DEVELOPMENT AND EVALUATION OF MULTIPLEX PCR ASSAY FOR THE DETERMINATION OF CAT, RABBIT, RAT AND SQUIRREL ELEMENTS IN FOOD PRODUCTS

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INSTITUTE OF GRADUATE STUDIES
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KUALA LUMPUR

2018

University of Malaya

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

University of Malaya

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Matric No: **HHC120008**

Name of Degree: **Doctor of Philosophy**

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DEVELOPMENT AND EVALUATION OF MULTIPLEX PCR ASSAY FOR THE DETERMINATION OF CAT, RABBIT, RAT AND SQUIRREL ELEMENTS IN FOOD PRODUCTS

ABSTRACT

Cat, rabbit, rat and squirrels are adulterated in meat and meat products for economic gain and exotic taste. However, most of these species are potential carriers of zoonotic threats and so pose huge threats to public health. Currently, several polymerase chain reaction (PCR) based methods have been proposed for authentication these species in separate assays which are costly and involved long-amplicon length biomarkers that breakdown during food processing. To overcome the need, for the first time, multiplex conventional PCR, PCR-RFLP and quantitative PCR assays with TaqMan Probes were developed here for the discriminatory identification of cat, rabbit, rat, and squirrel in food products. In conventional PCR and PCR-RFLP, rabbit (123 bp), rat (108 bp) and squirrel (243 bp) targets were amplified from ATP6 and cytb genes along with a eukaryotic internal control (141bp). The products were sequenced and cross-tested against 22 species. A total of 81 reference samples and 72 meatball specimens were screened to validate the assay. Analyte stability was evaluated through boiling, autoclaving and micro oven cooking. The lower limits of detection were 0.001ng DNA for pure meat and 0.1% for meatballs. Specificity was confirmed through sequencing and RFLP analysis. When PCR products were digested with *Bts*I*Mut*I and *Bts*CI enzymes, distinctive fingerprints (115 & 8 bp for rabbit; 64 & 44 bp for rat and 176 & 67 bp for squirrel) were obtained. The detection limit of the assay was 0.1% meat in frankfurter formulation. Finally, a novel pentaplex real- time PCR assay with TaqMan probes was developed for identification and quantification of the squirrel, rat, rabbit and cat species in a single assay platform. For real-time quantitative PCR, species specific

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primers and probes were developed against ATP6, and cytochrome b genes to amplify 108, 123, 161and 176 bp DNA fragments from rat, rabbit, squirrel and cat meat products, respectivly under various states. A141 bp internal amplification control (IAC) of 18S rRNA was used to avoid any false negative results. Specificity of the assay was evaluated against 22 non- target species but no cross-reactivity was found. Each of the target species DNA was quantified, and PCR efficiency was determined based on standard curve that was generated using 10-fold serially diluted mixed DNA extract (1:1:1:1) from squirrel, rat, rabbit and cat species. The assay was valid both under pure, processed and admixed states with 10-0.1% (w/w) adulterant from each species. The limit of quantification was 0.003 ng DNA from each species. Analyses of 18 model burgers (9 chicken and 9 beef) and 18 frankfurters (9 chicken and 9 beef) revealed 91 - 122% target recovery at 0.1 - 10% adulteration. Finally, 72 commercial burgers (36 chicken and 36 beef) and 72 frankfurters (36 chicken and 36 beef) were screened but no target species was detected except IAC. Although, the study was validated using different food matrices, the shorter-aspects of amplicon length, exceptional stability under veracious treatment conditions convinced that the developed methods could be a useful tool in the identification and quantification of cat, rabbit, rat and squirrel species in any food matrices.

Keywords: multiplex conventional PCR, PCR-RFLP, TaqMan probes, cytochrome b, ATP6.

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PEMBANGUNAN DAN PENILAIAN ESEI PCR MULTIPLEKS UNTUK PENENTUAN ELEMEN KUCING, ARNAB, TIKUS DAN TUPAI DALAM PRODUK MAKANAN

ABSTRAK

Kucing, arnab, tikus dan tupai dicampurkan di dalam daging dan produk daging untuk keuntungan ekonomi dan rasa eksotik. Namun, kebanyakan spesies ini berpotensi menjadi pembawa kepada ancaman zoonotik dan menjadi ancaman besar terhadap kesihatan awam. Kini, beberapa kaedah tindakbalas rantaian polimeras (PCR) telah dicadangkan untuk pengesahan spesies ini di dalam esei berbeza yang mahal dan menggunakan amplikon penanda-bio yang panjang dan akan terurai semasa pemprosesan makanan. Untuk mengatasi hal ini, buat kali pertama, esei konvensional multipleks PCR, PCR-RFLP dan PCR kuantitatif (qPCR) dengan prob TaqMan telah dibangunkan di sini untuk diskriminasi identifikasi kucing, arnab, tikus dan tupai di dalam produk makanan. Di dalam PCR dan PCR-RFLP, sasaran arnab (123bp), tikus (108bp) dan tupai (243bp) diamplifikasi daripada gen ATP6 dan cytb bersama dengan kawalan eukaryotik (141bp). Produk dijujukkan dan ujian silang dilakukan terhadap 22 spesies. Sejumlah 81 sampel rujukan dan 72 spesimen bebola daging diuji untuk mengesahkan esei. Kestabilan analit dinilai melalui pendidihan, autoklaf dan memasak di dalam mikro oven. Had pengesanan terendah adalah 0.01ng DNA untuk sampel daging tulen dan 0.1% untuk bebola daging. Kekhususan telah disahkan melalui penjujukan dan analisis RFLP. Apabila produk PCR di tindakbalaskan dengan enzim *BtsIMutI* dan *BtsCI*, cap jari (115 & 8bp untuk arnab, 64 & 44bp untuk tikus dan 176 & 76bp untuk tupai) telah diperolehi. Had pengesanan terendah esei adalah 0.1% daging di dalam formulasi *frankfurter*. Akhir sekali, esei PCR pentapleks masa nyata dengan prob TaqMan telah dibangunkan untuk identifikasi dan kuantifikasi spesies tupai, tikus, arnab dan kucing di dalam esei tunggal. Bagi qPCR masa nyata, primer spesifik spesies dan

prob telah dibangunkan terhadap gen ATP6 dan cytb untuk amplifikasi 108, 123, 161 dan 176bp fragmen DNA bagi produk daging tikus, arnab, tupai dan kucing di bawah pelbagai keadaan. 141bp kawalan amplifikasi dalaman (IAC) 18SrRNA telah digunakan untuk menghalang keputusan palsu negatif. Kespesifikan dinilaikan terhadap 22 spesies tetapi tiada pencemaran silang dijumpai. Setiap sasaran dikuantifikasi dan kecekapan PCR ditentukan berdasarkan lengkung standard yang dijana menggunakan 10-ganda pencairan bersiri campuran ekstrak DNA (1: 1: 1: 1) daripada spesies tupai, tikus, arnab dan kucing. Esei adalah terpakai di bawah keadaan tulen, terproses dan campuran dengan 0.1-10% pencemar daripada setiap spesies. Had kuantifikasi adalah 0.1-0.003 ng DNA untuk setiap spesies. Analisis 18 model *burger* (9 ayam dan 9 daging) dan 18 *frankfurter* (9 ayam dan 9 daging) menunjukkan 91-122% sasaran *recovery* pada 0.1-10% pencemaran. Akhir sekali, 72 *burger* komersial (36 ayam dan 36 daging) dan 72 *frankfurter* (36 ayam dan 36 daging) disaring tetapi tiada spesies sasaran dikesan melainkan IAC. Bahkan, ujian disahkan pada matriks makanan, saiz amplikon yang pendek dan kestabilan unggul di bawah kondisi yang pelbagai membuktikan bahawa kaedah yang dibangunkan boleh dijadikan alat yang berguna untuk identifikasi dan kuantifikasi spesies kucing, arnab, tikus dan tupai dalam pelbagai matriks.

Kata kunci: PCR multipleks konvensional, PCR-RFLP, prob TaqMan, cytochrome b, ATP6.

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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 Doctor of Philosophy Degree. First of all, I would like to express my heartfelt gratitude to my supervisors, Associate Professor 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 Dr. M.A. Motalib Hossain 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, University of Malaya. I am very thankful to have Surmin Sultana, Sharmin Quazy Bonni, Nina Naquia, Lavan, Cahalvi and Ismat as group members. I am also very grateful to Mr Mahbub Alam for his valuable time and support to encourage my work. For all the knowledge assistance, moral support, kindness and cooperation, thank you so much. 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 to express my greatest thank to my wife for her unlimited sacrifice, consolation and love which inspired me to hard effort in research. Last but not least, I would like to thank my mother, children and other family members for their continuous support, precious prayers and unconditional love.

Thank you so much.

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LIST OF SYMBOLS AND ABBREVIATIONS

$	:	US Dollar
%	:	Percent
°C	:	degree Celsius
µg	:	Microgram
µL	:	Microliter
µM	:	Micromole
pg	:	Picograme
≥	:	greater than or equal to
3D	:	three dimensional
A260/A280	:	ratio of absorbance at 260 and 280 nm
AIDS	:	acquired immune deficiency syndrome
BLAST	:	Basic local alignment search tool
bp	:	base pair
Ct	:	threshold cycle
Cytb	:	cytochrome b
dH2O	:	distilled water
D-loop	:	displacement loop
DNA	:	deoxyribonucleic acid
dsDNA	:	double stranded - deoxyribonucleic acid
EC	:	European Commission
ELISA	:	enzyme –linked immunosorbant assay
FAO	:	Food and Agriculture organization
FDA	:	Food and Drug Administration
g	:	Gram

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GC-MS	:	gas chromatography-mass spectroscopy
GMP	:	good manufacturing practice
h	:	Hour
HPLC	:	high performance liquid chromatography
IAC	:	internal amplification control
IDT	:	integrated DNA technology
LOD	:	limit of detection
MEGA 5	:	molecular evolutionary genetic analysis version 5
mg	:	Milligram
$MgCl_2$	:	magnesium chloride
Min	:	Minute
ml	:	Milliliter
mM	:	Milimoler
mPCR	:	multiplex polymerase chain reaction
mqPCR	:	multiplex real-time PCR
mt	:	Mitochondrial
NCBI	:	national center of biological information
ng	:	Nano gram
nt	:	Nucleotide
nDNA	:	Nuclear DNA
OECD	:	Organization for Economic Cooperation and Development
PCR	:	polymerase chain reaction
RFLP	:	restriction fragment length polymorphism
psi	:	pounds per square inch
RAPD	:	randomly amplified polymorphic DNA
rRNA	:	ribosomal ribonucleic acid

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s	:	Second
ssPCR	:	species specific PCR
Ta	:	annealing temperature
Tm	:	melting temperature
UN	:	United Nation
USDA	:	US department of Agriculture
UV	:	Ultraviolet
w/w	:	weight/ weight

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CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Maintaining food safety and quality from farm to fork is the heart of public health and nothing can compromise it. Regulatory laws, public awareness and authentication techniques must work in concatenation to meet this common goal. One of the main food quality and safety issues involves the verification of food components, which are frequently adulterated or substituted either partially or entirely by a cheaper and sometimes a pernicious material that is never expected or suspected. Recent market monitoring studies showed that 19.4% of all foodstuffs in the USA (Hsieh, Woodward, & HO, 1995), 22% in Turkey (Ayaz, Ayaz, & Erol, 2006), 8% in the UK and 15% in swezerland were falsely labeled (Ballin, Vogensen, & Karlsson, 2009). Additionally, a recent scandal over rat meat sold as lamb after chemical treatment in China; horse meat products in the school meals in Europe ; monkey and dog meat in soup products in Indonesia and India (Rahman et al., 2014) dog and cat meat as chevon in China (Singh, Pathak, Nayak, Verma, & Umaraw, 2014) are highly alarming to the public health and religious faiths because these meat items are either carrier of zoonotic threats or not permissible in many religions and cultures (Schoppe, 2008).

However, the growing demand for animal proteins, their associated high prices and also unequal distribution of food provisions across the world have made it inevitable that many regions of the world will hunt for local wild lives for food subtenance (Hoffman & Wiklund, 2006). Illegal trades of certain endangered animal populations are quite prevalent, threatening a multitude of species including primates, carnivores, ungulates and wild fowl in natural habitats (Fajardo et al., 2010). Although humans have hunted and eaten wild meat for millennia, consumption over the recent years has increased dramatically (Milner-Gulland & Bennett, 2003). The recent trend of meat preference shows higher interest in wild meat over the red meat due to its nutritional

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facts such as lower content total dietary and saturated fats (Hoffman & Wiklund, 2006). Because of the great appeal for exotic meals having wild meat, a lot of rodent species are hunted in western Africa and other parts of the world (Hoffman & Cawthorn, 2012). The rodent species such as rat and squirrel are of grave concern and highly alarming since most of these species are not only the potential carrier of infectious zoonosis but also they are prohibited in several religions such as Islam and Judaism. Thus food authentication is a major concern not only for preventing commercial frauds, but also for the safety risks arising from the undeclared introduction of any food ingredient that might be harmful to human health and social cultures; such as a potentially allergenic or toxic ingredient or an animal species that is sensitive to certain religious or vegetarian consumers (Pavord et al., 2012).

Due to the above circumstances, determination of species origin in the processed and unprocessed food products is very important and a reliable and precise method or technique needed to fulfill the requirement. According to European Regulation (178/2002) on food safety ‘‘the ability to trace and follow a food, feed, food producing animal or ingredients through all stages of production and distribution’’ (Dalvit, Marchi, & Cassandro, 2007). Many countries have their regulatory bodies to control the import and export of food products. For example, Malaysia, Singapore, Thailand, Indonesia and Australia have credible halal certification bodies to ensure the halal status of marketed foods (Nakyinsige, Man, Sazili, Zulkifli, & Fatimah, 2012). Thus, a reliable, sensitive, rapid and easily performable system is required to authenticate the labeling ingredients of food products. Up-to-this-date, various analytical approaches have been documented to detect fraudulent mixing of food products. Numerous lipid (Rohman, Erwanto, & Man, 2011), protein (Ayaz, Ayaz, & Erol, 2006) and DNA-based assays have been proposed for meat speciation (Ali et al., 2015b; Matsunaga et al., 1999). However, the lipid and protein based methods are often unsuitable because they

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are laborious, target-biomarker are often modified and thus cannot distinguish closely related species in highly processed food such as heated or chemically treated products, and these are of less sensitive than DNA-based approaches (Ali et al., 2012a; Herrero, Royo, Lago, Vieites, & Espiñeira, 2013). Moreover, these methods are unable to differentiate closely related species, such as cow and buffalo. In contrast, the DNA-based techniques, especially the short-length DNA biomarkers are thermodynamically more stable, more sensitive and more reliable over the longer ones even under extreme states such as degraded or naturally decomposed samples (Ali, Amin, Hamid, Hossain, & Mustafa, 2015a). Among the DNA-based methods, PCR approaches are highly appreciated since they can amplify target biomarkers from single copy to easily detectable quantities, offering a highly sensitive, robust and low-cost platform for the identification of biological ingredients. Several PCR-platforms such as species-specific PCR (Aida, Man, Wong, Raha, & Son, 2005; Karabasanavar et al., 2011), multiplex PCR (Ali et al., 2015c; Bottero & Dalmasso, 2011), PCR-RFLP (Ali, Uda, Mustafa, & Yaakob, 2011;Chen, Liu, & Yao, 2010), randomly amplified polymorphic DNA (RAPD) (Arslan, Ilhak, Calicioglu, & Karahan, 2005), PCR product sequencing (Dooley, Sage, Clarke, Brown, & Garrett, 2005), and real-time PCR (Drummond et al., 2013; Köppel, Daniels, Felderer, & Brünen-Nieweler, 2013) have already been documented for the authentication of meat species.

Recently, squirrels were subjected to phylogeographical investigation (Finnegan et al., 2008), cross-species chromosome painting for genome organizations (Li et al., 2004), and sequencing for the determination of genetic structure of the fragmented populations (Barratt, Gurnell, Malarky, Deaville, & Bruford, 1999) and interspecies hybridization (Spiridonova et al., 2005). However, these methods are not suitable for species identification for regular market surveillance because of their specialized needs, lengthy procedure and involved cost. Recently, a real-time PCR was proposed for the

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identification of red and gray squirrels; but it was not tested under food matrices (O'Meara, Turner, Coffey, & O'Reilly, 2012). On the other hand, several PCR-based molecular detection schemes have been proposed for the authentication of rat (Fang & Zhang, 2016; Rahmania, Sudjadi, & Rohman, 2015) and rabbit species (Amaral, Santos, Melo, Oliveira, & Mafra, 2014; Rafayova, Lieskovska, Trakovicka, & Kovacik, 2009 ; Hanapi, Desa, Ismail, & Mustafa, 2015). However, these methods are mostly based on a single species target and long DNA marker that breaks down under food processing treatments and thus they are less trustworthy and incur more cost (Ali et al., 2015b).

The multiplex Polymerase Chain Reaction (mPCR) assays are highly promising since they offer the opportunity of multiple target nucleic acid identification in a single assay platform, saving both analytical cost and time (Ali, Razzak, & Hamid, 2014b; Iwobi et al., 2015). Recently, mPCR assays have been reported for pig, dog, cat, rat and monkey species (Ali et al., 2015c), beef, pork, horse and sheep species (Koppel, Ruf, & Rentsch, 2011) and beef, pork, lamb, chicken, ostrich meat and horse species (Kitpipit, Sittichan, & Thanakiatkrai, 2014). However, a mPCR assay is yet to be developed for the simultaneously authentication of squirrel, rat and rabbit meat in food chain. To fill up this research gap, we report here a more reliable and cost-saving mPCR assay for the authentication of squirrel, rat and rabbit materials in food chain for the first time.

In this regard, the species-specific PCR-RFLP assays are especially amazing since they offer the opportunity of product authentication by restrictive digestion of the amplified PCR products using one or more restriction enzymes (RE) (Ali et al., 2011a; Lin & Hwang, 2007). Using the sequence variation that exists within a defined region of DNA, the differentiation of even closely related species is possible using a PCR-RFLP assay with appropriate REs (Fajardo, González, Rojas, García, & Martín, 2010). Such assays have been successfully applied to discriminate closely related species such as

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cattle, yak and buffalo (Chen et al., 2010), cattle–buffalo and sheep–goat (Girish et al., 2005) swine and wild boar (Fajardo et al., 2008; Mutalib et al., 2012) and various fish species (Wolf, Burgener, Hübner, & Lüthy, 2000). However, these methods are mostly based on long-length DNA target which are broken down by natural or environmental decomposition and food processing treatments and hence they are less trustworthy and inconclusive for forensic investigation (Ali et al., 2015b). To the best of our knowledge, no RFLP authentication has reported for mPCR products of squirrel, rat and rabbit, where multiple amplified products do exist. In this regards, multiplex PCR-RFLP (mPCR-RFLP) assay, short amplicon length would be specially interesting and trustworthy for the simultaneous detection of squirrel, rat and rabbit products in food chain. To address this issue, we report here PCR-RFLP assay with short amplicon length for the discriminatory authentication of squirrel, rat and rabbit materials in frankfurter formulation, a popular food item widely consumed across the globe.

Although conventional PCR is very simple and cost effective technique but it is unable to quantify the targets present in the samples and it requires post-PCR analysis for the detection of amplified products (Yusop, Mustafa, Man, Omar, & Mokhtar, 2012). However, real time PCR (RT-PCR) allows the detection and measurement of the PCR products accumulated with reaction progress by monitoring each amplification cycle using a fluorescent dye or fluorescent-labeled probe. The fluorescent signal intensity is directly proportional to the accumulated PCR products in each cycle, facilitating the detection in a RT-PCR system at initial stage (Ali et al., 2012a). Generally, two types of fluorescent dyes are used in the RT-PCR assay such as DNA-intercalating dyes which bind to the minor groove of the DNA double helix and oligonucleotide probe which is complementary to the part of target amplicon. However, oligonucleotide probes are greatly promising since they offer higher specificity, development of multiplex quantitative PCR (qPCR) and less possibility to the formation

of primer dimer. On the other hand, DNA-intercalating dyes are not suitable for qPCR because they detect all double-stranded DNA, including non-specific reaction products and unable to differentiate the signals from different products (Yusop et al., 2012). Several probes based multiplex qPCR have been documented for the detection and quantification of beef and pork (Iwobi et al., 2015), beef, pork, horse and sheep (Köppel et al., 2011) and beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat (Köppel, Zimmerli, & Breitenmoser, 2009). In this study, I introduced a multiplex qPCR for the detection and quantification of squirrel, rat, rabbit and cat for the first time in the research.

1.2 Project Rationale

Cat, rabbit, rat and squirrel species are very sensitive in food products because most of them are potential carriers of zoonotic threats and rejected in most religions and cultures. Rabbit meat has received increasing attention because it contains low fat, cholesterol and sodium but high content of digestible proteins that offer excellent nutritive and dietetic properties (Dalle et al., 2002; Hernàndez & Gondret, 2006). Consequently, rabbit meat is sold at higher prices over other regular meats such as chicken, mutton, lamb, beef and pork. In the last 50 years, the world's production of rabbit meat has increased by more than 2.5 folds that were about 1.6 million tons in 2009. On the other hand, while rat meat is rejected in most of the societies as a food taboo, some rat species are domesticated and consumed in certain communities because of their greater carcass yield (ca. 65%), soft bones and taste like bird's meat (Ajayi et al., 1974; Odebode et al., 2011). Some African communities also prefer wild rat meat as an exotic and aristocrat meal in their social events (Ajayi et al., 1974), giving rise to a frequent trade of wild rats along the road side markets of many African countries (Redhead et al., 1990). Approximately, 80 million pieces of cane rats are hunted per year only in western Africa, with a yield of 300,000 metric tons of meat (Hoffman et al.,

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2012). Recently, rat meat was chemically modified to change physical appearances and sold lamb or boneless chicken (Ali, Razzak, & Hamid, 2014). Additionally, cat meat was sold as mutton in China (South China Morning Post, 2006), and India (Raj, 2015). Recently, one ton of fresh and frozen carcasses of cat were seized in an enforcement operational raid at Shunjiang in China while being sold as rabbit meat (Fang, & Zhang, 2016). Squirrel meat is not so much popular but some Southeast Asian and African communities consume it in daily meals as a source of proteins (Davis et al., 1990) and for certain health benefits, such as distinctive flavor, high proteins, low fat, less cholesterol and the absence of health-threatening anabolic steroids (Redhead et al., 1990). Squirrel meat is also widely appreciated in the UK and US restaurants as an exotic meat item (Anonymous, 2018). When rabbit and squirrel meat are appreciated as exotic menus there is a high chance of substitution by the rat and cat meat which are almost free of charge.

