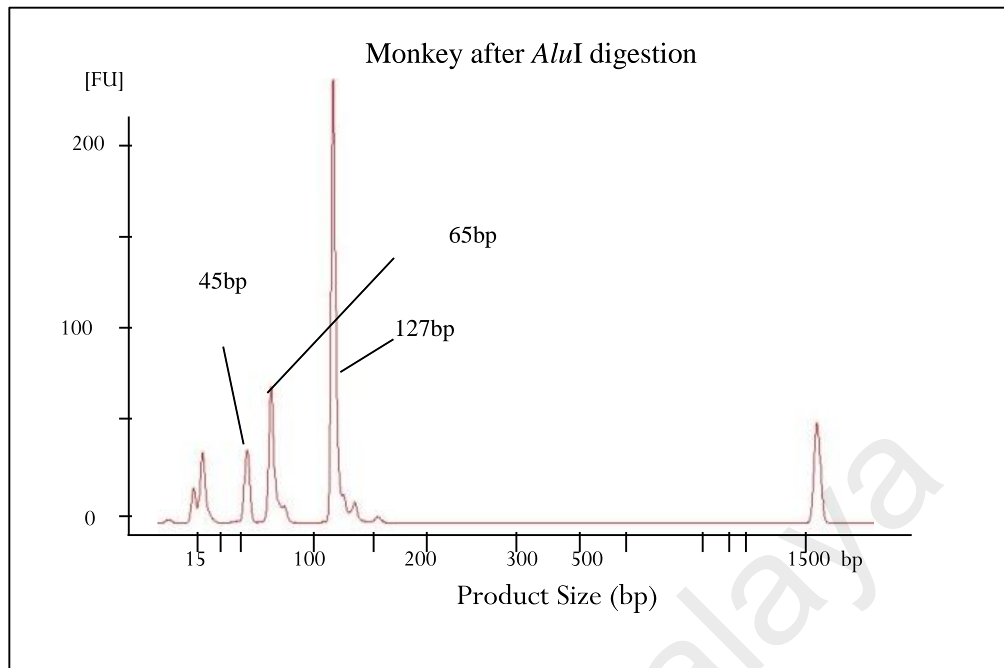


Figure 4.17: Restriction pattern analysis of monkey-target (120 bp) and endogenous control in pure state before digestion (Lanes 1-3) and after *Alu*I (Lane 4-6) and *CViKI*-I digestion (Lane 7-9). Lanes 1, 4 and 7: Monkey and Lanes 2, 5 and 8: Cow and Lane 3, 6 and 9: Goat.

University of Malaya



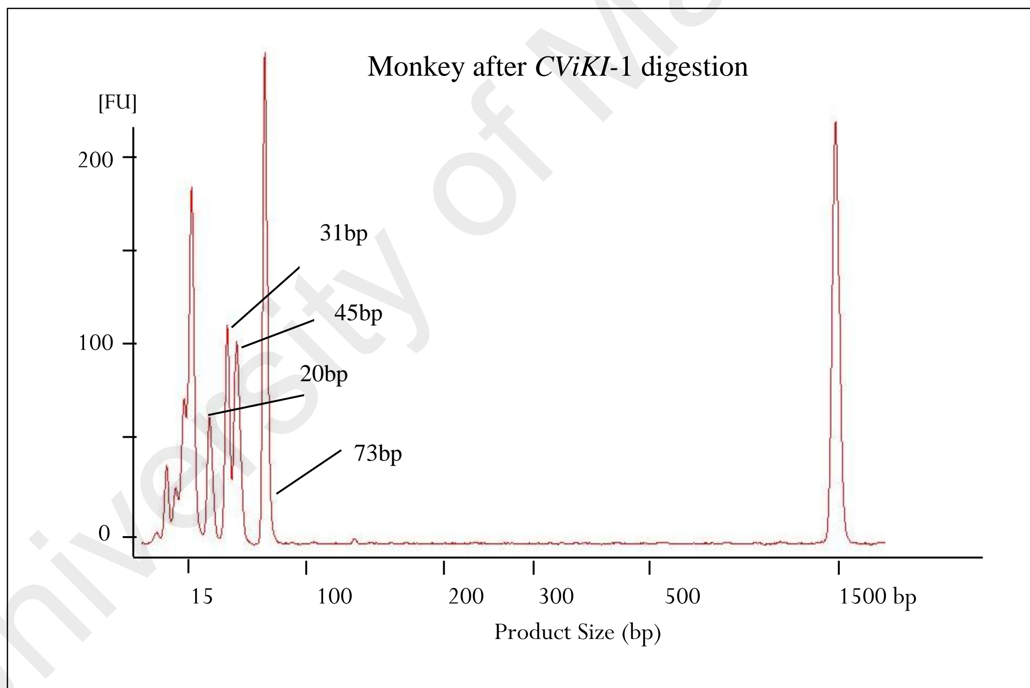


Figure 4.18: Electropherogram of amplified monkey DNA and endogenous control after *Alu*I and *CViKI*-I digestion. Peaks indicate the size of digested product; *Alu*I (127, 65, and 45 bp) and *CViKI-1* (73, 45, 31 and 20 bp).

University of Malaya

4.5.3 PCR-RFLP Analysis in Binary Mixture

RFLP analysis in meat mixture was also examined in order to define the ability of enzymatic process in complex background. For this purpose we only screen for monkey-goat mixture**. Figure 4.18** demonstrates restriction pattern analysis of monkey-target (120 bp) and endogenous control in binary admixed state with gradient percentage of monkey meat at 100% to 0.1% . Lane 1-4, and 7 shows 141 bp of control and 120 bp of monkey DNA band. *Alu*I digestion of the monkey PCR product give 65 and 44 bp (Lane 5 and 8) while *CViKI*-1 give (73, 45, 31 and 20 bp) in Lane 6 and 9. Lane 10 shows the 141 bp endogenous of 100% chevon.

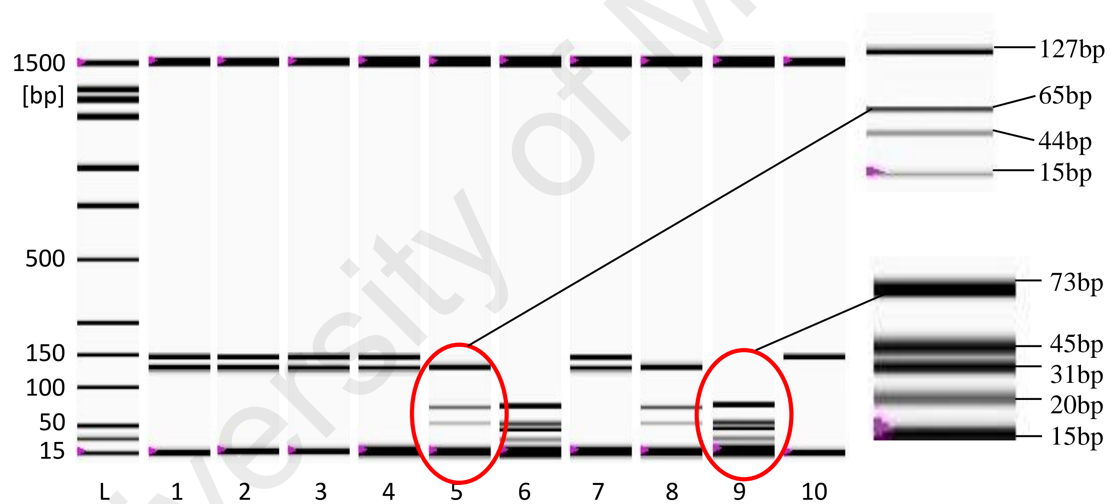


Figure 4.19: Specificity and sensitivity analysis in binary mixture of monkey and chevon. Lane L: DNA ladder; Lanes 1-4 and 7: 100%, 10%, 5%, 1% and 0.1% of monkey meat in balanced amount of chevon. Restriction patterns of PCR product obtained from 1% (Lanes 5 and 6) and 0.1% (Lanes 8 and 9) monkey in chevon after *Alu*I and *CViKI*-1 digestion. Lane 10: 100% chevon.

University of Malaya

Table 4.5 Restriction sites of *Alu*1 on 120 bp monkey mt-dloop gene.

Species	Forward Primer	*Alu* I Restricition Site	Reverse Primer
	10bp	65bp 76bp	110bp
Monkey	T G A A A T C A A T A T C C C G C A C A	A G C T A T A C T T G A G C T	C T C A G G G C C A T A A C A A C C A G
Cow	. . . . . C . . . C . A . . . . . T A G	T C G C T A T . C A A T . A A	T C T T C A . G G C C . T . T C A T C T
Sheep	. . . . . C . . . C . A . . . . . T . .	T A A C T A T T . A A T . A A	T C T T C A . G G C C . T . T C A T C T
Goat	. . . . . C . . G C . A . . . . . T T G	T A G C T A T T . A A T . A A	T C T T C A . G G C C . T . T C A . C T
Chicken	A . . G . . . . G C . A . . . C T G . C	T A . A C C C . . C . C C . .	G G T T C C T . G G . C . G G C A . . T
Buffalo	. . . . . C . . G C . A . . . T T C A G	T A G C T A T T C A A T . A A	T C T T C A . G G C C . T . T C A . C T
Pig	. . . . . C . . G C . A . . . . . T T G	T T T C T A T T G A T G A A C	. . T C A . . A . C A T C T C . . . T A
Deer	. . . . . C . . G C . A . . . . . T G G	T A G C T A T T . A A T . A A	T . T T C A . G G C C . T . T C A . C T
Duck	. . . . G C . A T . . T T G . .	T A A A C C C . . C . C C . .	T . . C T C . G T C A G G G C C A T C A
Salmon	A . . . C C G . C C . A . G A T T C . .	T A G G T C G . A . C T C G .	. C T . A . T . G . G G G . T . T . C T
Carp	A . . G . C . . C C . A . . A . T T T .	T . A G G G T T A C A C A A .	T . . C T A T T T C A G G G . C A T . A
Cod	A A C . T A A C C G G A . T T T . C T T	G A . C T A C A . C C C . T A	. . A G C T T . A G G T C . . T T G C T
Turtle	A . . G . . A . G C . A . . . T T G T T	G C G . . C . T A A C T . A .	T . T C A . . . A C A T . . T . T T G .
Rat	. . . . C . A . . . . . C . .	G A . . . . G A . A . .	T . . . . C . A T T G G T T
Cat	A . . . . C . . G C . A T . . T T G . T	G . T . T C T A . A A C . G A	T A . T T C A G G G C C . T . . A A T C
Dog	A . . . . C . . T C . A . . . T T G . T	G T T A C . . T C A T G A A A	A C T T C A . G G C C . T A . C . T T A
Quail	A . . G . . . . C C . A . . . C T G T C	C T A C . C C . C . C G C . C	G . T C C T C G G T C . G G C . . A T C
Pigeon	. . . . . C . . G C . A . T . . A C G C	C T A C . C C . . A . C A . G	T . . C T A T G T C A G G G C C A T . A

University of Malaya

Table 4.6 Restriction sites of *CViKI*-1 on 120 bp monkey d-loop gene

Species	Forward Primer		*CViKI*-1 Restriction Site / Reverse Primer	
	10bp	45bp	65bp / 76bp	107bp
Monkey	T G A A A T C A A T A T C C C G C A C A	G G C C	G T A G C T A T A C T T G A G C T	C T C A G G G C C A T A A C A A C C A G
Cow	. . . . . C . . . C . A . . . . . T A G	. . G .	. G T C G C T A T . C A A T . A A	T C T T C A . G G C C . T . T C A T C T
Sheep	. . . . . C . . . C . A . . . . . T . .	. . G .	. G T A A C T A T T . A A T . A A	T C T T C A . G G C C . T . T C A . C T
Goat	. . . . . C . . G C . A . . . . . T T G	. . G .	. G T A G C T A T T . A A T . A A	T C T T C A . G G C C . T . T C A T C T
Chicken	A . . G . . . . G C . A . . . C T G . C	T C A G	C C T A . A C C C . . C . C C . .	G G T T C C T . G G . C . G G C A . . T
Buffalo	. . . . . C . . G C . A . . . T T C A G	. . G .	. G T A G C T A T T C A A T . A A	T C T T C A . G G C C . T . T C A . C T
Pig	. . . . . C . . G C . A . . . . . T T G	. . G .	. G T T T C T A T T G A T G A A C	T . T T C A . G G C C . T . T C A . C T
Deer	. . . . . C . . G C . A . . . . . T G G	. . G .	. G T A G C T A T T . A A T . A A	. . T C A . . A . C A T C T C . . . T A
Duck	. . . . G C . A T . . T T G . .	T C A G	C C T A A A C C C . . C . C C . .	T . . C T C . G T C A G G G C C A T C A
Salmon	A . . . C C G . C C . A . G A T T C . .	. T . A	A . T A G G T C G . A . C T C G .	T . . . . C . A T T G G T T
Carp	A . . G . C . . C C . A . . A . T T T .	A . A A	T G T . A G G G T T A C A C A A .	T A . T T C A G G G C C . T . . A A T C
Cod	A A C . T A A C C G G A . T T T . C T T	. . T G	A A G A . C T A C A . C C C . T A	A C T T C A . G G C C . T A . C . T T A
Turtle	A . . G . . A . G C . A . . . T T G T T	T C A G	T G G C G . . C . T A A C T . A .	T . . C T A T T T C A G G G . C A T . A
Rat	. . . . C . A . . . . . C . .	. . . .	. . G A . . . . G A . A . .	. . A G C T T . A G G T C . . T T G C T
Cat	A . . . . C . . G C . A T . . T T G . T	C . G G	. G G . T . T C T A . A A C . G A	. C T . A . T . G . G G G . T . T . C T
Dog	A . . . . C . . T C . A . . . T T G . T	. . G .	. G G T T A C . . T C A T G A A A	G . T C C T C G G T C . G G C . . A T C
Quail	A . . G . . . . C C . A . . . C T G T C	T T . A	C C C T A C . C C . C . C G C . C	T . . C T A T G T C A G G G C C A T . A
Pigeon	. . . . . C . . G C . A . T . . A C G C	T T . A	C C C T A C . C C . . A . C A . G	T . T C A . . . A C A T . . T . T T G .

University of Malaya

University of Malaya

CHAPTER 5: DISCUSSION

5.1 Samples Collections

Despite abundance and wider availability, *Macaca fascicularis* are wildlife protected animals. However, they could be called for research purposes and hence we obtained permission and collected three individuals long-tailed macaque euthanised by Wild Life Malaysia in its premises for other research purposes. Other non-target species, such as chicken, duck, beef, buffalo, goat, lamb, venison, pork; quail, pigeon carp, cod and salmon, are regularly consumed and hence were procured from various markets in Selangor and Kuala Lumpur in Malaysia.

5.2 DNA extraction

Initially, DNA was extracted using Genomic DNA Mini Kit for Animal Tissues DNA. This DNA isolation kit, however, is not just specifically used to extract the whole genomic library, but also was designed for the purification of total DNA including mitochondrial DNA, viral DNA from the variety of animal tissues or cells. The kit come together with the micro-pestle which helps to homogenize tissue sample to shorten the time spent for cell lysis. As described in the protocol, this extraction method used proteinase K and chaotropic salt, guanidine hydrochloride to lyse cells and degrade protein. DNA in chaotropic salts binds to the glass fiber matrix of column and later on washed with elution buffer which actually is TE buffer, with contain low salt concentration.

The DNA extracted from the raw meat sample showed good quality and less contamination found when it produced an A260/A280 ratio of 1.70 to 2.00 (Table 4.1). The DNA concentration obtained as in Table 4.1.

University of Malaya

We obtained higher DNA concentration in treated samples (100-200 ng/µl in raw meats vs. 150-587ng/µl in boiled and autoclaved, and 600-700ng/µl in microwaved treated samples). The increase in DNA yield upon thermal processing of meat samples might be due to the dehydration process which results in increased number of cells for per unit weight of the sample (Karabasanavar et al., 2011b).