1.3 Problem Statements

To authenticate the meat and meat product, DNA - based PCR techniques have been evolved as the method of choice because protein and lipid based biomarkers are easily modified and so cannot offer so much reliability. Recently, squirrels were subjected to phylogeographical investigation (Finnegan et al., 2008), cross-species chromosome painting for genome organizations (Li et al., 2004), and sequencing for the determination of genetic structure of the fragmented populations (Barratt et al., 1999) and interspecies hybridization (Spiridonova et al., 2005). However, these methods are not suitable for species identification for regular market surveillance because of their specialized needs, lengthy procedure and high cost. On the other hand, several molecular detection schemes have been proposed for the authentication of rat, cat (Fang & Zhang, 2016; Rahmania & Rohman, 2015, Ali et al., 2015) and rabbit species (Rafayova, Lieskovska, Trakovicka & Kovacik, 2009; Amaral, Santos, Melo, Oliveira,

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& Mafra, 2014; Hanapi, Desa, Ismail, & Mustafa, 2015). However, these methods are mostly based on a single species target and long DNA marker that breaks down under food processing treatments and thus they are less trustworthy and incur more cost (Ali et al., 2015a). Moreover, there is no PCR assay that can detect cat, rabbit, rat and squirrel materials in a single assay platform. Furthermore, they are not validated for processed food analysis. Therefore, the development of multiplex PCR assay for authentication of cat, rabbit, rat and squirrel materials in meat product would be greatly adventurous.

1.4 Research Objective

1.4.1 General Objective

The objective of the overall study is to develop and validate multiplex PCR assay for the simultaneous detection of cat, rabbit, rat and squirrel species for the authentication of their ingredients in food products.

1.4.2 Specific Objectives

i. To develop primers and probes targeting mitochondrial gene of cat, rabbit, rat and squirrel species.

ii. To develop and validate a multiplex conventional and real-time PCR systems for the determination of rabbit, rat, and squirrel; and cat, rabbit, rat and squirrel species respectively, under various food processing treatments and complex matrices.

iii. To test the assay performance for the screening of cat, rabbit, rat and squirrel in processed meat products.

1.5 Scopes of Research

1.5.1 Development of Biomarkers

Nowadays, in addition to food authentication, DNA based techniques are applied for the molecular identification of pathogens in agriculture, environmental monitoring, bio

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diagnostics, bio terrorism and forensic analysis (Rahman, 2015). Recently, researchers have paid more attention to the short amplicon length biomarkers due to their extraordinary stability against severe food processing treatment since they still can traceable in the specimen which has been treated with high pressure and temperature (Ali et al., 2012a). In the previous literature reported that longer target DNA is susceptible to break down under harsh processing treatment causing there is a chance of false negative results (Ali et al., 2017). Although, longer amplicons are detectable but it has been proved that the shorter amplicon (≥ 150 bp) are more sensitive than longer ones (Ali et al., 2015b; Rojas et al., 2010). Due to the extensive sensitivity and stability of the shorter amplicon DNA target, it has vast application in forensic analysis, biochip and biosensor development.

Multiplex polymerase chain reaction (mPCR) is a greatly useful molecular biology technique by which multiple targets can be amplifled simultaneously from a single reaction mixture. They also reduce both analytical time and cost. In this regard, multiplex mPCR assay would be especially useful and trustworthy for the simultaneous detection of species in various food products. In this work, I have designed a total of four set of primers with amplicon sizes of 108 - 243 bp from, mitochondrial cytb gene for cat, rabbit, squirrel and ATP gene for rat species. Hence, *cytb* and ATP6 genes were targeted because of their higher degree of divergence and availability of sufficient conserved regions within the species but adequate polymorphism among the closely related species (Mohamad, Sheikha, Mustafa, & Mokhtar, 2013). Thus, the proposed activities will develop short length amplicon targeting mPCR assay for the discriminatory authentication of cat, rabbit squirrel, and rat materials in the food chain.

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1.5.2 Evaluation of the Biomarker specificity using PCR based Techniques

It is very important to evaluate the specificity of the developed biomarkers by using a well-known system to avoid ambiguity. The performance of the developed biomarkers of four target species (cat, rabbit, rat, and squirrel) was analyzed using PCR techniques. PCR is a powerful and authentic biochemical tool for the species identification in food products (Ali et al., 2012b). It is an *in vitro* amplification processes in which specific oligonucleotide primers hybridize to the complementary target region of the DNA template followed by the enzymatic reactions of Taq DNA polymerase were occurred to complete the process (Rahman, 2015). The amplified specific products are separated and visualized by using agarose gel under a gel image documentation system or automated capillary electrophoresis system to get better resolution (Tisza, Csikos, Simon, Gulyas, Javor, & Czegledi, 2016). Endpoint PCR system are not provided enough information to verity and authenticate the PCR products. Thus, sequencing of the PCR products coupled with restriction fragment length polymorphism (RFLP) was used to authenticate the amplified products if the amplicon contains appropriate restriction site (Chen, Liu & Yao, 2010). However, end point PCR assay is only qualitative detection scheme and unable to provide quantitative information such as amount of adulterant pressure in the specimen. In contrast, real-time multiplex PCR assay is a suitable tool for the identification, differentiation and quantification of many different target species using TaqMan probe containing fluorescent reporter dye (Molenkamp, Ham, Schinkel, & Beld, 2007). Therefore, this research proposed the developed oligonucleotide biomarkers with simplex and multiplex conventional PCR, PCR-RFLP and TaqMan probe real-time PCR assay for detection, differentiation and quantification analysis of cat, rabbit, rat and squirrel in the food chain.

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1.5.3 Assay Validation and Food Analysis

To check the validity of the developed authentication tool is a valid step because the reliability of the assay depends on the validity performance. For example, protein and lipid-based methods are not suitable for the analysis of extensive processed food due to their lack of stability and specificity. Hence, initial performance of the developed multiplex system was tested using the extracted DNA under raw state of target species and some other non-target species which were commonly used in meat products. Secondly, the assay was validated under different cooking conditions, namely, boiling, autoclaving and microwave cooking to realize the stability of the developed multiplex system. Subsequently, the assay sensitivity and specificity were evaluated by testing under binary and ternary admixture of the target meat analysis. Adulteration as well as fraudulent labeling in the meat products is an emerging and sensitive issue. However, to identify the origin of meat in the food chain has been a concern for the protection of consumer right, public health; religious believe etc. (Arslan, Ilhak, & Calicioglu, 2006). Therefore, it is a universal desire that does not substitute the high valued declared species, entirely or partially with other lower value ones (Mafra, Ferreira, & Oliveira, 2008). Rabbit meat is one of the significant meat of economic concern whereas cat, rat and squirrel meat are objectionable according to many religious aspect and health concern. Furthermore, the sensitivity and efficiency of the PCR assay often reduce in case of food and meat products due to the presence of various spices and additive which act as inhibitor for the binding of primers at specific site (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001a). Therefore, finally, the developed assay was validated under various laboratory made model and commercial food matrices such as burger, meatball and frankfurter which are popular and available. Thus, the novel assay demonstrated sufficient merits to be used by regulatory bodies for cat, rabbit, rat and squirrel authentication even in degraded specimens.

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1.6 Thesis Organization

This thesis comprises of six chapters namely introduction, literature review, materials and methods, result, discussion and conclusion and future recommendations. The contents of each chapter are described below:

Chapter 1 (Introduction): This chapter describes briefly the background of the study, project rational, problem statement, objective and scope of the present research. I described here important of the present research, with a short description of the drawback of the previous work and also explained the innovation of the present method to overcome the limitation of the previous reports.

Chapter 2 (Literature Review): This chapter consists of detailed literature review on importance of food authentication, prevalence and impact of food fraud, importance of cat, rabbit, rat and squirrel detection and current identification techniques.

Chapter 3 (Materials and Methods): All materials and protocols as well as bioinformatics tools used in this study are described in this chapter.

Chapter 4 (Results): Outcomes of the experiments are illustrated here. These include extraction of DNA, designed of biomarkers, specificity of biomarkers, sensitivity and validity of the assay in various matrices and PCR products authentication.

Chapter 5 (Discussion): The experimental findings outcomes are elaborately discussed and compared with previous reports.

Chapter 6 (Conclusion and Recommendation): Finally, finding summery of the present study including remarks and suggestion of future research were presented here.

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CHAPTER 2: LITERATURE REVIEW

2.1 Animals Materials in Foods Chain

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

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large animals because, it was probably makes up 40% or more of the diets and certainly a much larger proportion than in other primates (Wrangham, 2013). Subsequently, meat consumption opportunity was more expanded about 250,000 years ago when the earliest *Homo* invented fire (Goudsblom, 1992) and they made meat food more delicious through searing and roasting, and smoking and preserved it for later consumption. Results of paleontological and archaeological research supported theory that incorporation of larger amounts of animal proteins started with the earliest *Homo* and men started to domesticate animals and plants, which had begun 10,000 years ago, (Larsen, 2003) for adequate meat supply to their foods requirements. Thus, million years ago, animals were considered as valuable source of meat food, high biological value proteins and valuable minerals such as iron, vitamin as well as zinc, selenium and phosphorus.

2.2 Meat Consumption

Meat has played a crucial role in human evolution and is an important component of a healthy, well balanced diet because of its high nutritional values. Increasing populations and rapid income growth at the global scale has led not only to an increased demand for staple foods but also for the preferred foods such as meat products; this is because just meat can offer a lot such as the best source of proteins, fats, vitamins, minerals and micronutrients which are essential for human growth and development. The qualities of proteins from animal sources are greatly superior to those from the plant sources because all the eight essential amino acids for human growth and development are found in meats (Soares, Amaral, Oliveira, & Mafra, 2014). A 25 g of meat intake can provide 45% of child daily need for proteins and half of the vitamin B12. On the other hand, 100 g of meat to the total Zambian diet provides 50% proteins, 12% iron, 40% niacin and 25% energy demands (Jensen, 1981). Meat is important complement to the lysine deficiency diseases and promotes the absorption of iron from

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other foods. The top sources of meat our diet are domesticated animals such as cattle, pigs and poultry and to a lesser extent buffaloes, sheep, camels and goats. To a limited extent and regional regions basis, meat is also derived from wild animals such as turtle, deer, elk, rabbit, crocodiles, snakes and lizards (FAO, 2014; Klein, 2004). Moreover, various forms of animal meat products have got their entry into our commercial foods chain. Especially, the minced meat, sausages, burger patty and meatballs are the most common meat products and are being widely consumed around the world regardless of the brands, geographical and ethnical preferences.

Nevertheless, the consumers prefer to buy the processed meat products since they are ready-made, time-saving, and could be consumed without needing many efforts. Consequently, meat products in foods chain has gain huge consumers' popularity for their distinctive flavor and higher valued additives and desired taste. The current rate of the global meat consumption is 41.2 kg per capita per year (BBC, 2013) and it has been on the increasing trends somewhere for both domesticated and farming wild animals (Klein, 2004). Although it is a tough and complicated subject to account the exact figure of the global meat production and consumption, the figure often supports official policy and price support mechanisms. According to the Food and Agricultural Organization (FAO), global annual meat production is estimated to upsurge from 218 million tons in 1997 - 1999 to 376 million tons by 2030 (FAO, 2002). To keep up with this trend, food companies are vigorously competing to produce more meat supply including raw meat itself and various meat products. However, consumers nowadays becoming more anxious about their choice for healthy food and showing trends to avoid high content fat meat as well as increasing happenings of fraud labelling and adulteration in animal's meat products (Nicole, 2010). Thereby those who are concern about healthy diet regimes are trying to reduce high fat contenting meat, such as red meat in their dining table. As a response to consumer demands for healthy food, game/wild animals are

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being greatly integrated into common foods chain either legally and illegally adopted procedures. Because wild meat provides distinctive texture and flavor, low fat and cholesterol content and free from anabolic steroids or other drugs, they have become attractive selection in new and exotic delicacies (Hoffman & Wiklund, 2006; La Neve, Civera, Mucci, & Bottero, 2008) and it has created huge appeal to the consumers. According to the North American Elk Breeders Association's report, a tremendous growth of Elk animals farming industry was observed in USA from 1997 to 2003, where total market value was $ 150 million in that period. Similarly, the National Deer Farmer's Association, USA reported in 2003, about $1 billion worth deer meat were marketed by 11,000 U.S. farms (Klein, 2004). Recently, Rabbits' meat has got huge demands in US markets. More than 1.5 million commercial rabbits were sold for exotic meat across the USA in 2001 and at the same time USA earned $160,000 foreign currency by exporting rabbit meat. However, they were needed to import rabbit meat of about $ 1.5 million, reflecting an increase in consumer demand (Klein, 2004). Beyond the USA, many part of Africa consume bush meat and total consumption has been estimated to be 3.8 million tons of primate meat in per year. Only Tai region of the Ivory Coast contributes 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 bush meat animals are killed for consumption in each year (Bennett et al, 2002).

2.3 Food Adulteration

According to Food and Drug Administration (FDA), adulteration is the replacement of higher valued ingredients by cheaper ones for the purpose of economic gain. Thus food adulteration is defined as a deliberate act of degrading the quality of food products by fraudulent admixing or substituting lower-grade ingredients for its highest valued counterparts to for financial gain or additional profit.

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According to Federal Food, Drug, and Cosmetic (FD & C) Act (2002, Sec. 402) of the United States (Adulterated Food, 2002), a food shall be deemed to be adulterated if:

(a) It bears or contains any poisonous or deleterious substance which may render it injurious to health.

(b) It bears or contains a pesticide chemical residue, food additive, or a new animal drug (or conversion product thereof) that is unsafe for public health.

(c) It consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food.

(d) It has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health.

(e) It is, in whole or in part, the product of a diseased animal or of an animal which has died otherwise than by slaughter.

(f) Its container is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health.

(g) It has been intentionally subjected to radiation, unless the use of the radiation was in conformity with a regulation or exemption in effect.

(h) Any valuable constituent has been in whole or in part omitted or abstracted therefrom.

(i) Any substance has been substituted wholly or in part therefore.

(j) Damage or inferiority has been concealed in any manner.

(k) Any substance has been added thereto or mixed or packed therewith so as to increase its bulk or weight, or reduce its quality or strength, or make it appear better or of greater value than it is.

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The Government of Malaysia established the Department of Standards Malaysia whose aims include the protection of consumers' health and safety by assuring the standard of the manufacturing and trade of halal food (MS, 2009). According to the Department of Standards Malaysia food and drink and/or their ingredients permitted under the *Shariah* law must fulfill the following criteria:

(a) It does not contain any parts or products of animals that are non-halal by *Shariah* law or any parts or products of animals which are not slaughtered according to *Shariah* law;

(b) It does not contain najs (dogs and pigs and their descendents/non-halal contaminants) according to *Shariah* law;

(c) Food should be safe for consumption, non-poisonous, non-intoxicating or non-hazardous to health;

(d) Food not prepared, processed or manufactured using equipment contaminated with *najs* according to *Shariah* law;

(e) Food does not contain any human parts or its derivatives that are not permitted by *Shariah* law;

(f) During its preparation, processing, handling, packaging, storage and distribution, the food items a), b), c), d) or e) or any other things that have been decreed as *najs* by *Shariah* law.

2.3.1 Mislabeled Food

Food fraud is not a new practice but it has been started since the Roman and Greek Empires there were rules concerning the adulteration of wines with colors and flavors (Charlebois, Schwab, Henn, & Huck, 2016). A food control regulation was established in Germany and France in 13th century. At that time, King John prepared a circulation of penalties for bread adulteration in England (Shears, 2010). However, deceptive

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mislabeling of food products, especially meat and meat products, particularly the expensive one, has recently becomes a widespread issue (Rojas, González, García, Hernández, & Martín, 2012). For example, according to the Agriculture's Food Saftey and inspection Service (FSIS) about 12566 pounds of pork, beef and poulty products were recalled due to mislabeled in 2015 (FSW, 2015). In 2015, another mislbelled scandal, imported and farm raised about 25000 pounds of shrimp was sold as wild caught product (FSN2,2015). Moreover, Chuah et al., (2016) found 78.3% of tested samples were mislabeled in Malaysia.

These have increased consumer's concern about the composition and origin of food products, particularly in meat and meat products (Rojas et al., 2011a). Appropriate product labeling with proper description is very conclusive for consumers because it respects personal food choice, safeguards the public health, assures fair trade and religious belief (Ali et al., 2015c). The authenticity of the finished food products depends on their compliance with labeling rules and regulations, mainly in terms of the composition of ingredients, manufacturing methods and practices, genetic identity and technology (Charlebois et al., 2016).

2.3.2 Prevalence of Meat Food Fraud

The demand for meat and meat products are rapidly increasing with the increasing world's population. Unfortunately, despite having national and international rules and regulation in most of the countries (Kitpipit, Sittichan, & Thanakiatkrai, 2014), adulteration of meat and meat products is going on in rampant; this is just to make extra profit and outweigh the honest companies in the competitive markets (Ali et al., 2012c; Hou et al., 2015). The recently made grouper (*Epinephelus marginatus*) meals authentication studies in Madrid restaurant reflect that only 9 out of 37 samples contained authentic species (Asensio, 2008) and 22% meat products in Turkey were

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mislabeled (Ayaz et al., 2006), 19.4% in the USA (Hsieh, Woodward, & HO, 1995). Similarly, false or wrong labeling were found in about 8% meat products in the United Kingdom and 15% in Switzerland (Ali et al., 2014b). Fraud labeling was also found in the deer products, particularly blood, heart and antler products as elucidated by (Zha, Xing, & Yang, 2011; Ulca, Balta, Çağın, & Senyuva, 2013) demonstrated that chicken and turkey were found instead of beef in 100% beef labeled meatballs and no bovine DNA was found in sausages labeled as 5% beef in Turkey. Verification of beef and pasta products in the UK showed that 29 out of 2501 samples contained 1% horse DNA (Castle, 2013). The Food Safety Authority of Ireland also detected horse DNA in 37% of the tested beef burgers and 85% of them also contained pig DNA (Walkera, Burnsb, & Burns, 2013). Cawthorn et al. (2013) found that 68% (95 of 139) samples of burger patties, sausages and deli meats contained species which were not indicated on the product labeling. Pig DNA was detected in 30% of burger and patties, 52% of sausages, 32% of deli meats and 38% of minced meat products as undeclared species. Al-Nassir et al. (2014) identified undeclared species in 24% of beef burgers and minced meat samples. A total of 105 imported beef products were analyzed by Bourguiba-Hachemi et al. (2016) in the Arabian Gulf regions, and they found positive results for pig and horse species in 26% and 7% of the tested samples. Recently, police seized over 20 tons of fake beef which was made up from chemically treated pork in Shaanxi province of China, (Jeanette, 2013). In another incident, Chinese police arrested 904 suspects who were involved in the selling of processed rat meat as lamb (Beijing, 2013). In the recent years, Malaysia also faced some challenges such as porcine DNA in Cadbury chocolate (Rahman et al., 2015b), lard in bread and pig intestine casings in sausages (Man, Aida, Raha, & Son, 2007). Surprisingly, Clear Labs identified human and rat DNA in burger samples in northern California (Kowitt, 2016). The Clear Labs also found porcine DNA in beef burgers and beef DNA was found in ground lamb and pathogens DNA was

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found in 4.3% of tested food products (Kowitt, 2016). The above incidents are just some of the many examples of animal product adulterations that are taking places all over the world but sufficient to demonstrate that food products should be authenticated for their animal origins to promote fair-trade economic practices and prevent fraudsters from harming our public health, religious faith and personal budgets.

2.3.3 Impact of Food Fraud

Deceiving consumers by selling fraud foods is not a current issue. It not only causes an economic loss but also may put consumer on serious health risk because some people are allergic to certain food ingredients. In the 18th and early 19th centuries, numerous poisonous substances were used as food additives, for examples, chalk and alum were added as a whitening agent in bread; and sawdust, pipe clay or calcium sulfate was used to increase the volume or the weight of the bread (Tahkapaa, Maijala, Korkeala, & Nevas, 2015). During that time, lead was mixed with beer and wine; and sand, dirt and other leaves were regularly added to tea, coffee and spices (Schumm, 2014). In 1902, Dr. Harvey W. Wiley and co-workers who are known as the Poison Squad showed that food preservatives which were used at that time such as copper sulfate, sulfuric acid, borax and formaldehyde have the adverse effect in the body (Schumm, 2014). A remarkable incidence involving toxic oil syndrome that took 300 initial deaths and a total of 1663 lives out of 20,000 affected people in Spain in 1981 due to the consumption of industrial oil as olive oil (Borda et al., 1998; Gelpí et al., 2002). Another thunder like fiasco was the Chinese milk and baby formula adulteration with melamine in 2008 (Guan et al., 2009). After ingesting the melamine contaminated infant formula and milk, approximately 300,000 infants and children were affected with urinary tract stones and at least six were killed. In 1986, 23 persons were died due to methanol contamination with wine in Italy (Tahkapaa et al., 2015).

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Although meat and meat products forgery is not so much detrimental to health, it is a very sensitive religious and cultural issues that might provoke social unrest and extirpate certain endangered species from the world's natural habitats. Meat wholesaler of Japan mislabeled imported beef as domestic beef during the government buyback program after the bovine spongiform encephalopathy (BSE) crisis (Yeboah & Maynard, 2004). In 2005, Sudan (non-permitted color) was found in some meat products in China (Jia & Jukes, 2013). In 2003, inedible poultry meat of pet food plant got entry into the food chain in the UK. A severe food crisis was exposed in Belgium due to contamination of cancer-causing dioxin and polychlorinated biphenyls (PCBs) in 1999 (Covaci et al., 2008). The crisis happened due to the contamination of fat used for the production of animal feed with 1 gm dioxins and 50 kg PCBs; this resulted in adverse effect on domestic and export market of poultry and pork because about 2500 poultry and pig farms were affected (Buzby & Chandran, 2003) and the USA cancelled the import of certain types of food products from the entire European Union (Kennedy, Delaney, McGloin, & Wall, 2009). In Ireland, the Irish pork dioxin crisis in 2008 affected pork market because approximately 10% pig was affected due to the feeding of dioxin contaminated feed. Consequently, all pork products which were manufactured during this time were recalled, causing huge loses to the manufacturing industries (Kennedy et al., 2009). Poultry and beef of unknown sources were repackaged and marketed illegally as human food in Northern Ireland (Tahkapaa et al., 2015); in 2007 poultry were diseased and blanched, was marketed for human consumption in the UK and in 2006, approximately 150 tons of spoilt meat was distributed Germany (Rahman, 2015). These incidences reflect that adulteration and/or mislabeling of food was a common issue worldwide for many years ago to till date and conceivably a never-ending event. Consumers are not only the victim of food forgery, but also the government and even some time businessmen are greatly affected (Rahman, 2015).