In addition, the concentration also satisfactory when only about 30 mg of meat tissue is needed to yield 100 to 200 ng/ul of DNA. In short, the extracted DNA isolated was fine but for commercial meat product (meatball) gave unstable DNA concentration and low in purity due to the additional ingredients of meatball. This shortcoming has been overcome by adding more meat sample (50-100 mg), and double up the elution step in order to increase the DNA recovery.

5.3 Specificity Test and *In Silico* Analysis of Available Monkey Species

Species-specific PCR are often conclusive and have been widely used for the detection of beef (Calvo et al., 2002), chicken (Haunshi et al., 2009), pork and dog (Ali et al., 2012a; 2013) in singleplex and cattle, pig, chicken, sheep, goat and horse (Matsunaga et al., 1999) in multiplex PCR systems. Although the latter allow amplifying and detecting multiple targets at a time, reducing cost and time, it is often tedious and difficult to achieve optimum PCR condition with multiple target-species with uniform sensitivity. In contrast, singleplex PCR is easier, robust, accurate and highly sensitive to amplify a specific and single target (Ali et al.,2012a).

We retrieved the mt-DNA sequence of *M. facsicularis* (FJ906803.1) from NCBI and designed a set of primers to specifically amplify a short fragment 120bp of the d-loop region. We performed *in silico* analysis of 11 *Macaca* genus species to measure the possibility of detection with designed primers. The primer pairs were aligned with total of

University of Malaya

31 monkey species (11 *Macaca* species and 20 *Colobines*), 17 common meat species and *homo sapiens* DNA sequence. The result among all monkey species as in (Figure 4.2, Table 4.2, and Table 4.4). The results of ClustalW multiple alignment program revealed almost perfect matching (only 0-2 nt mismatching) with the d-loop gene of *M. fascicularis, M.arctoides, M. nemestrina, M.sylvanus, M.thibetana and M. fuscata;* and 0-4 nt mismatching for the rest of monkey species except for *M.radiata* (Table 4.2). Similar results were obtained from BLAST analysis in NCBI against non- redundant nucleotide sequences (result not shown), suggesting that the developed primers might be universal for the detection of macaque species. This also derived the cross testing of the primers with all the macaque species. Due to unavailability of macaque species in local markets as well as strong government's legislation, we did not get sufficient number of macaque species in commercial pet shops, raw markets. We had applied the Department of Wildlife and National Park Malaysia (PERHILITAN), all Macaca species but permission was given only for *Macaca fascicularis.* Although we could not test the ability of designed primers to amplify other macaque species, the comparison of primer mismatches predicted that at least 4 macaque species *(M.nemestrina, M.arctoides, M.sylvanus and M.thibetana)* could be amplified since they contained only one mismatch at the reverse primer. According to Wu et al., 2009, mismatch at the 3' end of primer binding region may hinder the successful PCR amplification. As for the rest of macaque species, the number of mismatch nucleotides was between 1 to 4 bases, which needs further verification using a practical PCR test.

On the other hand, multiple alignment between primers and common meats gave high nucleotide mismatch (10-14 nt) (Table 4.2); reflects the high specificity of macaque primer. Pairwise distance (Table 4.2) and phylogenetic tree (Figure 1b) among the closely related species were analysed using the maximum composite likelihood method (Ali et al., 2014; Mahfujur et al. 2014). The lowest distance was observed between *M.*

University of Malaya

fascicularis and buffalo, carp, dog, cat, rat and pig (1.43-1.44) and the highest was found between monkey and chicken (1.86), indicating a high genetic distance and unlikelihood of cross-species amplification in a real PCR run. The mismatched-bases in the primer binding sites of the studied species were between 50-70% which made the cross-species detection improbable (Ali et al., 2012). The 3D plot (Figure 4.3) clearly reflected high discrimination of *M. facicularis* from other animal species. We did the PCR test against 17 available species and it amplified only the *M. fascicularis* 120bp product (Figure 1a), further confirming the theoretical findings.

Four different PCR assays have been documented for the detection of monkey-species for the phylogenetic studies. However, the targets for those assays (Md-Zain et al., 2010) (cytochrome c, 850bp), (Abdul-Latiff et al., 2014) (cytochrome b, 383 bp), (Hayasaka et al., 1996) (mt-whole genome, 896bp) and (Blancher et al., 2008) (d-loop, 590bp), were very large which easily break down during food processing. Thus the documented assays are not suitable for meat-species detection in foods (Ali et al. 2012a; 2014). Meanwhile, Rönn et al. (2009) proposed a first generation microarray system for the detection of various primate species targeting the epsilon globin (341bp) and apoplipoprotein B gene (550 bp) sequences to trace out wild-meat trades. The latest studies appreciated short-amplicon-length PCR assays (<150 bp) targeting multi-copy mitochondrial genes for the detection of animal species in highly processed foods (Ali et al 2012a; 2014). Therefore, we documented here a 120-bp PCR assay targeting mt-d-loop gene for *M. fasicularis* detection in processed meats. To the best of our knowledge, such a short-amplicon-length PCR-RFLP method for macaque meat detection is the first report in literature.

In food industry, replacement of costly meats by cheaper products prevails to increase profit. Therefore, we screened here four different “Halal” branded chicken and

University of Malaya

four beef meatball brands and as result, all meatball tested was “free” from monkey meat contamination (Figure 4.2.2b). The screening was done triplicates on three different days to eliminate analyst biased. To mimic the real adulteration situation, the dummy model meatball was prepared following Rohman et al., 2011 and Rahman et al.,2014, and their composition is given in **Table 3.5**. While the monkey PCR-product was obtained from all positive controls, no commercial meatball collected from different outlets were found to be positive for monkey DNA (**Figure 4.11**), reflecting the absence of monkey-meat adulteration in meatball formulations in Malaysia. Amplification of endogenous eukaryotic control, reflected good quality DNA in all commercial products. The findings are acceptable in Malaysian perspectives since the country is committed to develop Halal-hub industry and strictly monitoring the Halal status of foods.

5.4 Limit of Detection

Generally, chicken, beef, goat, lamb and pork among the livestock, and deer and wild boar among the wild animals, are extensively examined for adulteration in foods. Up-to-this date, no detection technique for monkey species detection in foods have been tested and optimized. The currently available monkey specific PCR assays (Md-Zain et al., 2010; Abdul-Latiff et al., 2014; Hayasaka et al., 1996; Blancher et al., 2008) are suitable for evolutionary, taxonomy and phylogenetic studies among the species. Since these studies were not tested for meat authentication, their limit of detection (LOD) has remained undefined. Two sets of 10 fold serial dilution (10 to 0.0001 and 10 to 0.00001 ng) of DNA extracted from pure raw meat by three independent analysts on three different days was used to determine sensitivity under raw and pure states. Previously, Che Man et al. (2012) and Karabasanavar et al. (2014) tested their assay sensitivity for pork DNA by dilution method and detected as low as 0.001 ng DNA/µl. Here we clearly observed 120 bp PCR products from 0.0001ng macaque DNA template by conventional gel and as

University of Malaya

low as 0.00001ng of monkey DNA by automated electrophoresis due to its higher sensitivity instrument. Thus we defined the LOD for this assay under raw and pure states (**Figure 4.8**). Since spectrocopic measurement of DNA at low concentration is not reliable, the first concentration was measured at >100 ng level and then desired concetration was achieved by serial dilution of the average of triplicates

In order to simulate the real process of meat adulteration, we used base adulterated meat mixture (BAM) (Ali et al., 2012a). **Figure 4.9** shows PCR products from both monkey-beef and monkey-goat binary admixture and demonstrates that macaque-specific PCR assay developed in this study was highly sensitive since it can identify as low as 0.1% (w/w) monkey meat under mixed background. The intensity of the PCR product obtained from 0.1% monkey admixed suggested that the assay could detect much lower than 0.1%. Levels of adulteration or contamination down as low as 1% have been routinely detected and amounts of less than 0.1% have been shown to produce positive results (Lockley & Bardsley, 2000). Ali et al. (2012a) and Yusop et al. (2012) detected up to 0.01% (w/w) and 0.1% (w/w) respectively, of pork in meat mixture. The level of intentional adulteration are considered above 5% and inadvertent contamination is said to be in the range of 0.1 to 1% (Meyer & Candrian, 1996), therefore the sensitivity of this assay is appropriate to be applied for both detection of profit making adulteration and contamination of monkey DNA in meat products.