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Thus, authentication of food ingredients bears huge influences to safeguard our public health, food choice and preferences and of course religion compliances (Ali et al., 2014a).

2.3.4 Religious Prohibition and Social Factors

Religions have played a great role in the selection of menu throughout the human civilization (Rehman & Shahbaz Shabbir, 2010). The impact of religion on food consumption depends on the individuals who are following the teachings of the religion along with their understanding and interpretations of the religious tenet. Religious requirements and its adherence influence the feelings and attitudes of people towards food consumption (Jamal, 2003). Most of the religions have strict guidelines that determine the food consumption to show a respect to God as well as its health attributes (Meyer-Rochow, 2009). For the Islamic Shariah law of the Muslims is one of the most important foundations in social and cultural life. Thus, Muslim consumers strictly follow dietary laws enshrined in the holy Quran, Hadiths and in certain cases based on the opinions of a group of Islamic Scholars. According to the Islamic dietary laws, Muslims are prohibited from eating or using any product derived from pigs as well as prohibited body parts and ingredients such as blood and plasma even from halal animals, or permissible body parts such as flesh if the animal is slaughtered in a non-halal way such as shooting or electrical shock (Nakyinsige et al., 2012). Likewise, Jewish dietary laws consider animal's ingredients must comply with the kosher laws. Both the Muslim Halal and Jewish Kosher law require that animals must have chew their cud and split hooves, but pigs don't have chew their cud and thereby pork and pig derivatives are clearly prohibited as food materials in Islam and Judaism (Regenstein, Chaudry, & Regenstein, 2003). Although cow meat and its products are lawful as food for the Jewish, Christian and Muslim consumers, they are except milk are unacceptable

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to the followers of Hinduism; this is because cow is considered as a worship animal by them (Meyer-Rochow, 2009).

On the other hand, vegetarianism is strongly linked to a number of religions that originated from ancient India. While Judaism, Christianity and Islam have not strongly promoted vegetarian diet; religions that originated from ancient India such as Hinduism, Jainism and Buddhism, strongly practice vegetarianism in everyday menu. While vegetarianism is mandatory for everyone in Jainism (Burt, 2016), it is advocated by some influential scriptures and religious authorities of Hinduism and Buddhism (Davidson, 2003). Most of these religious tenets are to promote healthy lifestyle and preventing illness caused by food consumption. For example, halal is an all-encompassing concept which encourages a Muslim to seek and use products, ventures and services that promote cleanliness in all aspects of a person's life. Thus, halal food means that a product or service is safe for consumption, produced in a clean environment and health as well as the next benchmark for quality. Therefore, religion is one of the main factors determining food avoidance, taboos and special regulation particularly with respect to meat consumption. Culture and social lifestyle also play an important role in food selection. Lifestyle, such as practicing vegetarians consume only plant originated materials and some of the Buddhists think killing animals is a great sin. Considering to all of these points from the religious views, food and drug manufacturers should have loyalty and responsibility to provide reasonable information related to all aspects of food and drug production. Consumers also need to be assured that the information they are being provided by a company is accurate.

Malaysia is a multiracial country with various ethnic groups and religious tenets, but Islam is the official religion where 50% of the population practice Islamic doctrines (Fischer, 2008). Malaysia has shown great interest in halal industry development

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including food, pharmaceutical and personal care products as well as halal finance systems. Malaysia also imports food, cosmetic and medicinal products from several non-Muslim countries such as Australia, New Zealand, India and Thailand wherein the bulk market is not Halal compliant. Muslims around the world are facing similar problems when they purchase consumer items from non-Muslim country. These food and consumer products could contain haram substances because the manufacturers in the foreign countries and importers/exporters may not well understand the concept of Halal which a fundamental aspect in Islamic life is. Fortunately, Malaysia along with other Muslim countries have strong legislation and surveillance laboratories under halal regulatory board, JAKIM, to monitor the Halal markets and create trust to the Halal-consuming populations since 1982 (Othman, Ahmad, & Zailani, 2009). Thus, exporter and importers are required to meet with Malaysian standard for Halal Food Production, Preparation and Storage-General Guideline (MS 1500:2004) (Malaysian Standard). Despite strict monitoring of halal status, recently non halal beef was sold in Malaysia; this has put Muslim consumers in red alert in determining the presence of prohibited animal ingredients in marketed foods as well as medicinal products.

2.4 Regulatory Laws

The regulation of health products and food is an important activity that not only supports our health but also gives us the right tools for authentication. Nowadays, both in developed and developing countries, food and drug assurance systems are generally getting more stringent to ensure both the real and perceived food safety problems. Regulations are the rules issued by the Governor of a Council to carry out the intent of statutes (Acts or legislation) enacted by the Government. They are the instruments of legislative power and have the force of law. Regulations contain more specific information and requirements than Acts. These can include definitions, licensing requirements, performance specifications, exemptions, forms and other details. The

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complexity in regulations for the health and food product sectors reflect consumers' demand for safety food and drug, as well as firms' reputation for providing safe food and drug and maintaining global market shares. Even it turns into one of the potential engines for economic growth and societal development. The regulations also differ significantly across health product and food types, such as raw and processed food and drug, less and highly perishable food products, low or high incidence of risks for human health. The following countries regulatory system, statutes and rules provide a framework for Health to regulate health products and food in country

2.4.1 Malaysia Food Act 1983

Generally, "food law" is used to apply to legislation which regulates the production, trade and handling of food and hence covers the regulation of food control, food safety and relevant aspects of food trade. To protect the public from unhealthy food or health hazards and fraud in the preparation, sale and use of food, Malaysian Government enacted the Food Act law on 9th March 1983. Moreover, Malaysia is leading on the edge of making itself as a halal food hub of the world. It is evidenced by the fact that Malaysia is a Muslim-majority country and hence there is a demand for halal foods in the local markets and Malaysia also wants to earn revenues from the export of halal foods. Even, nowadays, halal foods have gain attention among non-Muslims community due to its quality attributes, hygiene and safety standard. Global halal food market value is estimated to be US$ 3.7 trillion by 2019(WAM, 2016). This big market may contribute as a potential engine for economic growth and societal development and Malaysia aspires to be is a leader in the halal food benchmarking. The United Nations has cited Malaysia as the world's best example of benchmarking of halal food in Geneva in 1997 (Bohari, Cheng, & Fuad, 2013). Thereby foods material including processed foods is stringently regulated by the Malaysia Food Act 1983 and Malaysia Halal Standard. This act protects the public from health risks and fraud in the food

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preparation, sale, use of food, and for matters incidental or connected in addition to that throughout Malaysia. Additionally, it is the first to announce a global halal center and create a restricted agency for halal monitoring at the national level.

2.5 Importance of Rabbit, Rat, Squirrel and Cat Detection

2.5.1 History of consumption:

2.5.1.1 Rabbit meat consumption

Rabbits are considered as small mammals belonging to the Leporidae of the order Lagomorpha family which are found in several parts of the world. Rabbit meat has got nutritive and dietetic properties as professed by Dalle Zotte (2002) (2004); Combes, (2004); Combes & Dalle Zotte (2005); Hernandez & Gondret (2006). Its proximate composition demonstrates its protein (about 22% when considering the loin – m. Longissimus dorsi or LD – and hindleg meat. Besides possessing a high protein content, rabbit meat also contains a high level of essential amino acid. The mineral content is also constant at around 1.2 -1.3 g/100 g meat and its lipid content is quite low (on average 3.4 g/100 g) compared to the other meats. Rabbit meat also possesses moderately high energy values (from 603 kJ/100 g in the loin to 899 kJ/100 g in the fore legs). Rabbit meat is a habitual diet in many European countries (Malta, Cyprus, Italy, Czech, Republic, Spain, Belgium, Luxembourg, Portugal, France) and certain north African countries such as Egypt and Algeria (Zotti, Szendro, 2011). During the past 50 years, the world production of rabbit meat had increased by 2.5 fold up to1.6 million tons in 2009. Currently, the world's leading producer is China (700,000 t/year). Over in Europe, the main rabbit meat producers are Italy (230,000 t/year), Spain (74,161 t/year) and France (51,400 t/year) (Zotti, Szendro, 2011). Latest statistics show that the countries with the highest rabbit meat consumption are as follows: Malta (8.89 kg per inhabitant), Italy (5.71 kg per inhabitant), Cyprus (4.37 kg per inhabitant), France (2.76

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kg per inhabitant), Belgium (2.73 kg per inhabitant), Spain (2.61 kg per inhabitant) and Portugal (1.94 kg per inhabitant) (Zotti, Szendro, 2011).

2.5.1.2 Rat meat consumption

Rats hail from the superfamily Muroidea, and comes in various medium sizes and are long-tailed rodents. Commonly black, red, brown and cane rat species are found in the south-east Asian regions and they are mostly considered as an agricultural pest. While rat meat is rejected in most of the societies as a food taboo, some rat species are domesticated and consumed in certain communities because of their greater carcass yield (ca. 65%), soft bones and taste like bird's meat (Odebode et al., 2011). Some African communities also prefer wild rat meat as an exotic and aristocrat meal in their social events, giving rise to a frequent trade of wild rats along the road side markets of many African countries (Redhead et al., 1990). Although rat is a carrier of zoonotic disease, approximately, 80 million pieces of cane rats are hunted per year only in western Africa, with a yield of 300,000 metric tons of meat (Hoffman et al., 2012). A historical outbreak of plaque disease in India in 1994 was linked to the rat meat consumption that took at least 60 lives out of 693 affected people (Deutsch et al., 2012). Similarly, 54 people were infected by plaque virus in 1967 in Vietnam and it was also linked to the consumption of rat meat as well (Conrad et al., 1968). Rat meat adulteration in common meat is also a very serious and sensitive social issue because it is a taboo in most societies and non-halal for the Muslims (Doosti et al., 2014). However, the wider availability of rat species all over the world and their cheaper prices might make them a preferred substitute in meat product.

2.5.1.3 Squirrel meat consumption

Squirrels come from the Sciuridae family, a family that includes small or medium size rodents. Squirrels are generally small size animals measuring about 7-10 cm.

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Squirrels can be found in almost every habitat from tropical rainforest to semiarid desert, except the high polar regions and the driest of deserts. They are predominantly herbivorous, living on seeds and nuts, but will eat insects and even small vertebrates (Wauters, & Dhondt, 1992). Squirrel meat is considered a favored meat in certain regions of the United States where it can be listed as wild game. Squirrel meat is not so much popular but some Southeast Asian and African communities consume it in daily meals as a source of proteins (Davis et al., 1990) and for certain health benefits, such as distinctive flavor, high proteins, low fat, less cholesterol and the absence of health-threatening anabolic steroids (Redhead et al., 1990). In many areas of the US squirrels are still hunted for food, as they were in earlier years (Kurlansky, 2009). Specifically, UK citizens are cooking with the invasive gray squirrel, which is being praised for its low fat content and the fact that it comes from free range sources (Colquhoun & Kate 2008).

2.5.1.4 Cat meat consumption

Podberscek (2009) highlighted that dating back to ancient times until now, feline meat has been consumed by humans. In the early period of 8,5000 BCE in Cyprus, domestic cat was consumed by human beings (Vigne, Guilane, Debue, Haye, & Gerard, 2004). Later, during the 17th century, it spread to teenhe United Kingdom (Thomas 1991) and then to China in the 14th century (Podberscek, 2009) and later to France and Germany in the 18th century (Ferrieres, 2006). Since, cat meat had been banned in certain countries and huge protection has been given by animal welfare group, the consumption of feline meat can only be seen in Cambodia, China, Thailand, Vietnam, South Korea, and some parts of Europe, Russia, Africa and Latin America (Podberscek 2009). In ancient history, cat meat has been consumed as part of traditional cultures, health benefits, and religious belief but in some countries its consumption has been reflected as a symbol of national pride (Podberscek 2009). For example, some people

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take cat meat as an aphrodisiac while others take it for treating rheumatism and arthritis (Podberscek 2009). In South Korea, about 100,000 cats are killed each year, whereas 4 million cats are consumed each year in China (Bartlett & Clifton 2003). In addition, a liquid or 'juice' is prepared from cats to be consumed as a 'tonic' for health benefits in South Korea and China as it keeps your body warm during the winter seasons (Podberscek 2009). In the absence of any available census data for cat population in many parts of the world and also there is no open market for trading in cat meat, thus, it could be considered as a highly potential adulterant in halal foods and meat products. Likewise, feline meats or materials are considered as an adulterant which is not permissible for consumption under the food consumption guidelines of Islam and Judaism. There have been instances where cat meat was sold as rabbit meat after repackaging by slaughterers in eastern China (Philips,2013) and in another case, cat meat was served as Indian curry in UK restaurant in 2013 (Chatterji, 2013). The Chinese police raided the illegal slaughterhouse and found thousands of cat meat mixed up with rabbit meat (Phillips 2013). Another shocking report which happened in Guangdong and Guangxi, southern provinces of China, where a large number of dead bodies of stray and domesticated cats were traded illegally and sold for profit. These dead bodies were sold at 10 yuan (= UK1 pound) which will be used as waste product in the regular food chain system (Phillips 2013).

2.6 Religious View and Health Issue

2.6.1 Zoonotic diseases

Zoonosis is the infection or disease that is naturally transmissible from animals to humans. According to Department of Agriculture and Fisheries of the Queensland Government over 200 zoonosis have been recognized these are caused by pathogenic agents such as viruses, bacteria, fungi, parasites and prions. Among this diseases 13 zoonosis are more fatal because about 2.2 million people were died due to the infection

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of these pathogens (Bryner, 2012). Now a days, endemic zoonoses cause an extra pressure of numerous diseases, specifically over the tropical zones and that also affect the livelihoods and food supply chain due to loss of livestock production (Halliday et al., 2015). Despite their detrimental effect, till date endemic zoonoses do not have proper recognition as well as understanding (Halliday et al., 2015). United States Department of Agriculture stated that about 60% of human pathogenic diseases are zoonosis and about 75% of infectious diseases are caused by animal origin. (USDA, 2016). Cat disease can be easily transmitted from cats to human beings although it is said that feline infectious diseases only affect cats. The chances of an average person contracting a zoonotic disease from a cat is low, but however, individuals with a weak or low immune systems are more susceptible to these diseases. Examples include infants, individuals with acquired immunodeficiency syndrome (AIDS), the elderly, and people undergoing cancer chemotherapy or receiving other drugs that may suppress their immune systems. Common feline zoonotic diseases are Campylobacteriosis, Bartonella henselae infection), Cryptosporidiosis, Dipylidium Infection (dog and cat flea tapeworm, Plague, Rabies, Ringworm, Salmonellosis, Sporotrichosis, Toxoplasmosis, Toxocara infection (roundworm) (August, & Loar, 1984). On the other hand, rats are a carrier of several zoonotic diseases. Some diseases are directly transmitted such as Hantavirus, Pulmonary Syndrome, Leptospirosis, Rat-bite Fever and Salmonellosis whereas some desease are indirectly transmitted such as Plague, Colorado Tick Fever, Cutaneous, Leishmaniasis (Himsworth, Parsons, Jardine & Patrick, 2013). Squirrels have no issues with humans as squirrels rarely bite unless they have been cornered or feel threatened. However, they do carry and transmit a handful of diseases, which makes their presence undesirable and occasionally dangerous. Moreover, squirrel is a carrier of several type of zoonotic desease such as

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Salmonellosis, Lyme disease, Tularemia, Leptospirosis, Rabies, Creutzfeldt–Jakob disease (CJD) (Rushton et al., 2006)

Majority of people believe that rabbits are very healthy companion pets and are free from any diseases. But one most common human health complaint involving rabbits is allergy either from their fur or their food and bedding. There are 4 major infectious diseases seen in pet rabbits. Two serious diseases caused by viruses may occur in rabbits, although they are rarely seen in indoor pets. They are myxomatosis and viral hemorrhagic disease. Because they are viral diseases, there are no effective treatments once the rabbit is infected. Two other infectious diseases of rabbits are Encephalitozoan cuniculi and Pasteurella multocida (Deplazes, Mathis, Baumgartner, Tanner, Weber, 1996).

2.7 Current Species Identification Technique

Researchers have paid more attention to the development of ideal and precise technique for the detection of several animal species due to ever-increasing meat and meat products fraudulent issues worldwide (Ali et al., 2014b). Although morphological test is used for the identification of some food like honey, but it is not appropriate for the detection of meat species particularly in processed meat products (Camma, Domenico, & Monaco, 2012). Moreover, microscopic technique also unsuitable for the meat product identification because it is unable to determine the accurate animal species in food staff (Ali et al., 2012d). However, numerous analytical approaches have been documented to detect the species origin in meat and meat products based on protein, lipid and DNA biomarkers. However, the lipid and protein based methods are often unsuitable because they are laborious, target-biomarker are often modified and thus cannot distinguish closely related species in highly processed food such as heated or chemically treated products, and are of less sensitive than DNA-based approaches (Ali et al., 2012e; Lago, Herrero, Madriñán, Vieites, & Espiñeira, 2011). Moreover, these

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methods are unable to differentiate closely related species, such as cow and buffalo. In contrast, the DNA-based techniques, especially the short-length DNA biomarkers are thermodynamically more stable, more sensitive and more reliable over the longer ones even under extreme states such as degraded or naturally decomposed samples (Ali et al., 2015b). The use of field and limitation of these methods are briefly presented here.

2.7.1 Lipid based assay

Lipid based techniques for analysis of meat species involves in the analysis of fatty acids positional distribution in triacylglycerol (TAG) and 2-monoacylglycerol (2-MAG) as all species stored n-6 polyenoic and monoenoic fatty acids in TAGs with unsaturation (except pigs) at the sn-2 position and larger chain length (Szabo, Febel, Sugar, & Romvari, 2007). Szabo et al., (2007) reported that rabbit and ruminants contain high amount of odd-chain-length fatty acids in their native TAGs which are the detectable markers of these two species. On the other hand, pigs can be detected by the analysis of 2-MAGs because they contain lower unsaturation in 2-MAGs. However, measurement of the fatty acid positional distribution provides information for the identification of the species but the content and varieties of the TAGs and 2-MAGs usually modified due to the processing and cooking treatments. Thus, these methods have very limited used for the identification of species in food and foodstuff due to its less reliability.

The Fourier transform infrared spectroscopy (FTIR) together with partial least square (PLS) or principal component analysis (PCA) is an important tool for the authentication of food species based on lipid (Rohman, Erwanto & Man, 2011). Infrared absorption spectrum of the samples was measured in the FTIR assays and this method is also able to collect high spectral resolution data (Griffiths & De Haseth, 2007). Analysis of fatty acids is important for the differentiation of fats from animal and plant sources. Therefore, analysis of fatty acids plays an important role in identification of adulteration

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or replacement of vegetable oils with lower priced lard in Kosher, halal and vegan food products.

2.7.2 Protein based assay

An overview of protein-based assays for the detection of species in meat and meat products are described below:

2.7.2.1 Histidine dipeptides based assay

Animal tissues, namely muscle, heart, kidney and liver naturally contain some dipeptides associated with histidine such as balenine (β-alanyl-L-3-methylhistidine, ophidine), anserine (β-alanyl-L-1-methylhistidine) and carnosine (β-alanyl-L-histidine). These dipeptides play an important physiological role in the tissue, such as antioxidant, buffering, vasodilatory activity, neurotransmitter action and enzyme modulator (Aristoy, Soler, & Toldrá, 2004; Carnegie, Hee & Bell, 1982). Histidine dipeptides are present only in animal tissues, but not in plant sources and these dipeptides are also animal specific (Aristoy et al., 2004). Thus, the species origin can be detected in the processed meat products by determining the ratio of these dipeptides particularly the ratio of carnosine and anserine or vice versa, because histidine dipeptides remain unaffected by heat treatment (Aristoy & Toldra, 2004). For example, Aristoy and Toldra (2004) showed that the height ratio of carnosine and anserine was in pork with 17.88 ± 3.74, followed by beef with 8.08 ± 1.91, lamb with 0.95 ± 0.26 and poultry with 0.20±0.08. Therefore, by measuring of these dipeptides can easily identify the existence of animal protein in feedstuff, as plant sources do not contain these dipeptides. This method was particularly developed for the detection of animal proteins in the animal feeds. Because bone meat meal, meat meal, fish meal etc. are the main source of calcium, amino acids and phosphorus, which play a role in the rapid growth of farmed animals (Aristoy & Toldra, 2004). Due to the Prevalence of

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mad cow disease (Bovine Spongiform Encephalopathy), the use of animal proteins was forbidden in the feed of ruminants in worldwide (Aristoy & Toldra, 2004). Although this technique can identify the origin of mammalian but is unable to determine the specific animal species, especially in complex matrices of various species (Aristoy & Toldra, 2004), reflecting the requirement of more specific and precise method for this analysis.