To challenge the assay detection limit, another lower concentration (0.00001 ng) of target DNA was prepared and run in Microfluid-Lab–Chip Experion automated system. Unlike gel electrophoresis where the sensitivity was found to be 0.0001ng, predictably, this system is able to detect as low as this quantity prove it more sensitive than conventional ones (**Figure 4.10**). This is because factors such as gel concentration, voltage, electrophoresis buffer and DNA stain did affect the separation and image of DNA

University of Malaya

in conventional agarose procedure in which produced different results and assay efficiency (Barakat et al.,2014). Therefore, automated electrophoresis system is a sustainable procedure that solved the drawback possessed by conventional gel.

5.5 Target DNA Stability Under Heat Treatment

The purpose of applying heat to meat sample was to study the effect of different thermal process on target DNA degradation (Arslan et al., 2006; Haunshi et al., 2009; Ilhak & Arslan, 2007). 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 steaming and canning process since it cooks at very high temperature (up to 300 °C) under pressurized conditions to kill any potential microbes present. **Figure 4.12-14**demonstrates that DNA extracted from all the heat-treated samples was successfully amplified by PCR. Boiling of meat samples at 60, 80 and 100 °C for 30 min did not affect the amplification of species-specific markers from *M.fascicularis* species (**Figure 4.12**). In a standard domestic practice microwave cooking is performed at 500 Watt (W) for 15-30 min. We cooked meat at three different conditions; low (300 W), medium (500 W) and extreme (700 W) microwaving and obtained PCR products from 10-0.001 ng template DNA (**Figure 4.13**). Meat cooked above 700 W for 30 min appeared to be dried out, burnt and thus was not suitable for consumption (data not shown). 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 in the present assay was sufficiently amplified up to 700 W microwaving for 30 min (**Figure 4.13**), reflecting the target-stability under extreme conditions.

University of Malaya

It is widely reported that the intensity of the heating and pressure of food-processing treatments, such as sterilization, clearly affects DNA fragmentation and can lead to false-negative results (Hird et al. 2006). Previously, Haunshi et al., 2009; Karabasanavar et al.,2011b and Mane et al., 2012 studied the effect of autoclave on DNA by treating various type of domestic meat at 121 °C for 15-30 min and they found their sample were stable and were not degraded at this condition. Meanwhile, Rojas et al. (2010) who carried out the quantitative study (real-time PCR technique) found out the positive signals were still observed in thermally treated samples containing though reducing in the amount of detectable small percentages of the target species DNA (Rojas et al., 2010). Here, we autoclaved monkey meat at 120 °C for 30, 90 and 150 min (extensive treatment) and found PCR products under all conditions. However, faded bands were obtained from 0.01 and 0.001 ng template under extreme autoclaving (Lanes 17 and 18 in **Figure 4.14**), reflecting some degree of target breakdown under extreme treatments. However, it did not affect the identification. This finding was in line with the established fact that shorter DNA targets are extremely stable under extreme processing treatments (Ali et al, 2014).

In order to adapt with real meat adulteration, we also analyzed the stability of the target DNA in commercial meat products, namely chicken (9 samples) and beef (9 samples) meatballs, collected from three different outlets in triplicates on three different days. While all the model meatballs of deliberate contaminations, amplified macaque specific PCR product, such product was absent in commercial meatball specimens, reflecting no adulteration of macaque meat in meatball products in Malaysia (Table 4.4). The changes of false negative detection was eliminated using a positive endogenous control that amplified 141bp from all specimens.

University of Malaya

5.6 RFLP Analysis

We successfully amplified 120 bp-site of mitochondrial d-loop gene of macaque monkey in the presence of a 141bp universal eukaryotic site of 18S rRNA as an internal control to evaluate the quality of the DNA used as well as to eliminate false negative detection (Ali et al., 2012a) Although, species-specific PCR assays are often conclusive, authentication of amplified PCR products would definitely increase the assay reliability. Occasionally, the end-point PCR assay can be unconvincing since it only shows the virtual data with lack of sequence information. This, however, can be overcome by other complementing analytical techniques namely, restriction analysis with at least two restriction endonucleases, probe hybridisation and DNA sequencing could verify authentic PCR products (Maede, 2006). Probe hybridisation is interesting since it can to detect multiple species simultaneously using more than 2 DNA probes in a single hybridisation reaction (labelled dyes) or separately. However, this procedure is laborious and requires high quality DNA which is less feasible for heat/chemical-treated DNA extracted from processed meats or meat products (Mafra et al., 2008). On the other hand, DNA sequencing is reliable but it is time-consuming, requires expensive laboratory set up and thus is not suitable for routine meat specification assessment (Girish et al., 2004; Lockley & Bardsley, 2000). Cooked or processed samples with degraded DNA and complex food matrices might further complicate it, hindering result interpretation. In contrast, PCR-RFLP has been extensively used to distinguish two or more closest species with simple instrumentation (Ong et al., 2007; Verkaar et al., 2002). It comprises of the generation of species-specific band profiles through restriction-digestion with one or more restriction endonucleases (Pereira, Carneiro, & Amorim, 2008) . These restriction enzymes cleave DNA molecule at recognition sites, originating a set of fragments with different lengths that could be separated according to their molecular size by electrophoresis (Pereira et al., 2008) PCR-RFLP has been proved to be a practical, highly

University of Malaya

repeatable and reliable technique meat species identification (Haider, Nabulsi, & Al-Safadi, 2012). In this work, we validated our assay by PCR-RFLP analysis since the amplicon size was small with adequate restriction sites but was short for DNA sequencing which requires larger sequences. RFLP also needs simple instrument and could be done in ordinary lab settings.

Thus, we digested the 120 bp monkey-specific PCR products by two different enzymes, *Alu*I and *CViKI*-1 since *in-silico* analysis showed available restriction sites for these enzymes with suitable fragment-lengths (New England Biolabs, http://nc2.neb.com/NEBcutter2/). Two-sites for *Alu*I (**Figure 4.15, Table 4.5**) and 4 sites for *CViKI*-1) (**Figure 4.16, Table 4.6**) were found within the amplified sequence (120 bpThe reliability of RFLP technique was screened first in pure sample. Lane 4 in **Figure 4.17** demonstrates 2 fragments of length 65 and 44 bp which resulted following *Alu*I digestion of the PCR product. However, a 11 bp fragment which was below the resolution capacity of the instrument (15 bp) could not be detected. Meanwhile, lane 7 of the same figure presents the *CViKI*-1 digestion product (3 fragments of length 45, 31, 20 bp). The other fragments (13 and 11 bp) were below the lower end resolution and hence remained undetected. On the other hand, endogenous control (141 bp) produced two *Alu*I (127 and 14bp) and four *CViKI*-1 fragments (73, 39, 15, and 14 bp). However, only 127 bp (Lane 4) and 73 and 39 bp (Lane7) fragments were detected.

Previously, we prepared two sets of mixed meat products (monkey–beef and monkey–chevon) to simulate the most potential forms of adulteration to detect adulterated monkey meats in processed meats. However, only monkey-chevon admixed is presented due to simplicity (**Figure 4.18**). Monkey specific PCR product (120bp) was obtained from all level of adulteration, even as low as 0.1 % (w/w) of spiked monkey meat in beef and chevon. An endogenous 141 bp eukaryotic targets were amplified from all admixed,

University of Malaya

reflecting good quality DNA in all admixtures, eliminating the chances of any false negative detection. We further confirmed the monkey-specific PCR product amplified from mixture backgrounds by digesting them with *Alu*I and *CViKI*-1. It has been reported that meat admixtures are not suitable for PCR-RFLP analysis since the digestion results might show a combination of miscellaneous restriction patterns for all possible species contained in the adulterated sample (Fajardo, 2007). However, we successfully amplified only the targeted products and its digestion products were similar to those from pure background. Therefore, we documented here a 120-bp PCR-RFLP assay targeting mt-d-loop gene for *M. fasicularis* detection in processed meats. To the best of our knowledge such a short-amplicon-length PCR-RFLP method for macaque meat detection is the first report in literature.