2.7.2.2 Analysis of muscle protein

Muscle protein can be originated by using isoelectric focusing (IEF) electrophoresis. Muscle proteins present in the sarcomeres or sarcoplasm are the target for the authentication of the species origin. The cytoplasmic part of the muscle cell (myocyte) is sarcoplasm and the structural unit of the muscle fibers (myofibers) is sarcomere (Hulland, 1993). Parvalbumins are present in high concentration in the fish muscle sarcoplasm, which are small, calcium-binding, acidic and heat-stable proteins. As these proteins are species specific and isoelectric P^H range is 3.8 to 5.3 in native state, the IEF profile of these proteins have been effectively introduced to discriminate the fish species (Addis et al., 2010; Berrini, Tepedino, Borromeo, & Secchi, 2006). Berrini et al., (2006) revealed that IEF profile is able to differentiate the inter-species polymorphic species but is not suitable for intra-species polymorphic species. Thus, two-dimensional electrophoresis (2-DE) can overcome this problem. 2-DE map of myosin light chain (MLC), a sarcomeric protein, can clearly distinguish the fish species as well as able to provide information of the preserve condition and freshness of the specimens (Martinez & Jakobsen Friis, 2004). Moreover, 2-DE method couple with proteomic assay, namely mass spectroscopy and in-gel digestion, are more suitable tool for discriminating the species-specific MLC in admixed and processed samples of different tissues and muscles of various species (Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009). Giometti et al. (1979) proposed that high-resolution two-dimensional

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electrophoretic technique can feasibly be applied for the analysis of biopsy samples of human muscle by resolving the major muscle proteins and enzymes. They successfully identified the ten enzyme components and actin, myosin, troponin and tropomyosin from the two-dimensional profile using rabbit muscle as a model. By comparing the human and rabbit muscle patterns found enormous similarities, but not confirm identifiable and additional modification is required for final results (Giometti, Anderson, & Anderson, 1979). Thus, electrophoretic and proteomics techniques are expensive, required skilled technicians, laborious and also not suitable for the investigation of admixed samples of different species (Addis et al., 2010; Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009).

2.7.2.3 Analysis of species-specific Osteocalcin

Osteocalcin (γ-carboxyglutamic acid-containing protein) is noncollagenous protein found in bone and dentin of most animals and play role in the formation of bone. According to EU Regulation ((EC) No 999/2001) feed containing meat and bone meal (MBM) is restricted for farmed animals. Furthermore, addition of animal proteins in the feedstuff of same species is also prohibited under the Regulation (EC) No 1774/2001. The permitted MBM source is only fish meal in the feed of fowl, pig and calves (Regulation (EC) No 999/2001). Thus, analysis of feedstuff to detect the contaminated animal MBM is mandatory by the EU Regulation ((EC) No 999/2001, (EC) No 1774/2002). The classical optical microscopic technique is the accepted official method for the identification of MBM in the feedstuff (EC, 2009). This method is reliable for the detection of animal origin, which are stable under processing treatment (133 °C and at 300 kPa for 20 min) required for MBM manufacturing, such as bone fragments, scales, gills teeth or hair (Kreuz et al., 2012). But microscopic method cannot apply in the quantitative approaches and to overcome this limitation spectroscopic (near infrared spectroscopy-NIRS) method was introduced (Abbas et al., 2010). To increase the

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performance of the spectroscopic method for analyzing the animal proteins in feedstuff, near infrared microscopic (NIRM) method has been developed. The NIRM is more useful because it possesses both spectroscopic and microscopic functions in one instrument (Abbas et al., 2010). The recent modified form of NIRM is NIR hyperspectral imaging, which allows both spectral and spatial characterizing information of the specimen simultaneously (Abbas et al., 2010). The sensitivity of the NIRM methods is up to 0.5% level of adulteration in feed specimen (Abbas et al., 2010). In addition, Fourier transform near infrared spectrometer (FT-NIR) couple with auto image microscope also have significant role for the differentiation of species contaminated in feedstuff. de la Haba et al. (2007) developed FT-NIR microscopic method for the discrimination of land-animal and fish particles in feed samples (de la Haba et al., 2007).

The protein, osteocalcin (OC) is a not suitable target molecule for the differentiation of species due to its conserved nature as well as very low variability in the sequences. But there is enough variation at the genus level of OC such as it contains amino acid sequence variation between the species which help to distinguish the different species (Balizs et al., 2011). Consequently, Balizs et al. (2011) developed a suitable method for detecting species-specific OC on the basis of mass differences due to the variation in amino acid sequences, by using the matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) and high-resolution hybrid mass spectrometry (HR-Q/TOF MS). This method was successfully applied for the differentiation of bovine and porcine materials in MBM samples (Balizs et al., 2011). In addition, Kreuz et al., (2012) developed sandwich ELISA technique to identify the MBM in feed, on the basis of raising antibody against the bovine osteocalcin. The developed method is stable under the heat treated samples (145 °C) and is very sensitive (1 ng for pure state and 0.1% for adulterated sample) and they also proposed that it may apply for the discrimination of

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bovine and horse species (Kreuz et al., 2012). However, these methods are highly expensive, required skilled operator to operate and unable to differentiate specific-species properly, particularly in the mixed matrices.

2.7.2.4 Detection of species specific proteins by ELISA

Although above described protein-based methods are suitable for the identification of feed and food ingredients but these are not applicable for the routine analysis of commercial feed and food products because they are comparatively expensive, laborious, complex to handle and time consuming (Asensio, González, García, & Martín, 2008). On the other hand, the immunological method, namely Enzyme-Linked ImmunoSorbent Assay (ELISA) has been more suitable and widely used tools for the authentication of food products due to its low cost, high specificity, sensitivity and simplicity (Asensio et al., 2008; Carrera et al., 2014).

Until now, various reports have been documented for the authentication of food using both MAbs and MAbs on the based on structural and soluble proteins of the muscle cell. Berger et al., (1987) raised PAbs against the antigen of chicken and pork muscle tissue, which are heat-resistance. They found that isolated antigens were immunoreactive under 120^{0} C for 15 min and sensitivity chicken and pork ELISA were 126 and 250 ppm level, respectively. Furthermore, Rencova et al., (2000) also developed ELISA method for the identification of heat treated samples. Poultry, rat, kangaroo and horse species were successfully identified with a sensitivity of 1-5% by developing the PAbs against muscular tissue which was heated at 100 or 1200 C for 30 min (Renčová, Svoboda, & Necidova, 2000).

Researchers also developed ELISA methods for the quantitative evaluation of adulterated meat samples. For examples, ELISA method was introduced for the quantitative measurement of the raw pork in the admixture of raw beef with the

quantification limit up to 1% (Martin, Chan, & Chiu, 1998). Chen and Hsieh (2000) reported quantitative ELISA technique for the quantification of pork in heat treated various meat products such as sausage bologna ham, salami spread franks and luncheon meat using MAbs which was raised against heat-stable muscle protein of pig. The limit of detection was found 0.5% (w/w) porcine material in various meat mixture and the accuracy of the developed method was confirmed by comparative study with commercial PAbs test kit (Chen & Hsieh, 2000)

2.7.3 DNA-based method

Recently, researchers have paid more attention to the DNA-based methods and these methods becoming more prominent and widely used for the verification, quantification and monitoring of adulterated species in meat and meat products because of its specificity, sensitivity, preciseness, robustness, rapidity and inexpensiveness (Darling & Blum, 2007). The DNA-based methods are considered as extraordinary and highly useful tools in practical fields due to the exceptional properties of DNA molecule such as codon degeneracy, superior heat stability, abundant presence in multiple copies in most cells along with intra-species conserved and inter-species polymorphic fingerprint etc. (Ali et al., 2014a; Mafra, Ferreira, & Oliveira, 2007). Stability of biomarkers is a key factor for successful species detection particularly in processed meat products, as these products are prepared under extreme heat and processing treatment. Unlike protein biomarkers which readily denature under heat processing treatment, DNA biomarkers are highly stable under severe processing condition (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2012). Furthermore, a small amount of sample is enough for the detection species in DNA-based methods because multiple copies of DNA are present per cell (Gupta, Rank, & Joshi, 2011; Mane, Mendiratta, & Tiwari, 2012). In addition, DNA also carry enormous information compared to proteins due to the genetic code degeneracy and the existence of large non-coding stretched (Pereira, Carneiro, &

Amorim, 2008). Due to the above advantages, DNA-based methods have become more favorable tool for the detection of species in complex background of heavily processed foods. However, among the DNA based assay, PCR has been gained increasing attention due to accuracy, higher sensitivity, reliable and rapid investigation scheme, where DNA is used as a detection target and a single DNA copy is amplified into multiple copies (Aida et al., 2005; Ali et al., 2014b). Design of specific biomarkers of the target species is a fundamental step of PCR assay development. According to the research requirement, both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been introduced in numerous studies for the design of biomarkers (Morin, Hedrick, Robertson, & Leduc, 2007). Researchers have gained particular attention to the mitochondrial DNA (mt-DNA) over nuclear DNA (n-DNA) especially, for the identification of meat products due to the following advantages:

(i) the absence of pseudogene or repetitive sequence, complicated intron which result in simpler in complexity than n-DNA,
(ii) rapid evolution of mt-DNA due to the higher base substitution rate than n-DNA, allowing the existence of more diversity in sequences and facilitating the differentiation of phylogenetically closely related species (Fajardo et al., 2010; Zha , Xing , & Yang , 2010),
(iii) sequence of mt-DNA is more conservative because of its maternal inheritance and lack of recombination in all vertebrates (Rokas, Ladoukakis, & Zouros, 2003)
(iv) more stable because mt-DNA is present in higher number per cell (800-1000) and surrounded by double membrane (Girish et al., 2004).

Thus, mt-DNA can survive under severe processing treatment, offering the target of biomarker design for the reliable detection of species in compromised samples and in

the admixture of closely related species (Ali, Hashim, Mustafa, & Man, 2011b; Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Mane et al., 2012).

2.7.3.1 Polymerase chain reaction (PCR) based assay

PCR is an in vitro process in which a specific target DNA fragment can be amplified from a single or small number of DNA to a large number of DNA under a simple enzymatic reaction (Garibyan & Avashia, 2013; Levin, Ekezie, & Sun, 2018). Kary Mullis, who is the pioneer of the PCR technique, explained the PCR assay as "lets you pick the piece of DNA you're interested in and have as much of it as you want" (Garibyan & Avashia, 2013). The major components of the PCR reaction includes, primers, template DNA, DNA polymerase and nucleotides (Garibyan & Avashia, 2013). Only simple three-steps cycling reactions are required for PCR assay, such as

(i) Double stranded DNA denaturation
(ii) Primers annealing
(iii) Primer extension

When amplification target is RNA, a complementary DNA (cDNA) of that RNA must be generated with the help of reverse transcription prior to PCR is started (Schochetman, Ou, & Jones, 1988). The key function of the PCR reaction is the association of individual building blocks nucleotides (adenine, guanine, cytosine and thymine) together by the enzymatic reaction of DNA polymerase for the synthesis of PCR products. The primers are short single stranded DNA sequences and complementary to the DNA of target species either from 5'-end or 3'-end of the desired sequence. Annealing of the primers with the dissociated DNA stands facilitate the DNA polymerase to start the extension of new stands. Thus, after completion of each cycle, the copy of DNA is become double, allowing the synthesis of large number of DNA after 30 to 40 cycles. After mixing the PCR all PCR reagents in the PCR tube or 96-well

plate is placed in the Thermal Cycler to run the three basic steps of repeated DNA amplification reaction (Garibyan & Avashia, 2013; Schochetman et al., 1988). For the detection of PCR amplified products, DNA visualization is accomplished under an electrophoresis system of agarose gel or polyacrylamide by staining with ethidium bromide or other non-carcinogenic DNA stain (eg. Florosafe DNA stain) and an appropriate DNA size marker under a gel image documentation system for only gel image (Lee, Costumbrado, Hsu, & Kim, 2012) or on automatic Capillary Electrophoresis System for both gel image and electroferogram (Doley et al., 2005; Fajardo et al., 2010). Among the DNA-based studies, PCR assays have occupied the central place because they can amplify a specific fragment of DNA from a minute quantity such as single copy to any detectable quatities (Reid, O'donnell, & Downey, 2006). Because of this feature a large number of PCR methods have been developed for the authentication of different species such as fish and meat species. A brief description of the different PCR-based assays is illustrated below under different subheadings:

2.7.3.2 PCR sequencing

Sequencing of PCR products clearly tells whether an authentic target has been detected or not without needing any enzymatic digestion or other post PCR analyses (Fajardo et al., 2010). Singleplex PCR products can be amplified from the DNA of a wide range of species by using one set of universal primer pair (Kocher et al., 1989) and the PCR product could be sequenced and analyzed to identify species origin of the amplified fragments and their intra- and inter-specific discrimination of even very closely related species (Fajardo et al., 2010). Mitochondrial cytb, 16S rRNA and 12S rRNA genes are most widely used for the development of genetic biomarkers for the differentiation of species by PCR sequencing because of their sequence availability in the databases and sufficient level of mutation (Karlsson & Holmlund, 2007). Chikuni et al., (1994) developed a PCR sequencing method using a set of universal primers

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targeting 646 bp fragment of mitochondrial *cytb* gene for the identification of 8 mammals such as cow, pig, goat, sika deer, horse, rabbit and Japanese serow as well as five birds namely, chicken, Japanese tree-sparrow, dusky thrushe, Japanese Quail and a thrush in Europe (Chikuni et al., 1994). In order to authenticate the meat species, Brodmann et al., (2001) also developed PCR sequencing technique and successfully identified the meat of red deer, roe deer, fallow deer, and chamois by amplifying the 428 bp products from mitochondrial cytb gene. However, the system was unable to discriminate the meat from domestic pig and wild boar (Brodmann et al., 2001). Chamois meat was also detected by sequencing of a 282 bp PCR product from mitochondrial cytb gene (Colombo, Cardia, Renon, & Cantoni, 2004). Kitano et al. (2007) designed two sets of primers targeting mitochondrial 12S rRNA and16S rRNA for the amplification of 215 bp and 244 bp PCR products, respectively. The sequence analysis of the PCR products confirmed that the developed primer sets properly amplified the desired DNA fragments of different types of vertebrates such as mammals, amphibians, reptiles, birds and fish. PCR sequencing method was also applied for the verification of processed meat products. A 402 bp PCR products from the 87 samples of processed meat products labeled as beef and pork were successfully identified and separated by sequence analysis (Hsieh et al., 2005). More recently, Spychaj et al. (2016) reported a PCR sequencing method for the precisely authentication of bovine, pig and duck in different heat treated meat products such as frankfurters and sausages. The buffalo species also detected by using PCR sequencing method (Venkatachalapathy, Sharma, Sukla, & Bhattacharya, 2008). Despite of its benefits, PCR sequencing systems need specialized instruments and operators and they are also expensive. Thus, it is not a suitable technique for routine analysis of meat products.

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2.7.3.3 DNA barcoding

DNA barcoding was introduced in 2003 and it has been applied as a reliable, fast and inexpensive method that can identify species without necessitating taxonomic analyses (Luo et al., 2011; Vernooy et al., 2010). DNA barcoding often amplifies about 650 bp fragment of mitochondrial cytochrome oxidase I (COI) gene and assign species based on sequence variation to make reference sequences that can act as a molecular detection tag for each of the species profiled by PCR (Fajardo et al., 2010). Identification of species are usually accomplished by comparing the sequences of target species with DNA barcodes of known species through alignment searching, distance-based tree construction, decision theory, the characteristic attribute organization system and the back propagation neutral network (Luo et al., 2011).

A Canadian national research network has developed the Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org) which currently accommodates barcode records for over 850,000 samples, representation about 100,000 species (Vernooy et al., 2010). The invention of DNA barcoding system seems to be promising in various area like forensic analysis, biosecurity and food authentication as well as protection of wildlife (Ferri, Alu, Corradini, Licata, & Beduschi, 2009). Most of the studies regarding food speciation using DNA barcoding system have focused on fishery and seafood products (Fajardo et al., 2010). For example, Barbuto et al. (2010) applied DNA barcoding method for the detection of shark slices sold (palombo) using 550 bp barcode sequence from coxI gene. The developed technique was able to identify adulteration in 80% of the tested samples of commercial palombo in Italy. Another approach for the authentication of seafood was introduced by Wong et al., (2008) wherein they used 652 bp sequence from the COI gene and successfully identified that 25% of the specimens were potentially mislabeled. Recently, Hajibabaei et al. (2006) developed a short length barcode (~100 bp) for the identification of museum specimens, as higher length barcode

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like 650 bp cannot recover with full length due to DNA degradation in highly decomposed samples. DNA barcode system was also developed for the detection of domestic animals. Ramada (2011) designed one set of universal primer targeting 422 bp mitochondrial 16S rRNA gene of buffalo. The developed system successfully identified buffalo as well as cattle, goat and sheep.

Although DNA barcoding system has gained wide spread support in the identification of species and biodiversity screening, it is not free from limitations. Firstly, DNA barcoding amplify long DNA fragment, like 650 bp segment of COI gene which often breakdown in heat and pressure treated foods and feeds samples (Ali et al., 2015b; Fajardo, González, Martín, Rojas, et al., 2008; Hird et al., 2006). Secondly, the system is applicable for only single species detection scheme and cannot be applied for the detection of multiple species in a single assay platform. Thirdly, this technique requires two major steps: one is PCR amplification and second one is the post-PCR sequencing of the amplified products reflecting that make it quite expensive. Fourthly or finally, the assay cannot generate quantitative data (Ali et al., 2011).

2.7.3.4 Species specific PCR

Recently, researchers have paid more attention to the species-specific PCR (SSP) targeting mitochondrial genes due to its simplicity, sensitivity, preciseness, cost-effectiveness and requirement of very lower amount of sample (Karabasanavar et al., 2011b). In this method, target DNA fragment is amplified using one set of primers (forward and reversed) by an enzymatic reaction of DNA polymerase followed by separation on agarose or polyacrylamide gel with ethidium bromide or other non-carcinogenic staining dye to visualized (Ali et al., 2011). Both simplex or singleplex (Barakat, El-Garhy, & Moustafa, 2014; Mane et al., 2012) and multiplex (Dalmasso et al., 2004; Hou et al., 2015) SSPCR assays have been documented.

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(a) *Simplex PCR*

Simplex PCR involves amplification or detection of single species in a reaction. Until now, enormous simplex PCR assays for the detection of various species with different target (amplicon) sizes have been documented due to its sensitivity, accuracy and robustness. For example, Mane et al., (2012) introduced beef specific PCR assay based on 513 bp amplicon sized from mitochondrial D-loop gene for the detection of raw, processed and autoclaved beef and beef products. Arslan et al., (2006) also reported beef specific PCR assay for the identification of various heat treated meat including boiling, pressure cooking, roasting and pan frying by amplifying 271 bp fragment of mitochondrial DNA. Various reports have also been documented for the authentication of buffalo species. Girish et al. (2013) developed a rapid detection method of buffalo species using mitochondrial D-loop gene for amplifying the 482 bp fragment. Another highly specific PCR assay was developed targeting the same gene for the identification of buffalo meat which amplified 534 bp PCR product (Karabasanavar et al., 2011). Kumer et al. (2011) reported buffalo mitochondrial D-loop specific PCR assay targeting 358 bp amplicon size. To authenticate the processed meat and meat products, a buffalo specific PCR assay was documented for the amplification of 537 bp amplicon from mitochondrial D-loop gene. The assay was sensitive up to 1% level of adulteration under autoclaved condition (Mane et al., 2012). Recently, Vaithiyanathan et al. (2016) developed beef and buffalo specific PCR methods with a common forward primer for both beef and buffalo and the species specific reverse primers from the mitochondrial D-loop region. The developed systems successfully amplified 126 bp and 226 bp PCR products for beef and buffalo species, respectively with a detection level of 0.47 ng for beef and 0.23 ng for buffalo DNA in simplex PCR assays. Numerous simplex PCR assays also introduced for the verification of porcine material in food chain. To developed pork specific PCR system, different types of mitochondrial genes

have been targeted with different amplicon sizes including cytb (Aida et al., 2005; Ali et al., 2011), D-loop (Che Man, Mustafa, Khairil Mokhtar, Nordin, & Sazili, 2012; Haunshi et al., 2009; Karabasanavar et al., 2014) and 12S rRNA (Man et al., 2007). Other species also detected by using simplex PCR assay such as goat (Kumar, Singh, Singh, & Karabasanavar, 2011; Rodríguez et al., 2004a), sheep (Rodríguez et al., 2004a), cat (Ali et al., 2016), dog (Rahman et al., 2014), monkey (Ali et al., 2016) and turtle (Ali et al., 2015b).

(b) ***Multiplex PCR***

The multiplex PCR is an extraordinary and relatively the latest addition in PCR technologies, where multiple target DNA fragments are amplified simultaneously in a single assay mixture, reducing both time and cost (Ali et al., 2015c). Both conventional (end-point) and real-time PCR assay have been introduced for the authentication of meat and meat products. Nowadays, these techniques have got great promise since they offer abundance advantages Matsunaga et al. (1999) were the first to introduce multiplex PCR technique for the detection of five meat species such as pig, cattle, goat, horse and sheep. They used a common forward primer from the mitochondrial cytb gene and reversed primer from species specific DNA sequences. Rea et al. (2001) developed a duplex platform for the detection of bovine and water buffalo milk and mozzarella cheese based on 113 bp and 152 bp fragments from cytb gene of bovine and water buffalo respectively. The sensitivity of the method was found to be 1 pg for raw and 1% level for adulteration. Gupta et al. (2012) optimized the same primer pairs which were developed by Rea et al. (2001) for the simultaneously detection of beef and buffalo meat with the similar sensitivity (1 pg). Duplex PCR was also introduced for the authentication of cattle and buffalo fat targeting mitochondrial D-loop gene of both species. 126 bp and 226 bp PCR products were successfully amplified for cattle and buffalo respectively and the limit of detection was 0.12 ng for buffalo 0.47 ng for cattle

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(Vaithiyanathan & Kulkarni, 2016). Bai et al. (2009) developed a multiplex PCR assay for the detection of cattle, pig, chicken and horse meats by amplifying 292, 412, 239 and 451 bp fragment, respectively. The sensitivity of the assay was found to be 0.1 ng. Multiplex PCR was also developed for the analysis of feedstuff to detect the species commonly used in rendering plants namely, ruminant, pork, poultry and fish. To carry out the authentication 104, 290, 224 and 183 bp PCR products of the mitochondrial genes (16s rRNA for ruminant and 12S rRNA for others) were amplified with detection limit of 0.002% for ruminants, pork and poultry and 0.004% for fish (Dalmasso et al., 2004). Mitochondrial cytb gene was targeted for the amplification of 398 and 439 bp sequences to identify pig and horse respectively in a single assay platform (Di Pinto et al., 2005). He et al. (2015) optimized multiplex PCR technique to detect four different species including pork, beef, duck and mutton. The identification was carried out by using 212 (pork), 116 (beef), 322 (duck) and 177 (mutton) bp fragments from cytb, cytb, ND2 and 16S rRNA, respectively. Recently, Ali et al., (2015c) developed a multiplex PCR method for the simultaneous identification of five species forbidden in Halal (Islamic) foods, such as pig, dog, monkey, cat and rat. The targeted genes were mitochondrial cytb for cat, ATPase 6 for rat and dog and ND5 for monkey and pig, for the amplification of 172, 108, 163, 129 and 141 bp DNA fragments respectively. Multiplex PCR also extended for the verification of genetically modified organisms (GMOs) in food and feed (Germini et al., 2004).