Previously, larger PCR product size (200-800 bp) used to be the target and caused no burden to researchers to find other alternatives as the RFLP analysis can be directly run in gel electrophoresis (Sun and Ling, 2003; Verkaar et al.,2006; Malisa et al., 2006). However, as the aim of this study to employed short DNA amplicon, few limitations came up such as the ability and availability of restriction enzyme. Smaller amplicon size tend to have few restriction site, or worst case to have near cutting site result in difficulty in analysis. Conventional gel procedure unable to differentiate closer gel bands since they have lower resolution. By using Experion Automated system, however, it provides excellent resolution (5-10 bp) over a broad dynamic range, hence able to discriminate near located band. This instrument also allow analysis of DNA fragments of 15–1,500 bp. In short, Experion Automated Electrophoresis system is a perfect instrument to analyze the PCR products in a systematic way which provides automatic documentation, rapid, sensitive, and reproducible results as well as quantification data..

University of Malaya

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Two types of DNA based specification methods successfully developed for the detection of shorter-length macaque-mitochondrial DNA in raw and processed mixed meats and commercial food products. The first one was the species-specific PCR assay which itself is conclusive and allowed monkey material identification under raw, pure, admixed and commercial matrices. The primers targeting a 120-bp sites of d-loop gene which is present in multiple copies in each cells were successfully designed. The macaque specificity was ensured by alignment analysis, mismatch comparison, phylogenetic tree and 3D plot. The primers set were challenged against 17 potential species and accurate target was amplified only from the macaque targets, confirming the specificity and self-standing ability of the designed primers. The specificty of the developed primers were theoretically analyzed with 51 different species, including 13 species of macaque genus. The results demonstrated conserved sequences for the most of the macaque species but huge mismatches with other primates and non-primate species, indicating that the developed primers might be universal for all macques. However, the findings could not be concluded due to the lack of samples from other macaque species.

Since the breakdown of target is quite common under food processing conditions, the monkey meats were subjected under boiling at 60 °C, 80 °C and 100 °C for 30 min, microwave oven at 300, 500 and 700 Watt for 30 min using commercial home microwave and autoclaving at 121 °C under 14.5 psi for 30, 60 and 150 min treatments. Extraordinary stability were revealed under all treatment conditions, reflecting the reliability of the targets under any compromised states such as natural decomposition or force full degradation of DNA by physical or chemical shocks of food processing. Further checked

University of Malaya

the specificity under ternary admixed and matrices of commercial foods such as burgers and meatballs and satisfactory results were obtained since macaque targets were amplified from all backgrounds.

Secondly, shorter targets often compromise specificity since number of species-specific fingerprints is reduced within a shorter-sequence regime. Consequently, the authentic PCR targets were verified by RFLP analysis. The PCR product was amplified in presence of a 141bp universal site of eukaryotic 18S rRNA gene and digested with *Alu*I and *CViKI*-1 restriction enzymes since *in-silico* analysis by NEB cutter demonstrated, two *Alu*I restriction sites with fragments lengths of (65,44 and 11bp) and 3 restriction sites with fragments lengths of *CViKI*-1 digestion product (45, 31, 20 bp). On the other hand, endogenous control (141 bp) produced two *Alu*I (127 and 14bp) and four *CViKI*-1 fragments (73, 39, 15, and 14 bp) digests were separated in a microfluidic-based lab-on-a-chip automated electrophoresis system incorporated in Experion Bioanalyzer. The fragments were separated with good resolution and it was reflected both in gel-image and electropherograms. The tested limit of detection was 0.00001 ng macaque DNA under raw states, 0.1% (w/w) in binary admixtures and complex matrix commercial food products (meatballs).

To the best of our knowledge, it is the first systematic study for macaque meat detection under complex matrices and compromised states. No study has developed a PCR assay with as low as 120 bp target with enough fingerprints for macaque species. The extraordinary stability and well-established sensitivity of the study reflects its application in food authentication or archaeological studies of macaque species. The study is relevant in Malaysian perspectives since the country is committed to build up

University of Malaya

halal hub industry and a macaque monkey, which considered as prohibited animal abundant in its tropical forests.

6.2. Recommendation for Future Work

Recently, real-time PCR has got popularity over other PCR-based methods because of its automation, rapidity and sensitivity and ability to quantify potential targets. Thus there is a clear scope to develop various real-time PCR assays such as SYBR Green, Evagreen, Molecular Beacon and TaqMan probe real-time PCRs. Most of the PCR assays have not been validated under various food matrices. It is also difficult to extract DNA from various matrices such as fat, dairy products, chocolates etc. Therefore, appropriate DNA extraction protocol should be developed, optimized and adapted for various food matrices. The PCR targets were developed here should be validated by comparing it with other exiting targets. Multiplex PCR assays are highly promising since they allow the detection of multiple species in a single assay platform, reducing cost and time. Therefore, the opportunity to develop such assays for multiple haram species could be explored.

University of Malaya

REFERENCES

Abdul-Latiff, M. A. B., Ruslin F., Fui V. V, Abu, M., Japning J., Rovie-Ryan, Abdul-Patah P., Lakim M., Roos C., Yaakop S., Md-Zain B. M. (2014). Phylogenetic relationships of Malaysia's long-tailed macaques, *Macaca fascicularis*, based on cytochrome b sequences. *ZooKeys*(407), 121-139. doi: 10.3897/zookeys.407.6982

Abdulmawjood, A., Schönenbrücher, H., & Bülte, M. (2003). Development of a polymerase chain reaction system for the detection of dog and cat meat in meat mixtures and animal feed. Journal of food science, 68(5), 1757-1761.

Abegg, C., & Thierry, B. (2002). Macaque evolution and dispersal in insular south-east Asia. Biological Journal of the Linnean Society, 75(4), 555-576. doi: 10.1046/j.1095-8312.2002.00045.x

Aida, A. A., Che Man, Y. B., Wong, C. M. V. L., Raha, A. R., & Son, A. R. (2005). Analysis of raw meats and fats of pigs using polymerase chain reaction for Halal authentication. Meat science, 69(1), 47-52. doi: http://dx.doi.org/10.1016/j.meatsci.2004.06.020

Ali, M. E., Hashim, U., Mustafa, S., Che Man, Y. B., Yusop, M. H. M., Bari, M. F., . . . F., H. M. (2011). Nanoparticle sensor for label free detection of swine DNA in mixed biological samples. Nanotechnology, 22(19), 195503.

Ali, M. E., Hashim, U., Mustafa, S., & Che Man, Y. B. (2012a). Swine-Specific PCR-RFLP Assay Targeting Mitochondrial Cytochrome B Gene for Semiquantitative Detection of Pork in Commercial Meat Products. Food Analytical Methods, 5(3), 613-623.

Ali, M. E., Rahman, M. M., Hamid, S. B. A., Mustafa, S., Bhassu, S., & Hashim, U. (2013). Canine-specific pcr assay targeting cytochrome b gene for the detection of dog meat adulteration in commercial frankfurters. Food Analytical Methods, 7(1), 234-241.

Ali, M. E., Razzak, M. A., & Hamid, S. B. A. (2014). Multiplex PCR in Species Authentication: Probability and Prospects—A Review. Food Analytical Methods, 1-17.

Ali, M. E., Razzak, M. A., Hamid, S. B. A., Rahman, M. M., Al-Amin, M., Rashid, N. R. A., & Rakhine, A. (2015). Multiplex PCR Assay for the Detection of Five Meat Species Forbidden in Islamic Foods. Food chemistry.

Arslan, A., Ilhak, O. I., & Calicioglu, M. (2006). Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat science, 72(2), 326-330.

Asensio, L., González, I., García, T., & Martín, R. (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control, 19(1), 1-8.

Ballin, N. Z., Vogensen, F. K., & Karlsson, A. H. (2009). Species determination–Can we detect and quantify meat adulteration?. Meat science, 83(2), 165-174. Ballin, N. Z. (2010). Authentication of meat and meat products. Meat science, 86(3),577-587.

University of Malaya

Barakat, H., El-Garhy, H. A., & Moustafa, M. M. (2014). Detection of pork adulteration in processed meat by species-specific PCR-QIAxcel procedure based on D-loop and cytb genes. Applied microbiology and biotechnology, 98(23), 9805-9816.

BBCNews. (2013, 2 February 2013). Traces of pork DNA found in halal prison meat. from http://www.bbc.com/news/uk-21302925Bennett, E. L. (2002). Is there a link between wild meat and food security? Conservation Biology, 590-592.

Bernués, A., Olaizola, A., & Corcoran, K. (2003). Labelling information demanded by European consumers and relationships with purchasing motives, quality and safety of meat. Meat science, 65(3), 1095-1106. Blancher, A., Bonhomme, M., Crouau-Roy, B., Terao, K., Kitano, T., & Saitou, N. (2008). Mitochondrial DNA sequence phylogeny of 4 populations of the widely distributed cynomolgus macaque (Macaca fascicularis fascicularis). Journal of heredity, 99(3), 254-264.

Bonne, K., & Verbeke, W. (2008). Religious values informing halal meat production and the control and delivery of halal credence quality. Agriculture and Human Values, 25(1), 35-47.

Bottero, M. T., & Dalmasso, A. (2011). Animal species identification in food products: Evolution of biomolecular methods. The Veterinary Journal, 190(1), 34-38.Calvo, J. H., Rodellar, C., Zaragoza, P., & Osta, R. (2002). Beef-and bovine-derived material identification in processed and unprocessed food and feed by PCR amplification. Journal of agricultural and food chemistry, 50(19), 5262-5264.

Brandon-Jones, D., Eudey, A. A., Geissmann, T., Groves, C. P., Melnick, D. J., Morales, J. C., . . . Stewart, C. B. (2004). Asian Primate Classification. International Journal of Primatology, 25(1), 97-164. doi: 10.1023/B:IJOP.0000014647.18720.32

Cammà, C., Di Domenico, M., & Monaco, F. (2012).Development and validation of fast real-time PCR assays for species identification in raw and cooked meat mixtures. Food Control, 23(2), 400-404.

Castle, S. (2013).Nestlé Removes 2 Products in HorseMeat Scandal, The New York Times. Retrieved from www.nytimes.com/2013/02/20/ world/europe/nestle-pulls-2-products-in-horse-meat-scandal.html?_r=0&pagewanted=print

Cawthorn, D.-M., Steinman, H. A., & Hoffman, L. C. (2013). A high incidence of species substitution and mislabelling detected in meat products sold in South Africa. Food Control, 32, 440-449.

Che Man, Y. B., Aida, A. A., Raha, A. R., & Son, R. (2007). Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control, 18(7), 885-889. doi: http://dx.doi.org/10.1016/j.foodcont.2006.05.004

Che Man, Y. B., Mustafa, S., Khairil Mokhtar, N. F., Nordin, R., & Sazili, A. Q. (2012). Porcine-specific polymerase chain reaction assay based on mitochondrial d-loop gene for identification of pork in raw meat. International Journal of Food Properties, 15(1), 134-144.

University of Malaya

Chen, F.-C., & Hsieh, Y. (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. Journal of AOAC International, 83(1), 79-85.

Chen, S.-Y., Liu, Y.-P., & Yao, Y.-G. (2010). Species authentication of commercial beef jerky based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene. Journal of Genetics and Genomics, 37(11), 763-769.

Chikuni, K., Ozutsumi, K., Koishikawa, T., & Kato, S. (1990). Species identification of cooked meats by DNA hybridization assay. Meat science, 27(2), 119-128.

Chomel, B. B., Belotto, A., & Meslin, F.-X. (2007). Wildlife, exotic pets, and emerging zoonoses. Emerging infectious diseases, 13(1), 6.

Colombo, F., Cardia, A., Renon, P., & Cantoni, C. (2004). A note on the identification of Rupicapra rupicapra species by polymerase chain reaction product sequencing. Meat science, 66(3), 753-755.

Colombo, F., Marchisio, E., Pizzini, A., & Cantoni, C. (2002). Identification of the goose species (Anser anser) in Italian "Mortara" salami by DNA sequencing and a Polymerase Chain Reaction with an original primer pair. Meat science, 61(3), 291-294.

Conlan, J. V., Vongxay, K., Khamlome, B., Gomez-Morales, M. A., Pozio, E., Blacksell, S. D., . . . Thompson, R. (2014). Patterns and Risks of Trichinella Infection in Humans and Pigs in Northern Laos. PLoS neglected tropical diseases, 8(7), e3034.

Davidson, J.A. (2003). World Religions and the Vegetarian Diet. Journal of the Adventist Theological Society, 14/2 (Fall 2003): 114–130

Delgado, C., Rosegrant, M., Steinfeld, H., Ehui, S., & Courbois, C. (2001). Livestock to 2020: the next food revolution. Outlook on Agriculture, 30(1), 27-29.

Dooley, J. J., Paine, K. E., Garrett, S. D., & Brown, H. M. (2004). Detection of meat species using TaqMan real-time PCR assays. Meat science, 68(3), 431-438.

Eudey, A. A. (2008). The Crab-Eating Macaque (Macaca fascicularis): Widespread and Rapidly Declining. Primate Conservation, 23(1), 129-132. doi: 10.1896/052.023.0115

European Commission (2000). Council Regulation (EC) No 1760/2000 of the European Parliament and of the Council of 17 July 2000 establishing a system for the identification and registration of bovine animals and regarding the labelling of beef and beef products

Engler, M., Parry-Jones, R., & Europe, T. (2007). Opportunity or threat: The role of the European Union in global wildlife trade: Traffic Europe.

Enser, M., Hallett, K., Hewitt, B., Fursey, G. A. J., & Wood, J. D. (1996). Fatty acid content and composition of english beef, lamb and pork at retail. Meat science, 42(4), 443-456. doi: http://dx.doi.org/10.1016/0309-1740(95)00037-2

Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., Garcı´a, T., & Martín, R. (2008). Differentiation of European wild boar (Sus scrofa scrofa) and domestic swine (Sus scrofa domestica) meats by PCR analysis targeting the

University of Malaya

mitochondrial D-loop and the nuclear melanocortin receptor 1 (MC1R) genes. Meat science, 78(3), 314-322.

Fajardo, V., González, I., Rojas, M., García, T., & Martín, R. (2010). A review of current PCR-based methodologies for the authentication of meats from game animal species. Trends in Food Science & Technology, 21(8), 408-421. doi: http://dx.doi.org/10.1016/j.tifs.2010.06.002

Fooden, J. (1995). Systematic review of Southeast Asian longtail macaques, Macaca fascicularis (Raffles, [1821] (Vol. n.s. no.81(1995)). [Chicago, Ill.] :: Field Museum of Natural History.

Fooden, J. (2006). Comparative review of fascicularis-group species of macaques (Primates: Macaca). Fieldiana Zoology, 1-43.

Fuentes, A (2011). Monkeys on the Edge: Ecology and Management of Long-Tailed Macaques and their Interface with Humans. New York, NY: Cambridge University Press, New York.

Girish, P., Anjaneyulu, A., Viswas, K., Anand, M., Rajkumar, N., Shivakumar, B., & Bhaskar, S. (2004). Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat science, 66(3), 551-556.

Girish, P., Anjaneyulu, A., Viswas, K., Shivakumar, B., Anand, M., Patel, M., & Sharma, B. (2005). Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat science, 70(1), 107-112.

Haider, N., Nabulsi, I., & Al-Safadi, B. (2012). Identification of meat species by PCR-RFLP of the mitochondrial COI gene. Meat science, 90(2), 490-493. doi: http://dx.doi.org/10.1016/j.meatsci.2011.09.013

Hambali, K., Ismail, A., Zulkifli, S. Z., Md-Zain, B. M., & Amir, A. (2012). Human-Macaque Conflict and Pest Behaviors of Long-Tailed Macaques (Macaca fascicularis) in Kuala Selangor Nature Park.

Hannah, R. (2013). Horsemeat found in some school dinners. Retrieved 15 February 2014, from http://www.bbc.co.uk/news/education-21475337

Hargin, K. D. (1996). Authenticity Issues in Meat and Meat Products. Meat science, 43, 277-289.