Thus, multiplex PCR assay is highly promising and useful technique discriminatory power of identifying several species under complex matrices. Thus it can save both labor and time. On the other hand, simplex PCR assay needs several different assays since each set of species specific biomarkers are used separately (Zha, Xing , & Yang, 2011). However, all of these assays are based on single gene targeted and most of them

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are long DNA targeted which are not suitable for the analysis of highly degraded samples due to the breakdown of the target amplicon.

2.7.3.5 PCR- Randomly amplified polymorphic DNA (PCR-RAPD)

Randomly Amplified Polymorphic DNAs (RAPD) are the fragments of DNA that are amplified by PCR assay with the help of synthetic short oligonucleotide primers complementary to random sequence. Therefore, PCR-RAPD method involves in the simultaneously amplification of many distinct DNA fragments due to the randomly binding of the single arbitrary short primer (generally 10 bp) at the many different location on the genomic DNA followed by carry out the gel electrophoresis for the separation and visualization of the amplified products depending on their sizes (Fajardo et al., 2010; Hadrys, Balick, & Schierwater, 1992). Samples identification are accomplished by comparison the DNA bands profile according to the expectation depending on experimental conditions, primer and DNA used as the produced band pattern from amplified products are characteristics of the template DNA (Fajardo et al., 2010; Kumar & Gurusubramanian, 2011).

Arslan et al. (2005) used PCR-RAPD technique for the identification of various animal species in raw and processed meat products. The method successfully identified the cow, pig, sheep, goat, wild swine, camel, dog, cat, donkey and rabbit or bear species using a short (10 bp) primer. The method is also applicable for the detection of species origin in the 1:1 mix of raw minced meat from beef-sheep, horse-beef or sheep-pork. This method was also applied for the detection of ten meat species namely beef, buffalo, pig, wild boar, horse, cat, dog, venison, kangaroo and rabbit by producing fingerprint patterns using 10 bp containing 29 primers. Although, some primers of this method can generate district fingerprints for the differentiation of the species but other cannot distinguish the species origin (Koh, Lim, Chua, Chew, & Phang, 1998). Martinez et al.,

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(1998) applied this technique for the authentication of beef, buffalo, pork, goat, elk, mule, ostrich, donkey, reindeer, kangaroo, horse, and lamb species in the various meat products such as frozen red meat, sliced, salmoni and Lammerull. Another approaches of PCR-RAPD for the identification of four meat species including cattle, buffalo, sheep and goat (Calvo, Zaragoza, & Osta, 2001b). PCR-RAPD technique have some advantages including simple, rapid, eliminating more complex analytical steps and no need previous knowledge of the target DNA sequence (Fajardo et al., 2010). However, the main limitation of this method is reproducibility, in practice it is very difficult to produce reproducible amplified DNA band pattern (Arif et al., 2010; Koh et al., 1998). Furthermore, this technique is not applicable for the analysis of extremely processed meat and meat products, as highly purified DNA is mandatory for the reproducible RAPD patterns. In addition, PCR-RAPD method is not suitable for the identification of species in mixed samples containing more than one species (Fajardo et al., 2010).

2.7.3.6 PCR-restriction fragment length polymorphism (PCR-RFLP)

PCR restriction fragment length polymorphism (PCR-RFLP) is one of the most important molecular techniques accomplished by numerous researchers. The PCR-RFLP assays are especially interesting because they offer the opportunity to authenticate a product by restrictive digestion of the amplified PCR products using one or more restriction enzymes (REs) (Chen et al., 2010). Using the sequence variation that exists within a defined region of DNA, the differentiation of even closely related species is possible using a PCR-RFLP assay (Hsieh & Hwang, 2004). However, the PCR-RFLP technique is very simple and inexpensive and easily applicable in the routine analysis (Farag, Alagawany, Abd El-Hack, Tiwari, & Dhama, 2015).

Species-specific PCR assay is often conclusive (Ali, et al., 2015b) but it has yet to be considered a definitive analytical method because of certain “hard-to-control” features

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of the amplification process (Focke, Haase, & Fischer, 2010; Yang, Kim, Byun, & Park, 2005). For example, it sometimes produces artifacts due to contamination by alien DNA at a minute scale (Doosti, Dehkordi, & Rahimi, 2014; Yang et al., 2005), but these ambiguities or doubts could be eliminated by the verification of the amplified product through at least one of three different methods, namely, PCR-RFLP assay, probe hybridization, and target product sequencing (Maede, 2006). Probe hybridization is an attractive technique because it can detect multiple species in a single experimental run through the use of multiple labeled probes (Nascimento, de Albuquerque, Monesi, & Candido-Silva, 2010) but this procedure requires purified DNA and is also laborious, expensive, and time- consuming (Chen et al., 2010). In contrast, DNA sequencing is a more efficient and reliable tool, but it requires an expensive laboratory setup and is often not suitable for the analysis of processed food under complex matrices because of the coextraction of the food ingredients that often bring errors into the final results (Girish et al., 2004; Mafra et al., 2007).

On the contrary, the PCR-RFLP assay can overcome all of these limitations and has been widely used to authenticate the original PCR product amplified from a particular gene fragment (Park, Shin, Shin, Chung, & Chung, 2007; Sharma, Thind, Girish, & Sharma, 2008). It comprises the generations of a specific fragment profile through restriction digestion with one or two endonucleases. A carefully selected restriction endonuclease cleaves the PCR product at specific recognition sites, producing a set of DNA fragments of different lengths that could be separated and visualized by gel electrophoresis (Ballin, Vogensen, & Karlsson, 2009); thus, it distinguishes the artificial PCR product from the original through the analysis of the restriction fingerprints (Doosti et al., 2014; Times, 2015b).

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Such assays have been successfully applied to discriminate closely related species such as cattle, yak, and buffalo; pig and goat (Chen, Liu, & Yao, 2010); cattle-buffalo and sheep-goat (Girish et al., 2005); swine and wild boar (Mutalib et al., 2012); and various fish species (Nebola, Borilova, & Kasalova, 2010). Kumar et al., (2014) developed RFLP assay for the authentication of five most commonly used meat species namely cattle, buffalo, pig, sheep and goat. Two different REs (*Alu1* and *Taq1*) were used for the digestion of PCR products and distinctive digestion profiles allowed to differentiate each species. RFLP assays were also developed and applied on the PCR products of cat (Ali et al., 2015a), and dog (Rahman et al., 2015a). Besides this method, an universal primers set was designed from the mitochondrial cytb gene for the amplification of 359 bp DNA fragments from six species including pig, beef, buffalo, goat, chicken, rabbit and quail. The species were discriminated from the restriction digestion pattern generated by the digestion of five Res such as *BsaJI, AluI, BstUI, MseI and RsaI* (Murugaiah et al., 2009). However, these methods are mostly based on single and long-length DNA targets which break down under natural or environmental decomposition and food processing treatments, making them less trustworthy and inconclusive for forensic investigation (Bottero & Dalmasso , 2011; Focke et al., 2010).

2.7.3.7 Real-Time PCR

In contrast to conventional PCR assays, real-time PCR techniques are especially promising genetic tools for the authentication of meat products since they offer the opportunity of fast, greater resolution, target quantification, automation, reproducibility, high sensitivity and real-time monitoring (Cheng, He, Huang, Huang, & Zhou, 2014). Moreover, multiplex platform of the real-time PCR assays allow the distinct advantages over singleplex PCR methods of detecting multiple target oligos in a single reaction, lower the cost and labor, together with a time-saving feature (Ali et al., 2015c; Iwobi et al., 2015). Particularly, real-time PCR involves in the directly monitoring the generation

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of PCR products during each amplification cycle and able to measure at the exponential phase of the reaction there is no need to complete the reaction. Unlike end-point (conventional) PCR assay, this system allows quantifying the PCR products at an initial stage of the reaction that is more precise and accurate. As fluorescent molecules are used to collect the real-time data, since there is high correlation between intensity of the fluorescent dye and the quantity of PCR products (Fajardo et al., 2010). Two general categories of fluorescent chemistries namely, double-standard (ds) DNA-interrcalating dyes such as SYBR Green (Asing et al 2016) or Eva Green (Safder & Abasiyanik,2013) and probe based chemistry such as TaqMan (Ali et al., 2012) or Moleculer Becon (Hadjinicolaou, Demetriou, Emmanuel, Kakoyiannis, & Kostrikis, 2009) probes are available for the real-time PCR systems. The main drawback of the DNA-intercalating dye system is its bind non-specifically to all dsDNAs produced during the PCR reaction such as primer-dimers or any non-specific products, resulting in increased fluorescent background or false positive (Manit Arya et al., 2014). Moreover, some dyes are known to inhibit the PCR reaction (Gudnason, Dufva, Bang, & Wolff, 2007). In contrast, TaqMan probe based method is particularly promising since specifically-designed probe and primer sets significantly enhance the specificity and reliability of the assay (Ali et al., 2012). Because fluorescent signal is generated only when hybridize the specific probe due to the DNA polymerase moves by and cleaves off the probe's quencher molecule (Arya et al., 2014). In addition, TaqMan probe based techniques significantly facilitate to develop the multiplex real-time PCR assays (m-qPCR) because specific probes can be labeled with distinguishable and different reporter dyes which allows the identification of amplifications formed by one or multiple primer sets in a single PCR assay tube (Arya et al., 2014).

Several simplex and multiplex qPCR reports have been introduced for the identification and quantification species in food products. For example, a SYBR Green I

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oriented qPCR method was developed for the quantification of bovine milk adulteration in buffalo cheese products. The technique successfully identified the adulterated bovine milk in most of the marketed buffalo cheese samples (Lopparelli, 2007). SYBR Green fluorescence also used for the detection and quantification of bovine, porcine, caprine, goose, turkey, chicken, and equine (Okuma & Hellberg, 2015); pork (Soares, Amaral, Oliveira, & Mafra, 2013).

On the other hand, Safdar et al. (2014) used the EvaGreen fluorescence dye, to develop a duplex qPCR assay for the reliable and rapid detection of bovine and caprine species in ruminant feeds. The method was optimized under heat treated (133^0 C and 3 bar for 20 min) bovine and caprine admixed meat. Safdar et al. (2013) also introduced another Eva Green approaches for the discrimination of beef and soybean in sausages. Iwobi et al. (2015) introduced TaqMan based m-qPCR assay for the quantification of beef and pork in minced meat. The sensitivity of the method was 20 genome equivalents and the validation of the method was carried out on various marketed minced meat products. Another TaqMan based m-qPCR approach for the differentiation of bovine and buffalo in dairy samples. The method was validated by the analysis of commercial products with satisfactory results (Drummond et al., 2013). A TaqMan probe qPCR assay was reported for the authentication of species and gender origin of beef. This method consists of two reactions: bovine-specific qPCR and Y-chromosome–specific m-qPCR. The technique is highly powerful tool for the discrimination of beef gender (Herrero et al., 2013). TaqMan probe based mqPCR also applied for the quantification of pork, beef, sheep and horse (Köppel et al., 2011); pork, duck, chicken, goose and turkey (Köppel et al., 2013); red deer, sika deer and fallow deer (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015) and pig, chicken and duck (Cheng et al., 2014). Although numerous m-qPCR have been documented, but to the best of our

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knowledge, no m-qPCR assays have been documented for the simultaneously detection and quantification of cat, rabbit, rat and squirrel in food products.

2.8 Validation of PCR Method

(a) Definition

According to Taverniers et al. (2004) "Validating a method is investigating whether the analytical purpose of the method is achieved, which is obtaining analytical results with an acceptable uncertainty level". Subsequently, Green (1996) depicted "Method validation is the process of proving that an analytical method is acceptable for its intended purpose". To fulfill this definition, the PCR method need to be properly optimized, standardized and developed so that it can be adapted to accomplish performance characteristics that are consistent with the purpose of the assay. (World Organization for Animal Health, 2009).

(b) Practical Evaluation of Parameters and Acceptance Criteria

Various parameters of the PCR assay have to be tested to check the fitness of the method performance. A method can be accepted for routine analysis, if it complies with the predetermined criteria. During development and in-house validation of singleplex and multiplex PCR methods the following parameters need to be evaluated (Broeders et al., 2014).

i) Applicability

In the applicability statement, the developer should clearly describe the scope of the method with complete information, such as name of target species, which matrix is intended, or the amount of DNA have been analyzed. The method need to be assessed using several matrices namely, raw and processed materials, food and feed, and genomic DNA and plasmid DNA. Moreover, to detect the probable PCR inhibitors,

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different amounts of DNA can be analyzed. Reproducible results need to be produced for as many matrices as possible (Broeders et al., 2014).

ii) Practicability

To evaluate the practicability of the assay, blind samples need to be tested by the routine laboratory. Herein, new method can be run in combined combination with existing methods that had been already applied in the laboratory under the same conditions. To further evaluate the practicability, the developed method need to be transferred to a second laboratory to confirm the reproducible results (Broeders et al., 2014).

iii) Optimization and Standardization of Reagents and Determination of Critical Control Parameters

Collection and preparation of sample as well as DNA extraction procedures are all critical parameters in assay performance and should be optimized for good results. Appropriate DNA extraction methods vary depending on sample types. For example, extraction of DNA from raw meat samples is relatively easy, while that from complex matrices is more difficult. It is essential to develop an efficient and reproducible extraction method prior to perform further validation of the PCR assay. All apparatus used during validation process must be calibrated according proper protocols.

It is also important to determine the ability of the assay to remain unaffected due to slight variations in the main parameters during the development of the PCR method. To assess the critical parameters of the method it is essential to achieve an optimized PCR assay. Examples of such parameters include: concentration of $MgCl_2$, primers, buffer, dNTP and DNA Taq polymerase as well as annealing time and temperature. To identify the critical points that must be entirely be controlled in the assay, critical control parameters characterization is mandatory (Belak, & Thorén, 2004).

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iv) Repeatability

Compliance between replicates within and between runs of the qPCR assay must be considered. This provides significant information about the method before further validation is performed. If excessive inconsistency is found, it should be perfected prior to continue the validation process. To check the PCR assay repeatability, each replicate should be considered as an independent sample. For example, for a replicate (e.g. a triplicate), three different aliquots of DNA extract are prepared for a specimen and amplified, and the variation from the mean value detected is determined as an indication of repeatability. Therefore, use of single DNA extract to analyze triplicate amplifications in not acceptable. Inter-run coefficient of variation of the qPCR assay can be determined by using the Ct-values generated from the replicated samples (Belak, & Thorén, 2004).

v) Determination of Analytical Specificity and Sensitivity

Specificity of the PCR assay is defined as the ability of the system to discriminate the target species from other non-target species. The specificity of the assay is determined by analyzing DNA extract from target and genetically related species. Allowable cross-reactivity is mainly dependent on the desired purpose of the assay and must be determined for each case.

Limit of detection (LOD) or sensitivity of the assay is defined as the lowest quantity of DNA detected by the assay. Serially diluted extracted DNA is used until the assay can no longer detect the target in question in more than 5% of the replicates to determine the assay's sensitivity (Belak, & Thorén, 2004).

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vi) Establishing Reproducibility of the Assay

Reproducibility plays an important role to evaluate the assay precision. An identical method (reagents, protocol and controls) is applied in various laboratories to determine the assay reproducibility. At least three laboratories test results of the same set of specimens (minimum of 20 samples) with identical aliquots are required to validate the assay reproducibility as well as ruggedness of the assay (Belak, & Thorén, 2004). For DNA-based procedures, the following additional information should be supplied in particular (Codex Alimentarius Commission, 2010):

- **Primer pairs**

"General methods have to provide the defined primer pairs and the sequence they target. Recommendations as to the efficiency/use of primer set have to be clearly stated, including if the primers are suitable for screening and/or quantification".

- **Amplicon length**

"Food processing will generally lead to a degradation of target DNA. The length of the amplified product may influence the PCR performance. Therefore, the selection of shorter amplicon sizes (within reason) will increase the possibility to get a positive signal in the analysis of highly processed foodstuffs. In general, the length of the amplified fragment for the taxon-specific DNA sequence and the target sequence should be in a similar size range".

- **Whether the method is instrument or chemistry specific**

"At the moment a number of different types of real-time instruments and chemistries are available. These instruments and chemistries may have different performance such

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as stability of reagents, heating and cooling characteristics, which affects ramp rates and affects the time necessary for a whole PCR run".

"Beside the differences in the heating and cooling system there are differences in the technique and software used to induce and subsequently to record the fluorescence. The detection and quantification of the fluorescence could also vary according to the recording instruments and software used. Qualitative methods generally tend to be less instrument-specific than quantitative methods".

"The methods are generally instrument and chemistries dependent and cannot be transferred to other equipment and chemistries without evaluation and/or modification".

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CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection

Fresh muscle tissues were obtained from rabbit (*Oryctolagus cuniculus*), squirrel (*Callosciurus notatus*), chicken *(Gallas gallus*), beef (*Bos Taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hiscus*) pig (*Sus scrofa*), duck (*Anas platyrnychos*), pigeon (*Columba livia*), crocodile (*Crocodylus niloticus*), donkey (*Equus asinus*), amboina box turtle (*Cuora amboinensis*), chinese edible frog (*Hoplobatracus rugulosus*), deer (*Cervus nippon yesoensis*), dog (*Canis lupus familiaris*), cat (*Felis catus*), tuna (*Thunnus orientalis*) and salmon (*Salmo salar*). Specimens were also taken from four commonly used plant species, namely, wheat (*Triticum aestivum*), cucumber (*Cucumis sativus*), onion (*Allium cepa*), and chili (*Capsicum Capsicum annuum*). Where available, meat, fish and plant spices specimens were collected from commercial wet (Pudu Raya) and super markets (Aeon, Tesco and Giant) at Kuala Lumpur on three different days. Deer (*Cervus nippon*) meat was procured in triplicates from the Faculty of Veterinary Sciences at the University of Putra Malaysia, located at Serdang in Selangor. Stray dog *(Canis lupus familiaris)*, cat (*Felis catus*) and rat (*Rattus rattus*) muscle were donated by Kuala Lumpur City Hall (KLCH/DBKL) at Air Panas in Kuala Lumpur. Monkey (*Macaca fascicularis sp)* meat was a gift from the Department of Wildlife and National Park Malaysia (DWNPM/ PERHILITAN) at Cheras in Kuala Lumpur. I would like to note that DBKL routinely kills rats, cats and stray dogs for population control and public security purposes in the town area; so no animals were killed for this study purposes but sufficient amount of muscle tissue samples were taken from the already killed animals following institutional and country laws. Details information of all the collected samples is given in Table 3.1. Commercial beef and chicken meatballs, frankfuter and burger of different brands were purchased in 3 samples, each from different stores in Kuala Lumpur, Malaysia. All samples were

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transported under ice-chilled conditions and were cut into the smallest possible pieces with surgical blades prior to storage at -20°C until further use.

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Table 3.1: Information of collected food samples

	Species	Sources	Geographic coordinates of the sources	Animal Sources	Sample	Number of samples
1	Squirrel	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	30
2	Rat	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	Dead	Meat	30
3	Rabbit	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	30
4	Chicken	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	30
5	Cow	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	30
6	Goat	Tesco supermarket	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	25
7	Pig	Wet market	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	20
8	Pigeon	AEON supermarket	PJ old town, Pataling jaya, Kuala Lumpur, Malaysia	Dead	Meat	20
9	Sheep	Wet market	PJ old town, Pataling jaya, Kuala Lumpur, Malaysia	Dead	Meat	25
10	Duck	Wet market	PJ old town, Pataling jaya, Kuala Lumpur, Malaysia	Dead	Meat	20
11	Buffalo	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	30
12	Crocodile	Purl point shopping centre	Old klang road, Selangor, Malaysia	Dead	Meat	25
13	Turtle	AEON supermarket	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	25
14	Donkey	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	25
15	Deer	Veterinary Department	Kuala Lumpur, Malaysia	Dead	Powder	30
16	Monkey	Wildlife and National Parks (DWNP)	Cheras, Kuala Lumpur, Peninsular Malaysia,	Dead	Meat	20
17	Dog	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	Dead	Meat	15

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Table: 3.1, continued

	Species	Sources	Geographic coordinates of the sources	Animal Sources	Sample	Number of samples
18	Cat	Dewan Bandaraya Kuala Lumpur (DBKL)	Kuala Lumpur, Malaysia	Dead	Meat	15
19	Chines frog	Wet market	Paser Borong, Pudu Raya and Selangor, Malaysia	Dead	Meat	15
20	Tuna	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	15
21	Salmon	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	Dead	Meat	15
22	Wheat	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	Powder	Fresh vegetable	1-2 kg
23	Cucumber	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Fresh vegetable	1-2 kg
24	Onion	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Fresh vegetable	1-2 kg
25	Chili	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Fresh vegetable	1-2 kg
26	Chicken Frankfurter, meatball, burger (Ramly)	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Meat products	2-3 Kg
27	Chicken Frankfurter, meatball, burger (Tesco)	Tesco supermarket	Kuala Lumpur, Malaysia	-	Meat products	3 Kg
28	Chicken Frankfurter, meatball, burger (Ayamas)	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Meat products	2 Kg
29	Chicken Frankfurter, meatball, burger (Prima)	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Meat products	2 Kg
30	Beef Frankfurter, meatball, burger (Ramly)	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Meat products	3Kg
31	Beef Frankfurter, meatball, burger (Figo Foods)	AEON BIG supermarket	Midvally, Kuala Lumpur, Malaysia	-	Meat products	2 Kg
32	Beef Frankfurter (Saudi Gold)	Tesco Supermarket	Kuala Lumpur, Malaysia	-	Meat products	3.5 Kg
33	Beef Frankfurter, meatball, burger (Farm's Best)	Tesco Supermarket	Kuala Lumpur, Malaysia	-	Meat products	3 Kg

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3.2 DNA Extraction

Total DNA was extracted from 30 mg of muscle tissue of each meat and fish species as well their admixed meat products using a Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan). Plant DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN GmgH, Hilden, Germany). DNA from commercial meatballs was extracted using NucleoSpin Food DNA kit (MACHEREY-NAGEL, GmbH & Co., Duren, Germany). The purity and concentration of all extracted DNA was determined using a UV-VIS spectrophotometer (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK) based on absorbance at A260/280 and calculated the ratios. All extracted DNAs were kept at -20 °C until further uses.

3.3 Development of Biomarker for Multiplex PCR

Proper design of primers is a vital factor for an efficient and successful PCR amplification. Higher efficiency and maximum specificity of PCR depends on the optimal matching of primer sequences and also adequate primer concentrations (He, Soini, Mertsola, & Viljanen, 1994). An inaccurately designed primers may lead to little product or formation of primer-dimer and/ or non-specific products (Abd-Elsalam, 2003). The development of multiplex PCR primer sets is more complex and complicated because all primers are annealed to their respective targets under a single set of PCR conditions. Specificity and Tm are also more important in a multiplex system over the conventional PCR (Vieux, Kwok, & Miller, 2002). In addition, PCR products length (amplicon size) should also be taken in account during the design of primers. The size of the amplicon depends on the resolution capability of the detection system, so that generated PCR products can distinguish easily from one another.

In order to design of the primers, whole genome sequences of the target species were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned using

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Molecular Evolutionary Genetics Analysis verson 5 (MEGA5) alignment tool (Tamura et al, 2011) for identifying the inter-species hyper-variable and intra-species conserved regions. A publicity available primer designing software, primer 3Plus was used to get the designed sequence of the primers.

The following criteria and guidelines were considered for the design of species specific primers for amplifying specific target sequence:

3.3.1 Primer Length

The length of primer plays an important role for the specificity as well as annealing time and temperature for the target binding; these parameters are for a successful PCR (Wu, Ugooli, Pal, Qioan, & Wallace, 1991). Too long primers may decrease the efficiency of template DNA binding at normal annealing temperature due to the chance of forming secondary structure; whereas, too short primers may be result in low specificity and non-specific amplification (Abd-Elsalam, 2003). The ideal primer length should be 18-28 nucleotide, but usually good activity is obtained with primer having 20-24 nucleotide in length (Dieffenbach, Lowe, & Dveksler, 1993).

3.3.2 GC Content

One of the most important characteristic of primer is its GC content which refers to its annealing strength. To get good PCR product, a reasonable GC content should be maintained. Tm and annealing temperature (Ta) fully depend on the percentage (%) of GC content (Rychlik, Spencer, & Rhoads, 1990). The ideal GC content is 40-60% and 3 or more G's or C's at the 3-'end should be avoided because it has adverse effect to the primer specificity. Primer should not have long poly G or poly C stretches which result in non-specific annealing (Dieffenbach, Lowe, & Dveksler, 1993).

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3.3.3 Melting and Annealing Temperature

Melting temperature (Tm) is an important parameter of primer, since it plays a vital role for primer annealing. Primer with Tm of 55-65 °C work best in most of amplification reactions. Tm of both forward and reverse primers should have similar as they are annealed simultaneously. Moreover, multiplex PCR efficiency is affected by a little difference of Tm between the primer sets. Since all targets are amplified in a multiplex PCR in a single reaction mixture, all primers should have very close Tm. The acceptable Tm variation is 3-5°C between the primers but to get good result $\leq$ 2°C Tm variation is preferable. Significantly lower Tm of primer than the PCR annealing temperature (Ta) may cause failure to anneal and extend, while significant higher Tm may lead to non- hybridization and can extend at an incorrect location along the DNA sequence (Ali et al., 2014b). The approximate Tm value of the primer can be calculated by using the formula (generally valid for oligos in the 18-30 base range) of Wallace et al (1979) Tm (°C) = 2x (n A+n T) + 4x (nG+nC), where nA, nT, nG, and nC are the number of respective nucleotide in the primer. Mismatching between template DNA and primers is the main feature for specificity and Tm of the designed primers. Because Tm value reduce by 1-1.5 °C for 1% mismatching of the bases in a double-stranded (ds) DNA (Matsunaga et al., 1999). However, the increasing of percent of mismatching with non-target species leads to the decrease of Tm value, but higher specificity. On the other hand, the Tm of the TaqMan probes of the real-time PCR must be 8-10 °C degree higher than that of primers to facilitate the preferential binding of the probes prior to the annealing of the primers to the template (Arya et al., 2005).

3.3.4 3`-end Specificity

For the design of primer to achieve a successful PCR experiment, 3'-end sequence is very important because during the extension step, DNA polymerase state to attach nucleotide from the 3'-end of a primer. Since, complete annealing of the primer 3’-end is

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mandatory and incomplete binding at the 3'-end result in lower PCR or often no PCR products (Yuryev, 2007). Therefore, primers should have mismatch with non-target species at 3'-end, as it prohibits the PCR amplification (Ali et al., 2014). It is well known that for the control of mis-priming, the 3'-end position of the primer plays an important role (Kwok et al., 1990).

3.3.5 Primer-Primer Interactions

Primer should have a minimum of intermolecular or intramolecular homology that can promote to the formation of each primer dimerization or heparins. Primers with nucleotide sequence that would allow anneal one primer to other primer (s), result in primer-dimer formation, particularly when 3'-end of the primers anneal to the each other. Inter primer homology in the middle position of two primers may also interfere with hybridization. Primer with a self-homology region result in "sanp back" or able to form partially double stranded structures, hairpin , which will interfere with annealing to the template. To overcome the formation of hairpin, it is recommended that intra-primer homology of 3 bp or more should be avoided (Abd-Elsalam, 2003) .

3.3.6 Specificity

Primer specificity is checked in three different ways. At first, primers were aligned by using online Basic Local Alignment Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.ctg) to screen the identical and distant species. Secondly, to determine the total number of mismatch between target and non-target species, the primers are multiple sequences aligned with some common species using an alignment tool such as ClusterW (http:www.genome.ip/tools/clustalW/) or MEGA5. Finally, each primer is assayed in experiment with template DNA of non-target species to confirm the specificity.

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3.3.7 Design of Oligonucleotide Primers

Species-specific primers of three target species (rabbit, rat and squirrel) were designed by targeting mitochondrial Cytb and ATP6 genes that are well protected by multilayer mitochondrial membrane (Xin et al., 2006). Usually, mitochondrial genes offer higher degree of divergence but sufficient conserved regions within the species due to their maternally inheritance and availability in thousands of copies per cell (Xin et al., 2006). Previously, cytb and ATP6 genes were also used to study inter and intra species discrimination (Brown, George, & Wilson, 1979; Thitika Kitpipit et al., 2014). The complete genome sequences of each species were retrieved form the National Centre of Biotechnology Information (NCBI). The MEGA5 (version 5.2) and ClustalW sequence alignment tools were used to identify the hyper-variable and conserved regions to study the presence of mismatched nucleotides (Tamura et al., 2011). The species specific conserved regions were used to design three sets of species-specific primers for rabbit, rat and squirrel species, using Primer 3Plus software. This software ensured the Tm value, GC% and accuracy of the primer. The theoretical specificity of the designed primers was confirmed by Basic Local Alignment Tool (BLAST) in NCBI. Total mismatch between the target and non-target species were determined by aligning the primer's sequences against 22 different non-target species (Tamura et al., 2011). One set of primers that amplified a 141-bp site of eukaryotic 18S rRNA gene were used as internal positive control (IAC) (Fajardo, González, Martín, Hernández, et al., 2008). The designed primers were supplied by the First Base (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) and kept at -20 °C until uses. All information about primers could be found in Table 3.2

Table 3.2: Information about primers used in the study

Species	Target gene	Sequence (5'-3')	Tm value	Amplicon size (bp)	Reference
Squirrel	Cytb	Forward-ATCTCCCCACTCCTTCCAAT Reverse- CGCGGCCTACATGTAAGAAT	59.8 °C 60.1 °C	243	This study
Rabbit	Cytb	Forward- TCCGATACCTCCACGCTAAC Reverse- GGAGGATGATGCCAATGTTTC	60.1 °C 61.6 °C	123	This study
Rat	ATP6	Forward- CATCATCAGAACGCCTTATTAGC Reverse- AGGTTCGTCCTTTTGGTGTATG	60.1 °C 60.3 °C	108	This study
Endogenous Control	18SrRNA	Forward GTAGTGACGAAAAATAACAATACAGGAC Reverse ATACGCTATTGGAGCTGGAATTACC		141	Fajardo et al. 2008

3.3.8 Construction of Pairwise Distance and Phylogenetic Tree

The pairwise distance and phylogenetic tree were constructed by aligning each amplicon sequence with the respective gene sequence of target and 45 non-target species using the neighbor-joining method of MEGA 5 software (Tamura et al., 2011). For example, the sequence of rabbit cytb amplicon was aligned along with the cytb gene of rabbit and other 45 non target species such as. Pairwise distance of other two amplicons were constructed in the same way.

3.3.9 Construction of 3D Plot

3D plot of each primer set was generated from three variables such as forward and reversed primer mismatch (section 3.3.7) and pairwise distance (Section 3.3.8) data of individual primer sets using XLSTAT 2014 software (Addinsoft, 2013).

3.4 Development of Simplex PCR Assay

3.4.1 Optimization of Simplex PCR Assay

Simplex PCR of individual primer was developed using DNA extracted from muscle tissue of relevant species (squirrel, rat and rabbit). Total volume of all amplification assays was performed in a 25 µL reaction mixture comprising of 5 µL 5X GoTaq Flexi Buffer, 0.2 mM each of dNTPs, 2.5 mM $MgCl_2$, 0.9 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, United States), 0.2 - 0.4 µM of each primer and 2 µL (20 ng/µL) DNA template. Negative control (PCR amplification without template DNA) was

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carried out for each PCR reaction to avoid any contamination with PCR mixture. In the simplex PCR specificity test, we also used 0.4 µl a universal eukaryotic primer (forward primer: 5'GGTAGTGACGAAAAATAACAATACAGGAC 3' and reverse primer: 5' ATACGCTATTGGAGCTGGAATTACC 3') of 141 bp amplicon sized from 18S rRNA gene (Muhammad Safdar & Junejo, 2015b). ABI 96 well verity Thermal Cycler (Appiled Biosystems, Foster City, CA, USA) was used for the PCR reaction following the cycling parameters of an initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 57-59 °C for 30 - 35 s, extension at 72 °C for 40 s and the final extension at 72 °C for 5 min (Table3.4). PCR products were kept at -20°C for further analysis.

Table 3.3: Concentration of simplex PCR components

Primer	dNTP (mM)	MgCl2(mM)	Taq pol (unit)	Primer (uM)
Squirrel	0.2	2.5	0.9	0.4
Rat	0.2	2.5	0.9	0.3
Rabbit	0.2	2.5	0.9	0.2

Table 3.4: Cycling parameters of simplex PCR reactions

PCR reaction	Initial denaturation	35 cycles			Final extension
		Denaturation	Anneling	Extension	
squirrel	94 °C for 3 min	95 °C for 3 s	58 °C for 35s	72°C for 40s	72°C for 5 min
Rabbit	95 °C for 3 min	95 °C for 3 s	57°C for 35s	72°C for 40s	72°C for 5 min
Rat	95 °C for 3 min	95 °C for 3 s	59°C for 35s	72°C for 40s	72°C for 5 min

3.4.2 Gel Electrophoresis

In order to detection of species- specific simplex PCR amplified products, DNA visualization was accomplished by conventional gel electrophoresis and finally on automatic QIAxcel Advanced Capillary Electrophoresis System.

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3.4.2.1 Conventional Gel Electrophoresis

To perform the conventional gel electrophoresis, 2% (w/v) agarose gel was prepared as follows:

150 ml of 1 X Tris-borate-EDTA (TBE) buffer was taken in a 250 ml beaker subsequently added 3 g of agarose in the buffer and mixed well followed by heating in a microwave oven to dissolve completely. After reaching the gel temperature at about 50-60°C, 5-6 µl of Florosafe DNA stain (1st Base Laboratories, Selangor, Malaysia) was added and mixed gently folloded by molton gel mixture was cast in a horizontal electrophoresis try containing the well comb and wait for 20-30 min to solidify the gel. After placing the gel tray inside the 1x TBE buffer containing tank, 6 µl PCR products and 50 bp DNA ladder (Promega, USA) were loaded into the gel wells. After that, the gel electrophoresis (SUB13, Hoefer, Inc., California, USA) was carried out at 120 volts for about 90 min resulting the PCR products were separated on the basis of moleculer size. Finally, the PCR products banding profile was visualized under a gel image documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA)

3.4.2.2 QIAxcel Advanced Capillary Electrophoresis System

The QIAxcel Advanced Capillarly Electrophoresis System offer rapid, fully automatic, very sensitive and high resolution (can separate the products with 3-5 bp differences), required low amount of sample and convenience due to the use of ready- to-use gel cartridge. Due to the automatic system, minimum handling interaction is required for sample analysis resulting in little manual error and excluding the laborious gel preparation. The system is able to generate both gel image and electropherograms of the analyzed sample in a single analysis platform by applying the electrical current to a gel-filled capillary cartridge via individual electrode of each capillary. Unlike conventional

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gel electrophoresis, electropherograms of the PCR products can determine the accurate sizes of the amplicon.

3.4.3 Specificity Test of Simplex PCR Assay

Specificity of the simplex PCR assays were analyzed by cross-amplification with the extracted DNA of three target species (squirrel, rat and rabbit), 22 non- target of terrestrial and aquatic animal species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, turkey, chinese frog, tuna, salmon species) and 4 plant species (wheat, cucumber, onion, chili) which are commonly used in food products. In the simplex PCR specificity test, I also used 0.4 µl a universal eukaryotic primer of 141 bp amplicon sized from 18S rRNA gene.

3.4.4 PCR product sequencing

Extracted DNA of three target species (rabbit, rat and squirrel) were amplified using specific primer sets and were visualized on agarose gel. The amplified products were sequenced after cloning into the pJet1.2 blunted Vector (Integrated DNA Technologies (IDT), Singapore). Briefly, the blunt-end of the purified PCR products was ligated into the cloning site of pJet1.2 blunted Vector by proofreading DNA polymerases and the recombinant plasmid was introduced into living *E. coli* cells. The lethal gene of the vector was disrupted by the insertion of PCR product and thus the propagation of only the recombinant plasmid containing bacterial cells was facilitated because the plasmid was contained in transcription promoter T7. A single transformation colony of the recombinant plasmid containing cells is produced due to the expression of ampicillin-resistance gene that was encoded into the plasmid. After purification, the recombinant plasmid containing insert was separated by digestion with restriction enzyme. Finally, the PCR products were sequenced to determine the original order of the nucleotides in the amplified PCR products. The derived sequences were then compared with GenBank

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sequences by nucleotide basic local alignment search tool (BLAST) to evaluate potential species matching and also aligned with specific gene sequence using MEGA5 software to determine the similarity with specific species.

3.5 Development of Multiplex PCR Assay

3.5.1 Multiplex PCR optimization

After simplex PCR optimization, the duplex and finally multiplex PCR were developed step by step. All amplifications were perfumed in a total volume of 25 ul containing 5 µl of 5X Go Taq Flexi Buffer in Thermal cycler (Applied Biosystems, Foster City, CA, United States). The concentration of primer and other reagent and cycling parameter were given below in the Table 3.5 and Table 3.6 respectively. In each reaction IAC was used to eliminate the false negative detection. All PCRs were performed in an ABI 96 Well Verity Thermal Cycler (Applied Biosystems, Foster City, CA, United States). PCR products were visualized in 2% agarose gel stained with Florosafe DNA stain (First Base Laboratories Sdn. Bhd., Selangor, Malaysia) under a gel documentation system (AlphaImager HP, Alpha Innotech Corp., California, United States).

Table 3.5: Concentration of PCR components of various PCR Assays.

PCR	dNTP (mM)	MgCl2(mM)	Taq pol (Unit)	Primer (µM)
Simplex	0.2	2.5	0.9	0.2-0.4
Duplex	0.25	3.5	1.0	0.12-0.4
Multiplex	0.25	4.0	1.25	0.12-0.7

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Table 3.6: Cycling parameter of various PCR reactions.

PCR	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Simplex	94 °C for 3 minutes	95 °C for 30 seconds	58 °C for 30 seconds	72 °C for 45 seconds	72 °C for 5 minutes
Duplex	94 °C for 3 minutes	95 °C for 45 seconds	58 °C for 60 seconds	72 °C for 45seconds	72 °C for 5 minutes
Multiplex	95 °C for 3 minutes	95 °C for 45 seconds	58 °C for 60 seconds	72 °C for 60 seconds	72 °C for 5 minutes

3.5.2 Specificity Test of Multiplex PCR Assay

The specificity of the designed primers was checked by three different procedures that complement one another. Initially, the primer sequences were BLAST (Basic Local Alignment Tool) against non-redundant nucleic acid sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to screen the similar and distant species. Secondly, the primers were multiple aligned with 45 target and non-target species, using an online ClustalW software (http://www.genome.jp/tools/ clustalw/) to measure the total mismatch between the target and non-target species. Finally, the practical PCR assay was run against the DNA of 22 different non-target species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili) to confirm the theoretical specificity (Morf et al., 2013). The pairwise distances were calculated using MEGA5 and a phylogenetic tree was constructed to study the maximum and minimum genetic distances between the target and non-target species.

3.5.3 Limit of Detection of Multiplex PCR Assay

The sensitivity of the assay was determined under pure states. The DNA was extraction from the pure meat tissue of target species (squirrel, rabbit and rat). The DNA concentration was adjusted to 10 ng/ul which was 10-fold serially diluted to 1 ng, 0.1

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ng, 0.01 ng, 0.001 ng, 0.0001 ng. The lower limit of detection (LOD) was determined using serially diluted DNA extraction.

3.5.4 Stability Evaluation

The pure meat sample of the targets species were cooked individually in three different ways: firstly, approximately 8 - 10 g of each meat sample was cooked separately at 100 °C in a water bath for 60, 90, 120 and 150 min to simulate traditional cooking; secondly, meat samples were extremely autoclaved at 121 °C under 15 psi for 2.5 h to simulate canning process and finally, they were cooked in a micro oven at 500, 600 and 700 W for 30 min to simulate modern cooking practices (Table 3.7). All the heat-treated samples were stored at -20 °C until further uses. About 30 mg of the heat-treated samples were used for DNA extraction and the purified DNA was kept at -20 °C until further uses.

Table 3.7: Different thermal processes applied to target meat samples.

Heat Treatment	Condition	Time (min)	Pressure (psi)	References
Boiling	98°C	90	-	Ali et al., 2015b
Autoclave	121°C	20, 150	-	Ali et al., 2015d
Microwave	500, 600, 700 W	30	15	Ali et al., 2015b

3.5.5 Sensitivity Test of the Multiplex PCR Assay under Meat Admix (meatball)

To simulate the adulteration effect on commercial meat products, the chicken meat ball was prepared following Rohman et al. (2011) Typically, each type of meatball was made by adding the following ingredients: 500g of minced meat, 1g of egg, 1 g of chopped onion, one teaspoon of cumin seed, ¼ teaspoon of cayenne pepper and 6g of finely chopped sun-dried tomato and mixed well. A negative control was prepared using only pure ground chicken meat blended with a blender (Panasonic Super Blender- PB-3205, 13600 Prai, Penang Malaysia) along with a rational amount of fats and other

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culinary ingredients (Table 3.8). Two types of positive controls were made; the first type was made by spiking 0.1% of each target species individually and the second type included all the three targets (rabbit, rat and squirrel) species at 1 %, 0.5% and 0.01% in chicken meatballs. The prepared meatball was placed in a fridge for 1 h to firm up. All the prepared meat products were subjected to autoclaving at 120 °C under a pressure of 15 psi for 2.5 h and were stored at −20 °C for further DNA uses.

Table 3.8: Model meatball formulation.

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

[a] 1%, 0.5, 0.1% and 0.01% of deboned rabbit, rat and squirrel meats were mixed with a balanced amount of deboned chicken in total of 100 g specimen, chopped, mixed and minced prior to make meatball.

3.5.6 Sensitivity Test of Multiplex PCR Assay under Meat Admixture (Frankfuter)

For the screening of commercial products using developed mPCR system, two types of ready to eat model frankfuter chicken and beef were prepared as per Ali et al. (2014a). The prepared beef and chicken products were deliberately adulterated by spiking of 1%, 0.5% and 0.1% of target species (rabbit, rat and squirrel) (Table 3.9).

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Thus, frankfurters spiked with 0.1% of target species were autoclaved at 121°C for 2.5 h under 15-psi pressure. All samples were stored at -20°C until DNA was extracted.

Table 3.9: Composition of model frankfurters used in this study.

Ingredient	chicken	beef	squirrel	rat	rabbit
Minced meat	45[a]	45[a]	45	45	45
Soy protein	7.5	7.5	7.5	7.5	7.5
Starch/breadcrumb	6.5	6.5	6.5	6.5	6.5
Chopped onion	2.5	2.5	2.5	2.5	2.5
Chopped ginger	0.15	0.15	0.15	0.15	0.15
Cumin powder	0.75	0.75	0.75	0.75	0.75
Garlic powder	0.5	0.5	0.5	0.5	0.5
Black pepper	0.23	0.23	0.23	0.23	0.23
Tomato pest	2.0	2.0	2.0	2.0	2.0
butter	2.5	2.5	2.5	2.5	2.5
salt	SA	SA	SA	SA	SA
othersb	SA	SA	SA	SA	SA

[a]To prepare ≥70 g frankfurter specimens,10%, 1%, , and 0.1% of squirrel, rat, and rabbit were mixed with a balanced amount of respective minced meat. [b]Flavoring agents and enhancers. [c]SA, suitable amounts

3.6 Enzymatic Digestion and RFLP Analysis

The amplicon sequence of each target species was retrieved from NCBI data base and was checked with NEB Curtter Version 2.0 software to find out the restriction sites. Based on the software assessment, two restriction endonucleases were chosen for RFLP analysis that cut only the target amplicon but did not have any restriction sites in IAC. *Bts*CI was selected for squirrel (243 bp) and rabbit (123 bp) amplicon; but *Bts*I*Mut*I was chosen for rat (108 bp) amplicon (Table 3.10). The restriction digestion was performed in a reaction mixture composed of 15 µL unpurified PCR product from each of the simplex PCRs, 2.5 µL buffer, 1 µL of RE and 0.25 µL BSA and balance amount of nuclease free water. The reaction mixture was gently mixed and spun down and incubated at 37°C first for 60 min for *BtsC*I digestion and then 55°C for another 60 min for *Bts*I*Mut*I digestion in a shaking water bath. Finally, the reaction mixture was heated at 80°C for 20 min to denature the enzymes and stop restriction digestion shown in

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Table 3.11. However, for multiplex PCR, restriction digestion was executed in 25 µL reaction mixture having 15 µL unpurified PCR products, 2.5 µL digestion buffer, 2.5 µL *BtsC*I enzyme and 1.5 µL *Bts*I*Mut*I enzyme. The digested PCR product was visualized using a QIAxel DNA High Resolution Kit in an automated QIAxel Advanced Capillary Elecrophoresis System (QIAGEN GmbH, Hilden, Germany).