Hanzaee, K. H., & Ramezani, M. R. (2011). Intention to halal products in the world markets. Interdisciplinary Journal of Research in Business, 1(5), 1-7.

Haunshi, S., Basumatary, R., Girish, P., Doley, S., Bardoloi, R., & Kumar, A. (2009). Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. Meat science, 83(3), 454-459.

Hayasaka, K., Fujii, K., & Horai, S. (1996). Molecular phylogeny of macaques: Implications of nucleotide sequences from an 896-base pair region of mitochondrial DNA. Molecular Biology and Evolution, 13(7), 1044-1053. Hird, H., Chisholm, J., Sánchez, A., Hernandez, M., Goodier, R., Schneede, K., ... & Popping, B. (2006). Effect of heat and pressure processing on DNA

University of Malaya

fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. Food additives and contaminants, 23(7), 645-650.

Hoffman, L. C., & Wiklund, E. (2006). Game and venison – meat for the modern consumer. Meat science(74), 197-208. doi: 10.1016/j.meatsci.2006.04.005

Hoffman, L., & Cawthorn, D. (2012). What is the role and contribution of meat from wildlife in providing high quality protein for consumption. Animal Frontiers, 2(4), 40-53.

Ilhak, O. I., & Arslan, A. (2007). Identification of meat species by polymerase chain reaction (PCR) technique. Turkish Journal of Veterinary and Animal Sciences, 31(3), 159-163.

Ikeda, J. P. (1999). Culture, food, and nutrition in increasingly culturally diverse societies. In J. Germov & L. Williams (Eds.), The Sociology of Food and Nutrition: The Social Appetite. South Melbourne: Oxford University Press.

J.D. Wood, M. E., A.V. Fisher, G.R. Nute, P.R. Sheard, R.I. Richardson,, & S.I. Hughes, F. M. W. (2007). Fat deposition, fatty acid composition and meat quality: A review. Meat science, 78, 343-358. doi: doi:10.1016/j.meatsci.2007.07.019

Karabasanavar, N. S., Singh, S. P., Umapathi, V., Girish, P. S., Shebannavar, S. N., & Kumar, D. (2011a). Authentication of carabeef (water buffalo, Bubalus bubalis) using highly specific polymerase chain reaction. European Food Research and Technology, 233(6), 985-989.

Karabasanavar, N. S., Singh, S. P., Umapathi, V., Kumar, D., & Shebannavar, S. N. (2011b). Identification of goat meat using highly species-specific polymerase chain reaction. Journal of Food Quality, 34(2), 142-149.

Karabasanavar, N. S., Singh, S. P., Kumar, D., & Shebannavar, S. N. (2014). Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. Food chemistry, 145, 530-534.

Karlsson, A. O., & Holmlund, G. (2007). Identification of mammal species using species-specific DNA pyrosequencing. Forensic Science International, 173(1), 16-20. doi: http://dx.doi.org/10.1016/j.forsciint.2007.01.019

Kaiman, J. (2013). China fake meat scandal: telling your rat from your mutton. from http://www. theguardian.com/world/2013/may/03/china-fake-meat-rat-mutton

Kesmen, Z., Sahin, F., & Yetim, H. (2007). PCR assay for the identification of animal species in cooked sausages. Meat science, 77(4), 649-653.

Kumar, D., Singh, S. P., Singh, R., & Karabasanavar, N. S. (2011). A highly specific PCR assay for identification of goat (Capra hircus) meat. Small Ruminant Research(97), 76-78.

Lever, J., & Miele, M. (2012). The growth of halal meat markets in Europe: An exploration of the supply side theory of religion. Journal of Rural Studies, 28(4), 528-537.

Lin, J. (2005). Tackling southeast asia's illegal wildlife trade. SYBIL, 9, 191.

University of Malaya

Lockley, A., & Bardsley, R. (2000). DNA-based methods for food authentication. Trends in Food Science & Technology, 11(2), 67-77.

Maede, D. (2006). A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences. European Food Research and Technology, 224(2), 209-217.

Mafra, I., Ferreira, I. M., & Oliveira, M. B. P. (2008). Food authentication by PCR-based methods. European Food Research and Technology, 227(3), 649-665.

Malisa, A., Gwakisa, P., Balthazary, S., Wasser, S., & Mutayoba, B. (2006). The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. African Journal of Biotechnology, 5(18).

Mane, B., Mendiratta, S., & Tiwari, A. (2009). Polymerase chain reaction assay for identification of chicken in meat and meat products. Food chemistry, 116(3), 806-810.

Mane, B., Mendiratta, S., Tiwari, A., & Bhilegaokar, K. (2012). Detection of adulteration of meat and meat products with buffalo meat employing polymerase chain reaction assay. Food Analytical Methods, 5(2), 296-300.

Martín, I., García, T., Fajardo, V., Rojas, M., Hernández, P. E., González, I., & Martín, R. (2007). Technical note: Detection of cat, dog, and rat or mouse tissues in food and animal feed using species-specific polymerase chain reaction. Journal of animal science, 85(10), 2734-2739.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., & Shinmura, Y. (1999). A quick and simple method for the identification of meat species and meat products by PCR assay. Meat science, 51(2), 143-148.

McKie, R. (2013). Horsemeat does carry a health risk, but it's incredibly small, The Guardian. Retrieved from http://www.theguardian.com/world/2013/feb/09/horsemeat-in-ready-meals-health-risk

Md-Zain, B., Mohamad, M., Ernie-Muneerah, M., Ampeng, A., Jasmi, A., Lakim, M., & Mahani, M. (2010). Phylogenetic relationships of Malaysian monkeys, Cercopithecidae, based on mitochondrial cytochrome c sequences. *Genet. Mol. Res, 9*, 1987-1996.

Meyer, R., & Candrian, U. (1996). PCR-based DNA analysis for the identification and characterization of food components. LWT-Food Science and Technology, 29(1), 1-9.

Milner-Gulland, E., & Bennett, E. L. (2003). Wild meat: the bigger picture. Trends in Ecology & Evolution, 18(7), 351-357.

Mohamad, N. A., Sheikha, A. F. E., Mustafa, S., & Mokhtar, N. F. K. (2013). Comparison of gene nature used in real-time PCR for porcine identification and quantification: A review. Food research international, 50(1), 330-338.

Murali, R. S. N. (2013). Uproar over macaque culling. The Star. From http://thestar.com.my/news/story.asp?file=/2013/3/18/nation/12850143&sec=nationMeyer-Rochow, V. B. (2009). Food taboos: their origins and purposes.

University of Malaya

Journal of Ethnobiology and Ethnomedicine, 5:18. doi: 10.1186/1746-4269-5-18

Murugaiah, C., Noor, Z. M., Mastakim, M., Bilung, L. M., Selamat, J., & Radu, S. (2009). Meat species identification and Halal authentication analysis using mitochondrial DNA. Meat science, 83(1), 57-61.

Nasi, R., Brown, D., Wilkie, D., Bennett, E., Tutin, C., Van Tol, G., & Christophersen, T. (2008). Conserva-tion and use of wildlife-based resources: the bushmeat crisis. Secretariat of the Convention on Biological Diversity, Montreal. and Center for International Forestry Research (CIFOR), Bogor. Technical Series(33), 50.

Nakyinsige, K., CheMan, Y. B., & Sazili, A. Q. (2011). Halal authenticity issues in meat and meat products. Meat science, 91, 207-214.

Nejad, F. P., Tafvizi, F., Ebrahimi, M. T., & Hosseni, S. E. (2014). Optimization of multiplex PCR for the identification of animal species using mitochondrial genes in sausages. European Food Research and Technology, 239(3), 533-541.

Nijman, V. (2010). An overview of international wildlife trade from Southeast Asia. Biodiversity and conservation, 19(4), 1101-1114.

Ong, S., Cheah, Y. K., Robin, T., Wolmon Gunsalam, J., Mat Isaa, Z., Chai, L. C., . . . Radu, S. (2007). Meat molecular detection: sensitivity of polymerase chain reaction-restriction fragment length polymorphism in species differentiation of meat from animal origin. ASEAN Food Journal, 14(1), 51-59.

Pereira, F., Carneiro, J., & Amorim, A. (2008). Identification of species with DNA-based technology: current progress and challenges. Recent patents on DNA & gene sequences, 2(3), 187-200.