Table 3.10: Restriction digests of the PCR products.

Target species	Amplicon size	Restriction enzyme	Restriction site	Fragment size
Squirrel	243 bp	BtsCI	GGATG▲NN▼	176 bp, 67 bp
Rabbit	123 bp	BtsCI	GGATG▲NN▼	115 bp, 8 bp
Rat	108 bp	BtsIMutI	CAGTG▲NN▼	64 bp, 44 bp

Table 3.11: Restriction enzyme reaction conditions for the digestion of simplex PCR product.

Target	Restriction enzyme	Amount of PCR product	Incubation temp. and time	Deactivation temp. and time
squirrel	BtsCI	1.0	37 °C for 60 mim	80°C for 20 min
rabbit	BtsCI BtsIMutI	1.0	37 °C for 60 mim	80°C for 20 min
rat	BtsIMutI	1.0	55 °C for 60 mim	80°C for 20 min

3.6.1 Authentication of PCR Products of Frankfurters by RFLP Analysis

To authenticate the three PCR products of rabbit, rat and squirrel (cytb and ATP6) by RFLP analysis, chicken and beef frankfurters were adulterated by spiking of 10% of target species (rabbit, rat and squirrel) and were heat treated by boiling at 100 °C for 90 minutes and autoclaving at 121°C under 15 psi pressure for 2.5 h. and RFLP analysis was performed.

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3.7 Multiplex (pentaplex) Real Time PCR Assay

3.7.1 Primers and Probes Design

The oligonucleotide primers and probes used in the present study were designed targeting mitochondrial cytb gene of squirrel, rat, rabbit and cat (Table 3.12). The 5′ and 3′ ends of each probe for squirrel, rat, rabbit and cat were labeled with ROX and TAO/3IAbRQSp; HEX and ZEN/3IABkFQ; Cy5 and TAO/3IAbRQSp; and TAMN and TAO/3IAbRQSp, respectively. Eukaryotic 18S rRNA specific primers and TaqMan probe were used as internal amplification control (IAC) for the normalization and specificity test of the developed multiplex (pentaplex) qPCR assay.(Ali, et al., 2012d) The IAC probe was labeled with FAM at the 5' end and ZAN/IOWA BLACK FQ at the 3'end (Table 3.12). The designed primers and probes were supplied by Integrated DNA technologies (IDT), Singapore.

Table 3.12: Sequence and Concentration of Primer and Probes Used in This Study.

Species	Target gene	Sequence (5'-3')	Tm value	Amplicon Size (bp)	Reference
Squirrel	Cytb	Forward- TCCGACCTCTAAGCCAATG Reverse ACTAACAGCTGGCATAAATAGAAGG Prob5'-56-ROXN/GCCTGTAGA/TAO/ATACCCCTTTATCACAATCGG/3IAbRQSp/-3'	58.8 °C 59.3 °C 69.3 °C	161 bp	this study
Rabbit	Cytb	Forward- TCCGATACCTCCACGCTAAC Reverse- GGAGGATGATGCCAATGTTTC Prob-5'-/5Cy5/GTAGGCCGC/TAO/GGAATCTACTATGGATCATAC/3IAbRQSp/-3'	60.1 °C 61.6 °C 69.4 °C	123	this study
Rat	ATP6	Forward- CATCATCAGAACGCCTTATTAGC Reverse- AGGTTCGTCCTTTTGGTGTATG Prob-5'-/5HEX/CGCCTCCAC/ZEN/ACATTTCAACACTGACTAAT/3IABkFQ/-3'	60.1 °C 60.3 °C 69.6 °C	108	this study
Cat	Cytb	Forward- GGAATAATGTTTCGACCACTAAGC Reverse- TGCCTGAGATGGGTATTAGGAT Prob- 5'-/56-TAMN/TCTGACTCCT/TAO/AGTAGCGGATCTCCTAACCC/3IAbRQSp/-3	60.3 °C 59.8 °C 69.1 °C	172	this study
Endogenous Control	18srRNA	Forward-GGTAGTGACGAAAAATAACAATACAGGAC Reverse-ATACGCTATTGGAGCTGGAATTACC Prob-FAM-AAGTGGACTCATTCCAATTACAGGGCCT-ZEN/IOWA BLACK FQ		141	Ali et al.2012

Table 3.13: Concentration of the primer and probes used the qPCR assay.

Target	Primer (nM)	Probe (nM)
squirrel	500	150
rat	250	100
rabbit	250	100
cat	250	100
IAC	150	100

3.7.2 Conditions Applied for multiplex (pentaplex) Real-Time PCR.

Multiplex real-time PCR assay of squirrel, rat, rabbit, cat and IAC were carried out in a Quant Studio 12 K flex real time PCR system in a 20 µL reaction volume, containing Prime time Gene Expression Master Mix (2X), Primer and probes (IDT.USA), total DNA template of each target species and nuclease free water. In the total volume of reaction mixture, the concentration of each species DNA template and master mix were 30ng/µl and 1X, respectively. The primers and probes information is given in

Table 3.13. The amplification was performed using an initial denaturation step at 95°C for 20s and annealing and extraction at 57°C for 60s.

3.7.3 Specificity Test.

The specificity of the developed real time multiplex PCR assay was tested against 22 non-target species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, turkey, chinese frog, tuna, salmon species, wheat, cucumber, onion, chili). The multiplex qPCR and reference PCR system were carried out simultaneously to determine the specificity of the developed system.

3.7.4 Limit of Detection.

In order to determine the limit of detection (LOD), the multiplex qPCR assay was calibrated with a serially diluted DNA extract from a mixture of equal amounts of squirrel rat, rabbit, and cat meat. The mixture of extracted DNA which was consisted of

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1:1:1:1 ratio of each target species was adjusted to the concentration of 30 ng/µl. After that, the DNA mixture was 10-fold serially diluted [1] and the concentration of the diluted DNA samples were 3, 0.3, 0.03, 0.003 ng. In this assay, 4 µl of DNA mixture from each diluted sample was added to the 20 ul reaction mixture. As a result, the final volume of each reaction mixture contained the same amounts of DNA from all target species and the serially diluted five reaction mixture contained the 30, 3, 0,3 ,0.03,0.003 ng of DNA, respectively. The multiplex qPCR of each dilution was assayed in 5 replicates.

3.7.5 Generation of Standard Curve

Standard curve was constructed to determine qPCR efficiency and quantify PCR targets. In order to generate the standard curve of the multiplex qPCR system for squirrel, rat, rabbit, cat and IAC, the DNA was extracted from the admixture (1:1:1:1) of squirrel, rat, rabbit and cat. The mixed DNA template contained the equal amount of DNA from all target species and the concentration of the DNA mixture was adjusted to the 30 ng/µl. After that it was 10-fold serially diluted to 3.0.3, 0.03, 0.003 ng/µl with nuclease free water. This resulted in mixtures containing 100% to 0.001% of DNA from each species. So 4 µl of each diluted DNA was added to 20 µl of reaction mixture. After performing the multiplex qPCR assay, the Ct values of each target species was plotted against the logarithmic concentration of DNA from each species. (Ali, et al., 2012; Cheng et al., 2014; Iwobi et al., 2015). Subsequently, the standard curve was built up and the efficiency of the assay was calculated based on the slope of the curve according to the equation (Druml, Mayer, Cichna-Markl, & Hochegger, 2015) as stated below:

$$\mathrm{E}(\%) = [10(-1/\mathrm{slope}) - 1]\,\mathrm{x}100 \quad (3.1)$$

The acceptance range of qPCR efficiency was between 90 and 110% that corresponded to a regression slope between -3.1 and -3.6 and an R^2 value of ≥ 0.98. (Iwobi et al., 2015)

The quantity of squirrel, rat, rabbit and cat in unknown specimens was determined based on respective Ct (López-Calleja, Cruz, González, García, & Martín, 2016) values according to the formula (Rojas et al., 2010) as give below:

$$Ct = m\log[\,] + c \quad (3.2)$$

where m is the slope and c is the intercept.

3.7.6 Sensitivity and Validity

To evaluate the sensitivity and suitability of the multiplex qPCR assay for food product analysis, two different types of model meat products, namely, burger and frankfurter of beef and chicken origins were prepared in laboratory. Beef and chicken products were deliberately adulterated with 10, 1 and 0.1 %(w/w) of squirrel, rat, rabbit and cat meat. The DNA was extracted from both the pure and adulterated meat products and concentration was adjusted to 30 ng/μL with nuclease free deionized water.

3.8 Location of work

I did my laboratory work in Nanotechnology and Catalysis Research Centre (NANOCAT) and Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya. DNA extraction, PCR and RFLP assay were performed in NANOCAT and measurement of DNA concentration, Gel documentation and real-time PCR were performed in CEBER. DNA sequencing was done by commercially by the First BASE Laboratories Sdn Bhd (IDT, Singapore).

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CHAPTER 4: RESULTS

4.1 DNA Extraction

Total genomic DNA was extracted from pure, admixed and meat products under raw and processed (boiled, autoclaved and microwaved) states on three different dates. The concentration of the extracted DNA was determined based on the absorbance reading at 260 nm and its purity was evaluated based on the ratio of absorbance at 260 nm and 280 nm. The absorbance at 260 nm indicates the absorbance maxima of nucleic acid and that at 280 nm reflects the absorbanc maxima of proteins. Finally, the A260/A280 ratio provides the DNA purity indicates with respect to the protein concentration (Oliveira, Paim, Reiter, Rieger, & D'azevedo, 2014). The average concentration and purity of the DNA extract from animal, fish, plant and meat product were given in the Table 4.1 .

Table 4.1: Concentration and purity of the extracted DNA.

Sample	Average concentration (ng/ul)	Purity (A260/A280)
Animal tissue (raw)	150-210	1.85-2.0
Animal tissue (Boiled)	80-120	1.80-1.94
Animal tissue (Microwaved)	35-57	1.75-1.79
Animal tissue (Autoclaved)	50-69	1.77-1.85
Fish tissue (raw)	120-250	1.85-2.0
Plant tissue (raw)	115-240	1.82-1.91
Meat product (raw)	80-125	1.77-1.91
Meat product (Boiled)	50-70	1.72-1.89
Meat product (Autoclaved)	42-65	1.70-1.85

4.2 Development of Biomarker

In this study, four pairs of primers were designed targeting cytb and ATP6 genes of cat, rabbit, rat and squirrel species to developed multiplex PCR assay with short length of amplicon. (Table3.2). To develop the multiplex PCR assay with successful PCR products the designed primers must have the particular criteria including short length

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amplicon, fully matching with target DNA and not matching with non-target DNA, with 40-60% GC content and Tm between 55-60 °C.

4.2.1 In-Silico Analysis of Biomarkers using Bioinformatics Tools

The designed primer set of each target species were aligned in silico against 45 species including own species and non-target species. The 100% matching sequence was found only with target species (rabbit, rat and squirrel) whereas (6 - 37) nucleotide (14.63 - 84.77%) with other species. By using of neighbor- joining method the pairwise distance showed the lowest and highest distance between the target species and non-target species. The genetic distance amoung the targets and other non-targets were significantly higher (0.2 - 4.10) suggesting very little or no provavility of cross target amplification (Table 4.2 - 4.7). This result indicated adequate genetic distance between the target and non-target species and also reflected no chance of any cross - target detection. Furthermore, Phylogenetic tree and 3D-plot also recommended the similar findings which supported the other result of silico test shown in Figure 4.1 and Figure 4.2.

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Table 4.2 Mismatch comparison of 123 bp amplicon of rabbit (Oryctolagus cuniculus) against 45 number of target & non-target species.

	Forward Primer		Reverse Primer	
Name of species	T C C G A T A C C T C C A C G C T A A	Mismatch	G A A A C A T T G G C A T C A T C C T C C	Mismatch
Oryctolagus cuniculus	. . . .	0	. . . .	0
Pentalagus furnessi	. . . . T	1	. . . . T . . . . . T . . T . . . . . T T	5
Bunolagus monticularis	. T . . . . T . . C . .	3	. . . . T . . C	2
Romerolagus diazi	. . . . T A . T . . . . . C . .	3	. . . . T . . C . . G . . . C . T . . A .	6
Brachylagus idahoensis	. . . . T . . T . . T . . . . . A . .	4	. . . . T . . . . A .	2
Callosciurus notatus	. T . . C . . . A . A . . . . . C . .	5	. . . . T . . . . . A G . T . . T . . . T	6
Callosciurus inornatus	. . . . C . . T A . A . . T . . C . .	6	. G . . T . . C . . A G . T . . T	7
Callosciurus prevostii	. T . . T . . T A . A . . . . . C . .	6	. G . . T . . . . . A G . T . . . . . T .	6
Callosciurus caniceps	. . . . C . . . T . A . . T . . C . .	5	. . . . C . . G G	3
Callosciurus erythraeus	. . . . C . . T A . A . . T . . C . .	6	. G . . . . . C . . A G . T G	6
Callosciurus finlaysonii	. . . . C . . T A . A . . T . . C . .	6	. . . . C . . A G . . G	4
Callosciurus nigrovittatus	. . . . T . . T A . A . . . . . C . .	5	. G . . T . . C . . A G . T . . . . . T T	8
Callosciurus orestes	. . . . T . . T A . A . . T	5	. . . . A G . T . . . . . T .	4
Rattus rattus	. . . . T . A . . T . . C . .	4	. . . . A . . T . . . . . A .	3
Rattus exulans	. T . . . . . . T . A . . T . . C . .	5	. . . . C . . A . . . G . . . . A .	4
Rattus norvegicus	. . . . A . . . . . C . .	2	. . . . G . . . . A .	2
Rattus tanezumi	. . . . T . A . . T . . C . .	4	. . . . A G . T G . . . . A T	6
Rattus tiomanicus	. . . . T . A . . T . . C . .	4	. . . . A G . . G . . . . A .	4
Rattus leucopus	. T . . . . . . T . A . . T . . C . .	4	. . . . T . . T . . . . . A .	3
Rattus niobe	. T . . . . A . . T . . C . .	5	. G . . . . G G . T . . . . . A .	5
Rattus praetor	. T . . . . . T . . A . . T . . C . .	4	. . . . C . . A G . T . . . . . A .	5
Rattus fuscipes	. . . . A . . T . . C . .	5	. . . . C . . A G . . . . T . . A .	5
Rattus lutreolus	. T . . . . . . T . A . . T	3	. . . . A . . T . . T . . A .	5
Crocodylus porosus	. . . . . A G . . . . . . T . . A . .	4	. . . . C . . A G . A T . A T . G T	5
Sus scrofa	. T . . C . . T . . A . . T . . A . .	4	. . . . A G . A G . . . . A .	4
Bos tauras	. . . . A . A . . . . . A . .	6	. . . . T . . . . . A G . A . . . . . T .	9
Gallus gallus	. . . . G A . T . . . . A . .	3	. . . . . . C A . . A G . A	5
Maleagris gallapavo	. . . A T A . . . . T . . G . .	4	. . . . T . C A . . A G . A G . T . . A .	5
Columba livia	. . . . . A . . . . T . . A . .	5	. . . . . . C A . . A G . . G	9
Anas platyrhynchos	. . . . C A . . . . C . .	3	. . . . T . C A . . A G . A . . . . . A .	5
Bubalus bubalis	. T . . . . . . A . A . . . . . A . .	3	. . . . C . . A G . A . . . . . A .	7
Ovis aries	. . . . T A . A . . . . . A . .	4	. . . . C . . A G . A	5
Capra hircus	. . . . A . A . . . . . A . .	4	. . . . A G . A	3
Cervus nippon yesoensis	. T . . . . . . A . A . . . . . A . .	3	. . . . C . . A G . A	4
Felis catus	. . . . T T . A . . . . . C . .	4	. . . . A . . . . . A . . A T	4
Canis lupus familiaris	. . . . C . . T A . G . . . . . A . .	5	. . . . A . . T G . A . . A T	6
Macaca fascicularis	C . . . C . . T . . . . C . .	4	. . . . C . . . . . T G . A	4
Equus asinus	. T . . C . . . . T . . C . .	4	. . . . A . . T . . . . . A .	3
Cuora amboinensis	. . . . C A . T A C A . . . . . C . .	7	. . . . . . C A . . A . . . . A .	4
Thunnus albacares	. . . . G A . . . . A . .	3	. . . . C . . A G . A G . A	6
Oncorhynchus nerka	. T . . . A . . A . . . . T . . C . .	5	. . . . T . . C . . A G . T G . A . . . T	8
Capsicum annuum cultivar Jeju	. A A A . A . G G G T A . A . . C . .	11	T C T G G C A C A A T . . G . G G . G G G	17
Cucumis sativus	. A G A . A . A G G T A . A . . C . .	11	T C C G G C A C A A T . . G . G G . G G G	17
Allium cepa	. A A A . A . G G G T A . A . . C . .	11	T C C G G C A C A A T . . G . G G . G G G	17
Triticum aestivum	. . . . T . . T A . G . . T		5 . T T C G G . G T C T C G G . G T T G T .	17
Mesocricetus auratus	. T . . C . . T . . T . . T	5	. . . . . G . . . . A . . T G . T T . A .	7
Cavia porcellus	. . . . T . . A . . T . . C . .	4	. . . . T . . . . . A . . T G C T . . T .	7

Table 4.3: Mismatch comparison of the 108 bp amplicon of rat (Rattus rattus) against 45 numbers of target & non-target species

Name of species	Forward Primer		Reversed Primer	
	C A T C A T C A G A A C G C C T T A T T A G C	Mismatch	C A T A C A C C A A A A G G A C G A A C C T	Mismatch
Rattus rattus	. . . .	0	. . . .	0
Rattus exulans	. . . . G	1	. . C . . . . T .	2
Rattus norvegicus	. . . . A . . C . . .	2	. . C	1
Rattus tanezumi	. . . . G . A	2	. . . .	0
Rattus tiomanicus	. . . . T . A . . . . . T	3	. . . .	0
Rattus leucopus	. . . . T . . A	2	. . C	1
Rattus niobe voucher	. . . . A	1	. . C . . . . . G	2
Rattus praetor	. . . . G . . . . . A	2	. . . .	0
Rattus fuscipes	. . . . G	1	. . . T	1
Rattus lutreolus	. . . . G . . . . . G . . . . C .	3	. . . .	0
Callosciurus notatus	. C . G G A . . . . T G T . . . . T . G . . G	10	A . . C T G A G G C G G . T T . T C . G T C	17
Callosciurus inornatus	. T . G G A . . A . T A T . . . . C . G . . G	11	A . . C T G A G G C G G A T T T T C . G T A	19
Callosciurus prevostii	. C . G G A . . A . T A T . . . . C . G . . G	11	A . . T T G G G G C G G . T T . T C . G T C	17
Callosciurus caniceps	. T . G G A . . A . T G T . . . . C . G G . G	12	A . . C T G A G G C G G A T T . T C . G T .	17
Callosciurus erythraeus	. C . G G A . . A . T A T . . . . C . G . . G	11	A . . C T G A G G G G G C T T . T C . G T C	18
Callosciurus finlaysonii	. C . G G G . . A . T A T . . . . C . G . . G	11	A . . C T G A G G G G G T T T . T C . G T .	17
Callosciurus nigrovittatus	. T . G G A . . A . T A T . . . . C . G . . G	11	A . . C T G A G G T G G T T T . T C . G T .	17
Callosciurus orestes	. T . G G A . . A . T A T . T . . C . G . . G	12	A . . C T G A G G T G G A T T . T C . G T .	17
Oryctolagus cuniculus	. C . . C C . T A G C . . A . . A . . . . A .	10	. . . T . C . . C	3
Pentalagus furnessi	. . . G G C . . A . T A T . A . . T . G . . G	11	A . . C T G A G G C G G A T T . T C . G T .	17
Bunolagus monticularis	. . . G G C . . A . T A T . A . . C . G . . G	11	G . . C T G A G G C G G A T T . T C . G T C	18
Romerolagus diazi	. . . G G A . . A . T A T . A . . C . G . . G	11	A . . T T G A G G G G G C T T T T C . G T .	18
Brachylagus idahoensis	. . . G G C . . . . T A T . T . . C . G . . G	10	G . . T T G A G G C G G . T T T T C . G T C	18
Crocodylus porosus	A T A A C C . G C . . G A T . G C T G A C T A	19	. C A G T . A A T . . G C C . G . . C A T A	14
Sus scrofa	. . A . . C . C A . . . . A . . C . . . . A T	8	. . C . A C . A . . . . . . C . A	6
Bos tauras	. . A . . . . . A . C . . A . . A G . A . . .	7	. . C . A T T . T . . . . A . . . A .	7
Gallus gallus	. . . . . C . . . G . A A . . G A T G G . T .	10	. C C C T . A A C . . G . C . G . T C A . A	14
Meleagris gallopavo	. T . . . C . . . G . . . T . G A T G G G T .	11	. C A C T . A A T . . . . C T G . T C A . A	14
Columba livia	. . . . C C . T A . C A A . . G . T G A . T T	13	. C A T T . A A C . . . A A C G . C C A . A	15
Anas platyrhynchos	. . . . C C . . . G C A A . . G A T G A . T .	12	. C A T T . A A C . . . A A C G . C C A . A	15
Bubalus bubalis	. . A . T C . . A . T . . A . . A . . A . . T	9	. . C . A T A . C . . G . . . . A . . . A .	8
Ovis aries	. C A . . . . . A . C . . A . . A G . C . A .	9	. . . . A T A . C . . . . A G . . A .	7
Capra hircus	. C . . . . . . A . C . . A . . A . . . . A .	6	. . . . A C A . C . . . . A . . . A .	6
Cervus nippon yesoensis	. . A . . . . . A . T . . T . . A G . A . A T	9	. . C . A T G . C . . . . A . . . A .	7
Felis catus	. T . . . C . T A . C . . A . . A . . . . A T	9	. . . . A T . A T . . . . A	5
Canis lupus familiaris	. . A . . C . C A G T . . . . . A . . C . A T	10	. . . . A C . A . . . G	4
Macaca fascicularis	T . A . C . . T A . . . A . . C C . . . . A T	10	. . C . A C G . T . . G . . . . A	7
Equus asinus	. C . . . . . C A . C . . A . . A . . . . A .	7	. . . . A C A A T . . . . A	6
Cuora amboinensis	. . A . C C . . A . C A A . . G A T G A C T A	15	. C C . T T A A C . . . A C . G . . C A T A	14
Thunnus albacares	. . A . . C . . A C . T C . . G A T G A C T A	14	. C . G T T A A T C T G C C C G . C C A . A	18
Oncorhynchus nerka	. T A . C C . C T C T G C . . G A T G A C T A	18	. C A C T . A A T T T . . . C G . T C A . A	15
Capsicum annuum cultivar Jeju	A . A A G G G . . G . G . A A A C T C A G T A	18	. . A . T . G G T G G T C T T T C C G G A A	18
Cucumis sativus	A . A A C G G . . G . G . A A A C T C A G T A	18	. . A . T . G G T G G T C T T T C C G G A A	18
Allium cepa	A . A A G G G . . G G G . A A A G T C A G T G	19	. . A . T . G G T G G T C T . G A T G T G A	17
Triticum aestivum	A . A A G G G . . G T G . A A A G T C A G T G	19	. . A . T . G G T G G T C T T T C C G G A A	18
Mesocricetus auratus	. . . . T . . T A . C . . T . . C T . A . . .	8	. . . T . G . . . . A G . . T .	5
Cavia porcellus	. C . . C C . C A C . . A . T . A . . C . A T	12	. . . . A C A T C . . G . . . . . T . . T .	8