PERHILITAN (2011) Annual Report, Department of Wildlife and National Parks (PERHILITAN) Page 36

PERHILITAN (2012) Annual Report, Department of Wildlife and National Parks (PERHILITAN) Page 38

Rahmah, G. (2014). Cadbury recalls two products following report of porcine DNA detected in chocolates. Retrieved 24 May, 2014, from http://www.thestar.com.my/News/Nation/2014/05/24/Cadbury-Choclate-porcine-DNA/

Rahman, M. M., Ali, M. E., Hamid, S. B., Mustafa, S., Hashim, U., & Hanapi, U. K. (2014). Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation. Meat Sci, 97(4), 404-409. doi: 10.1016/j.meatsci.2014.03.011

Ramanzin, M., Amici, A., Casoli, C., Esposito, L., Lupi, P., Marsico, G., . . . Russo, C. (2010). Meat from wild ungulates: ensuring quality and hygiene of an increasing resource. Italian Journal of Animal Science, 9(3), e61. Regenstein, J. M., Chaudry, M. M., & Regenstein, C. E. (2003). The Kosher and Halal Food Laws. Comprehensive Reviews In Food Science And Food Safety, 2, 111-127.

University of Malaya

Regenstein, J. M., Chaudry, M. M., & Regenstein, C. E. (2003). The Kosher and Halal Food Laws. Comprehensive Reviews In Food Science And Food Safety, 2, 111-127.

Reid, L. M., O'Donnell, C. P., & Downey, G. (2006). Recent technological advances for the determination of food authenticity. Trends in Food Science & Technology, 17(7), 344-353. doi: http://dx.doi.org/10.1016/j.tifs.2006.01.006

Riaz, M. N. (1999). ‘‘Examining the halal market.’’ Prepared Foods 168(10): 81–85.

Riaz, M. N. and M. M. Chaudry (2004). Halal Food Production.Boca Raton, Louisiana: CRC Press.

Rodríguez, M. A., García, T., González, I., Asensio, L., Hernández, P. E., & Martín, R. (2004). PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixtures. Journal of Food Protection®, 67(1), 172-177.

Rohman, A., Sismindari, Erwanto, Y., & Che Man, Y. B. (2011). Analysis of pork adulteration in beef meatball using Fourier transform infrared (FTIR) spectroscopy. Meat science, 88(1), 91-95. doi: http://dx.doi.org/10.1016/j.meatsci.2010.12.007

Rönn, A. C., Andrés, O., López-Giráldez, F., Johnsson-Glans, C., Verschoor, E. J., Domingo-Roura, X., . . . Bosch, M. (2009). First generation microarray-system for identification of primate species subject to bushmeat trade. Endanger Species Res, 6, 133-142.

Rojas, M., González, I., Pavón, M. Á., Pegels, N., Lago, A., Hernández, P. E., . . . Martín, R. (2010). Novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of meat and commercial meat products from game birds. Food Additives and Contaminants, 27(6), 749-763

Sahih Muslim (1932 b.) The Book of Hunting, Slaughter, and what may be Eaten. Book 34, Hadith 18.

Shaykh (Mufti) Muhammad Ibn Adam (HA) The Fiqh of Halal and Haram Animals.Darul Iftaa.UK http://www.central-mosque.com/index.php/General-Fiqh/the-fiqh-of-halal-and-haram-animals.html

Singh, V., & Neelam, S. (2011). Meat species specifications to ensure the quality of meat: a review. International Journal of Meat Science, 1(1), 15-26.

Sofos, J. N. (2008). Challenges to meat safety in the 21st century. Meat science, 78(1), 3-13. Stamoulis, P., Stamatis, C., Sarafidou, T., & Mamuris, Z. (2010). Development and application of molecular markers for poultry meat identification in food chain. Food Control, 21(7), 1061-1065.

Street, S. L., Kyes, R. C., Grant, R., & Ferguson, B. (2007). Single nucleotide polymorphisms (SNPs) are highly conserved in rhesus (Macaca mulatta) and cynomolgus (Macaca fascicularis) macaques. BMC genomics, 8(1), 480.

Sun, Y.-L., & Lin, C.-S. (2003). Establishment and application of a fluorescent polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for identifying porcine, caprine, and bovine meats. Journal of agricultural and food chemistry, 51(7), 1771-1776.

University of Malaya

Trowbridge, L. (2010, 28 April 2010). No soup for you! Rare monkey meat for soup gets couple arrested. from http://www.digitaljournal.com/article/291317

Unseld, M., Beyermann, B., Brandt, P., & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. Genome Research, 4(4), 241-243.

Valentin-Weigand P., Heesemann J., & Dersch P. (2014). Unique virulence properties of Yersinia enterocolitica O:3 – An emerging zoonotic pathogen using pigs as preferred reservoir host. International Journal of Medical Microbiology, 304(7), 824-834. doi: http://dx.doi.org/10.1016/j.ijmm.2014.07.008

Verkaar, E., Nijman, I., Boutaga, K., & Lenstra, J. (2002). Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat science, 60(4), 365-369.

Walker, A., Smith, S., & Smith, S. (1987). Mitochondrial DNA and human evolution. Nature, 325, 1-5.

Wilkie, D. S., & Carpenter, J. F. (1999). Bushmeat hunting in the Congo Basin: an assessment of impacts and options for mitigation. Biodiversity & Conservation, 8(7), 927-955.

Wolf, C., Burgener, M., Hübner, P., & Lüthy, J. (2000). PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. LWT-Food Science and Technology, 33(2), 144-150.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., Richardson, R. I., ... & Whittington, F. M. (2008). Fat deposition, fatty acid composition and meat quality: A review. Meat Science, 78(4), 343-358.Wyler, L. S., & Sheikh, P. A. (2008). International illegal trade in wildlife: Threats and US policy.

Wu, J.-H., Hong, P.-Y., & Liu, W.-T. (2009). Quantitative effects of position and type of single mismatch on single base primer extension. Journal of microbiological methods, 77(3), 267-275.

Wyler, L. S., & Sheikh, P. A. (2008). International illegal trade in wildlife: Threats and US policy.

Yusop, M. H. M., Mustafa, S., Man, Y. B. C., Omar, A. R., & Mokhtar, N. F. K. (2012). Detection of raw pork targeting porcine-specific mitochondrial cytochrome B gene by molecular beacon probe real-time polymerase chain reaction. Food Analytical Methods, 5(3), 422-429.

Zhou C., Liu Z., Jiang J., Yu Y., & Zhang Q. (2012). Differential gene expression profiling of porcine epithelial cells infected with three enterotoxigenic Escherichia coli strains. BMC genomics, 13(330). doi: 10.1186/1471-2164-13-330

University of Malaya

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Rashid, N.R.A., Ali M.E, Hamid, S.B.A, Rahman, M.M, Razzak, M.A, Asing, Amin, M.A (2015). A suitable method to detect a potential fraud of bringing Macaque monkey meat into the food chain. Food Additives & Contaminants. doi:10.1080/19440049.2015.1039073.

2. Ali M.E., Rashid N.R.A, Hamid S.B.A., Asing, (2015). Development and Validation of Short-Amplicon Length PCR Assay for Macaque Meat Detection under Complex Matrices. *Food Analytical Method.*. (Submitted).

3. Ali, M. E., Razzak, M. A., Hamid, S. B. A., Rahman, M. M., Al-Amin, M., Rashid, N. R. A., & Rakhine, A. (2015). Multiplex PCR Assay for the Detection of Five Meat Species Forbidden in Islamic Foods. *Food chemistry.*

Conference Paper

1. Hamid S.B.A., Rahman M.M., Rashid N.R.A., Ali M.E. (2014). Halal food authentication platforms: Potential and perspective in nanotechnology. *3rd Halal Gulf Conference.*

2. Ali M.E., Rashid N.R.A, Hamid S.B.A. (2014). Polymerase Chain Reaction Assay Targeting Mitochondrial D-Loop Gene for the Detection of Monkey (*Macaca fascicularis sp.)* Meat. *International Conference On Food Innovation(INNOVAFOOD).*

University of Malaya