Table 4.4: Mismatch comparison of the 243 bp amplicon of squirrel (Callosciurus notatus) against 45 number of target & non-target species

Name of Species	Forward Primer		Reversed Primer	
	A T C T C C C C A C T C C T T C C A A T	Mismatch	A T T C T T A C A T G T A G G C C G C G	Mismatch
Callosciurus notatus	. . . .	0	. . . .	0
Callosciurus inornatus	. . . . T . . . . . C . . C	3	T . . . C . C . . C . . . . T .	5
Callosciurus prevostii	. C . . T . . . G . . . . A . . T . . .	5	C . . T C . T . . . . A . . T .	6
Callosciurus caniceps	. C . . T . . T . . C . . C	5	T . . . C . T . . . . T	4
Callosciurus erythraeus	. . . . T . . C . . C C	4	G . . . C . G	3
Callosciurus finlaysonii	. C . . T . . T . . C . . C	5	. . . . . . G	1
Callosciurus nigrovittatus	. . . . T . . . . A	2	C . . . C . T . . . . A . . T .	5
Callosciurus orestes	. C . . . . . T G . . . . A . . T . . C	6	C . . T C . C . . . . . C . . A . . T .	7
Rattus rattus	. C . . T . . . G . C . . A C	6	. . . . C . C . . . . A .	3
Rattus exulans	. C . . T . . . G . . . . A C	5	. . . . C . C . . C . . . . . A . . A .	5
Rattus norvegicus	. C . . . . . . G . C . . A . . T . . C	6	. . . . C . C . . . . . G . . A . . A .	5
Rattus tanezumi	. . . . T . . A G . C . . A C	6	. . . . C . C . . . . A .	3
Rattus tiomanicus	. C . . T . . . G . C . . A C	6	. . . . C . C . . . . A .	3
Rattus leucopus	. C . . . . . . G . C . . A C	5	G . A . C . C . . . . A . . A .	6
Rattus niobe	. C . . T . . T G . C . . A C	7	. . A . C . C . . C . . . . . A . . A .	6
Rattus praetor	. C . . T . . . G . C . . A C	6	. . A . C . C . . . . A . . A .	5
Rattus fuscipes	. C . . T . . . G . C . . A	5	. . A . C . C . . C . . . . . A . . A .	6
Rattus lutreolus	. C . . T . . . G . C . . A C	6	T . A . C . C . . C . . . . . A . . A .	7
Oryctolagus cuniculus	. C . . T . . T G . . . . A . . A . . C	7	C . A . A . . . . C	4
Pentalagus furnessi	. C . . T . . T G . C . . A . . A . . C	8	C . A . A . . . . C . . . . T .	5
Bunolagus monticularis	. C . . . . . . G . C . . A . . A . . C	6	T . A T A	4
Romerolagus diazi	. C . . . . . . G . C . . A . . A . . C	6	G . A . A . G . . . . T .	5
Brachylagus idahoensis	. C . . . . . . G . C . . A . . G . . .	5	C . A T A . G . . C . . . . T .	7
Crocodylus porosus	. C T . G . . . . . A . . A C	6	C . . . C . C . . C A . C . . A . . T .	7
Sus scrofa	. C . . . . . A G . C . . C . . A . . C	7	. . . . A . C . . C . . . . A .	4
Bos tauras	. C . . T . . A G . C . . A . . A . . C	8	. . A T A . G . . C . . . . . A . . A .	7
Gallus gallus	. C . . . . . A G . C . . A C	6	C . . . C . T . . C A . C . . A . . A .	8
Maleagris gallapavo	. C . . . . . A . . C . . A C	5	C . . . C . . . . C A . T . . A	6
Columba livia	. C . . A . . A . . C . . C . . A . . C	7	T . A . C . . . . C A . C . . A . . A .	8
Anas platyrhynchos	. C . . T . . . G . A . . C . . T . . .	6	C . A . C . G . . C A . C . . A . . A .	9
Bubalus bubalis	. C . . . . . T G . . . . A . . A . . C	6	. . A T A . . . . C . . . . . A . . A .	6
Ovis aries	. . . . A G . . . . A . . A . . .	4	. . . T A . G . . . . A . . A .	5
Capra hircus	. C . . . . . A . . C . . A . . A . . C	6	. . . . A . . . . . A . C . . A . . A .	5
Cervus nippon yesoensis	. C . . . . C . . A . . A . . .	4	. . . . A . . . . A . . A .	3
Felis catus	. . . . A . . . G . C . . A . . T . . C	6	G . A . A . . . . A . . G .	5
Canis lupus familiaris	. C . . . . . A G . G . . G . . T . . C	7	. . . . C . . . . A . . A .	3
Macaca fascicularis	. . T . A . . . . . C . . G C C	6	T . . . C . G . . C A . C . . . . . A .	7
Equus asinus	. C . . G . . A . . C . . C . . A . . C	7	C . . T A . C . . C . . . . . G	6
Cuora amboinensis	. C . . . . . A . G C . . . . . T . . C	6	C . A . C . T . . . A . T . . T . . A .	8
Thunnus albacares	. C . . T . . T . . C . . C . . T . . .	6	C . A . . . C . . C A . C . . . . . A .	7
Oncorhynchus nerka	. C . . . . . A G . A . . A . . T . . C	7	T . A T A . G . . C A . C . C . . . A .	10
Capsicum annuum cultivar Jeju	G C G . T A T G G . A A A G A A . . . G	15	C C . . C C G A T . T G T C A G G T A T	17
Cucumis sativus	G C G . T A T G G . A A A G A A G . . G	16	C C . . C C G . T . T G T C A G G T A T	16
Allium cepa	G C G . T A T G G . A A A G A A . . . G	15	C C . . C C G A T . T G T C A G G T A T	17
Triticum aestivum	. . T A T . . A . . C . . G A G	8	T C A . C . T . . . A . T T T T . . T .	11
Mesocricetus auratus	. . . . T . . A . . . . . A . . T . . .	4	. . . T C . T . . . . . C . . T . . G .	6
Cavia porcellus	. C . . . . . A G . . . . A . . . . G C	6	. . A T C . . . . C A . C . . A . . A .	8

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Table 4.5: Pairwise distances between 123-bp amplicon of rabbit (Oryctolagus cuniculus) and 45 number of target & non-target species

	O. cuniculus	*P. furnessi*	*B. monticularis*	*R. diazi*	*B. idahoensis*	*C. notatus*	*C. inornatus*	*C. prevostii*	*C. caniceps*	*C. erythraeus*	*C. finlaysonii*	*C. nigrovittatus*	*C. orestes*	*R.rattus*	*R. exulans*	*R. norvegicus*	*R. tanezumi*	*R. tiomanicus*	*R. leucopus*	*R. niobe*	*R. praetor*	*R. fuscipes*	*R. lutreolus*	*C. porosus*	*S. scrofa*	*B. tauras*	*G. gallus*	*M. gallapavo*	*C. livia*	*A. platyrhynchos*	*B. bubalis*	*O. aries*	*C. hircus*	*C.n yesoensis*	*F. catus*	*C.lfamiliaris*	*M. fascicularis*	*E. asinus*	*C. amboinensis*	*T. albacares*	*O. nerka*	*C. annuum*	*C. sativus*	*A.cepa*	*T. aestivum*
Oryctolagus cuniculus	0.00																																												
Pentalagus furnessi	0.15																																												
Bunolagus monticularis	0.15	0.17																																											
Romerolagus diazi	0.23	0.26	0.24																																										
Brachylagus idahoensis	0.15	0.16	0.19	0.21																																									
Callosciurus notatus	0.35	0.29	0.31	0.33	0.35																																								
Callosciurus inornatus	0.30	0.29	0.30	0.32	0.30	0.18																																							
Callosciurus prevostii	0.37	0.35	0.34	0.36	0.32	0.20	0.20																																						
Callosciurus caniceps	0.28	0.36	0.29	0.32	0.39	0.22	0.16	0.20																																					
Callosciurus erythraeus	0.36	0.33	0.33	0.31	0.37	0.16	0.11	0.20	0.16																																				
Callosciurus finlaysonii	0.33	0.30	0.28	0.30	0.33	0.15	0.15	0.22	0.14	0.06																																			
Callosciurus nigrovittatus	0.37	0.34	0.41	0.37	0.36	0.20	0.15	0.09	0.19	0.19	0.21																																		
Callosciurus orestes	0.33	0.30	0.35	0.36	0.32	0.21	0.18	0.11	0.19	0.20	0.18	0.14																																	
Rattus rattus	0.26	0.23	0.27	0.32	0.24	0.22	0.22	0.30	0.25	0.25	0.23	0.29	0.23																																
Rattus exulans	0.25	0.25	0.26	0.29	0.26	0.28	0.27	0.31	0.25	0.28	0.24	0.35	0.29	0.10																															
Rattus norvegicus	0.24	0.29	0.28	0.25	0.24	0.26	0.33	0.30	0.25	0.30	0.29	0.32	0.24	0.14	0.15																														
Rattus tanezumi	0.32	0.22	0.30	0.37	0.30	0.23	0.25	0.35	0.29	0.23	0.22	0.31	0.27	0.05	0.12	0.19																													
Rattus tiomanicus	0.25	0.24	0.27	0.30	0.22	0.23	0.25	0.31	0.26	0.23	0.20	0.34	0.26	0.06	0.07	0.13	0.07																												
Rattus leucopus	0.24	0.22	0.26	0.28	0.24	0.30	0.27	0.30	0.25	0.28	0.29	0.32	0.27	0.10	0.10	0.16	0.14	0.13																											
Rattus niobe	0.28	0.28	0.32	0.33	0.29	0.29	0.23	0.31	0.28	0.28	0.31	0.31	0.28	0.14	0.15	0.18	0.17	0.17	0.10																										
Rattus praetor	0.29	0.29	0.30	0.28	0.28	0.29	0.23	0.31	0.28	0.26	0.26	0.29	0.25	0.14	0.13	0.18	0.17	0.15	0.09	0.07																									
Rattus fuscipes	0.23	0.24	0.28	0.25	0.23	0.28	0.21	0.33	0.24	0.26	0.24	0.31	0.27	0.12	0.10	0.14	0.15	0.11	0.09	0.09	0.08																								
Rattus lutreolus	0.23	0.21	0.29	0.28	0.24	0.27	0.24	0.31	0.28	0.28	0.30	0.35	0.27	0.12	0.10	0.18	0.17	0.13	0.07	0.11	0.11	0.07																							
Crocodylus porosus	0.49	0.45	0.58	0.57	0.52	0.47	0.43	0.52	0.48	0.40	0.43	0.48	0.43	0.46	0.43	0.49	0.43	0.41	0.48	0.49	0.45	0.37	0.41																						
Sus scrofa	0.26	0.32	0.33	0.37	0.25	0.30	0.24	0.32	0.30	0.24	0.22	0.34	0.30	0.19	0.19	0.24	0.18	0.15	0.21	0.23	0.21	0.19	0.20	0.41																					
Bos tauras	0.32	0.26	0.29	0.33	0.25	0.28	0.36	0.28	0.39	0.36	0.30	0.33	0.29	0.23	0.24	0.25	0.24	0.20	0.27	0.27	0.27	0.24	0.25	0.49	0.26																				
Gallus gallus	0.43	0.42	0.48	0.49	0.40	0.35	0.34	0.38	0.37	0.33	0.34	0.33	0.30	0.37	0.41	0.32	0.40	0.39	0.44	0.40	0.38	0.36	0.41	0.35	0.37	0.40																			
Maleagris gallapavo	0.46	0.41	0.45	0.49	0.48	0.44	0.46	0.47	0.46	0.40	0.40	0.45	0.40	0.46	0.44	0.39	0.41	0.44	0.54	0.50	0.47	0.45	0.50	0.36	0.43	0.44	0.19																		
Columba livia	0.32	0.37	0.37	0.45	0.38	0.40	0.34	0.51	0.35	0.37	0.37	0.46	0.39	0.34	0.31	0.34	0.35	0.31	0.35	0.34	0.32	0.26	0.31	0.32	0.37	0.41	0.15	0.24																	
Anas platyrhynchos	0.42	0.35	0.40	0.43	0.38	0.35	0.36	0.40	0.43	0.37	0.38	0.37	0.37	0.38	0.41	0.35	0.37	0.39	0.41	0.40	0.38	0.33	0.39	0.37	0.42	0.34	0.13	0.16	0.18																
Bubalus bubalis	0.28	0.25	0.26	0.30	0.27	0.26	0.37	0.34	0.39	0.33	0.31	0.38	0.33	0.20	0.17	0.23	0.20	0.19	0.22	0.22	0.20	0.16	0.18	0.46	0.21	0.11	0.38	0.45	0.36	0.37															
Ovis aries	0.28	0.34	0.28	0.24	0.27	0.30	0.32	0.29	0.28	0.29	0.24	0.32	0.25	0.22	0.22	0.20	0.25	0.22	0.27	0.29	0.22	0.23	0.28	0.55	0.20	0.15	0.35	0.46	0.42	0.45	0.12														
Capra hircus	0.25	0.28	0.27	0.32	0.31	0.30	0.34	0.35	0.34	0.32	0.27	0.38	0.28	0.20	0.22	0.20	0.20	0.20	0.25	0.27	0.24	0.23	0.26	0.46	0.19	0.17	0.33	0.39	0.33	0.39	0.14	0.09													
Cervus nippon yesoensis	0.27	0.30	0.26	0.33	0.32	0.28	0.34	0.33	0.29	0.30	0.27	0.34	0.35	0.23	0.21	0.21	0.24	0.21	0.23	0.26	0.21	0.20	0.22	0.48	0.21	0.19	0.37	0.45	0.35	0.40	0.11	0.11	0.11												
Felis catus	0.22	0.23	0.28	0.25	0.25	0.26	0.28	0.33	0.29	0.28	0.26	0.31	0.28	0.17	0.20	0.23	0.20	0.18	0.16	0.23	0.20	0.18	0.17	0.45	0.28	0.27	0.43	0.56	0.38	0.44	0.24	0.27	0.26	0.27											
Canis lupus familiaris	0.38	0.35	0.45	0.38	0.35	0.27	0.32	0.31	0.37	0.26	0.24	0.30	0.30	0.23	0.26	0.25	0.22	0.23	0.27	0.31	0.25	0.28	0.25	0.45	0.19	0.30	0.37	0.39	0.40	0.39	0.27	0.22	0.23	0.26	0.23										
Macaca fascicularis	0.33	0.31	0.32	0.35	0.37	0.39	0.29	0.42	0.34	0.28	0.29	0.40	0.35	0.35	0.34	0.30	0.34	0.38	0.38	0.40	0.35	0.33	0.35	0.49	0.36	0.42	0.31	0.37	0.36	0.31	0.40	0.35	0.33	0.36	0.39	0.31									
Equus asinus	0.21	0.25	0.23	0.29	0.24	0.28	0.27	0.28	0.27	0.29	0.30	0.34	0.26	0.21	0.20	0.20	0.26	0.23	0.20	0.23	0.23	0.20	0.18	0.45	0.18	0.28	0.39	0.40	0.41	0.36	0.24	0.25	0.28	0.27	0.27	0.29	0.31								
Cuora amboinensis	0.35	0.41	0.40	0.33	0.38	0.40	0.33	0.36	0.34	0.34	0.34	0.33	0.37	0.32	0.39	0.33	0.40	0.37	0.35	0.42	0.35	0.32	0.36	0.45	0.37	0.45	0.23	0.31	0.27	0.22	0.41	0.43	0.39	0.42	0.36	0.35	0.35	0.35							
Thunnus albacares	0.34	0.38	0.42	0.44	0.39	0.36	0.30	0.43	0.33	0.35	0.29	0.36	0.32	0.38	0.38	0.41	0.39	0.35	0.41	0.38	0.38	0.33	0.39	0.30	0.32	0.39	0.17	0.29	0.21	0.22	0.41	0.40	0.37	0.39	0.40	0.38	0.30	0.39	0.29						
Oncorhynchus nerka	0.35	0.38	0.33	0.35	0.41	0.41	0.35	0.44	0.42	0.42	0.42	0.42	0.42	0.47	0.41	0.48	0.45	0.43	0.47	0.46	0.42	0.43	0.43	0.45	0.41	0.41	0.36	0.37	0.26	0.33	0.42	0.38	0.39	0.48	0.48	0.46	0.35	0.41	0.38	0.24					
Capsicum annuum cultivar Jeju	1.78	1.56	1.41	1.67	1.86	1.70	1.57	1.88	1.78	1.87	1.83	1.87	1.79	1.69	1.59	1.69	1.93	1.72	1.64	1.87	1.73	1.64	1.69	1.65	2.12	1.89	1.93	1.89	1.69	1.53	1.90	2.15	1.87	2.10	1.72	2.16	1.76	1.84	1.39	1.62	1.68				
Cucumis sativus	1.90	1.60	1.48	1.77	1.98	1.80	1.66	2.02	1.89	2.00	1.95	2.00	1.91	1.79	1.68	1.80	2.00	1.83	1.74	2.00	1.84	1.73	1.80	1.82	2.25	2.02	2.18	2.07	1.89	1.64	2.04	2.19	2.11	2.24	1.82	2.24	1.91	1.97	1.51	1.79	1.87	0.04			
Allium cepa	1.86	1.62	1.46	1.74	1.94	1.77	1.64	1.97	1.86	1.96	1.91	1.96	1.87	1.76	1.66	1.77	2.02	1.80	1.71	1.96	1.81	1.71	1.77	1.64	2.20	1.97	1.92	1.88	1.68	1.53	1.99	2.14	1.96	2.18	1.79	2.24	1.84	1.93	1.44	1.62	1.67	0.03	0.06		
Triticum aestivum	1.00	0.97	1.00	0.86	1.06	0.96	0.82	0.89	0.86	0.90	0.96	0.82	0.81	1.03	1.22	1.02	1.05	1.09	1.06	0.99	1.00	1.02	0.99	1.27	1.00	0.97	0.94	0.99	1.04	0.98	1.07	0.92	0.94	1.07	1.09	0.98	0.91	1.05	0.85	1.00	0.90	1.54	1.70	1.64	0.00

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Table 4.6: Pairwise distances between 108-bp amplicon of rat (Rattus rattus) and 45 numbers of target & non –target species

	R.rattus	R. exulans	R. norvegicus	R. tanezumi	R. tiomanicus	R. leucopus	R. niobe	R. praetor	R. fuscipes	R. lutreolus	C. notatus	C. inornatus	C. prevostii	C. caniceps	C. erythraeus	C. finlaysonii	C. nigrovittatus	C. orestes	O. cuniculus	P. furnessi	B. monticularis	R. diazi	B. idahoensis	C. porosus	S. scrofa	B. tauras	G. gallus	M. gallapavo	C. livia	A. platyrhynchos	B. bubalis	O. aries	C. hircus	Cn yesoensis	F. catus	C.lfamiliaris	M. fascicularis	E. asinus	C. amboinensis	T. albacares	O. nerka	C. annuum	C. sativus	A.cepa	T. aestivum
Rattus rattus	0.00																																												
Rattus exulans	0.09																																												
Rattus norvegicus	0.09	0.11																																											
Rattus tanezumi	0.06	0.10	0.13																																										
Rattus tiomanicus	0.07	0.11	0.14	0.04																																									
Rattus leucopus	0.09	0.09	0.05	0.13	0.14																																								
Rattus niobe	0.12	0.08	0.11	0.12	0.13	0.11																																							
Rattus praetor	0.06	0.10	0.06	0.10	0.11	0.08	0.11																																						
Rattus fuscipes	0.09	0.12	0.10	0.15	0.16	0.08	0.15	0.08																																					
Rattus lutreolus	0.10	0.11	0.08	0.15	0.16	0.08	0.15	0.06	0.07																																				
Callosciurus notatus	1.72	1.94	2.01	1.81	1.94	1.97	1.81	1.87	1.90	1.95																																			
Callosciurus inornatus	1.94	2.06	2.79	2.15	2.11	2.72	2.10	2.15	2.03	2.71	0.14																																		
Callosciurus prevostii	1.86	1.89	2.11	1.92	1.89	2.07	1.92	1.97	1.98	2.06	0.11	0.17																																	
Callosciurus caniceps	2.08	2.03	2.73	2.07	2.03	2.66	2.07	2.12	2.00	2.65	0.12	0.11	0.15																																
Callosciurus erythraeus	2.18	2.04	2.73	2.12	2.09	2.67	2.07	2.12	2.00	2.66	0.15	0.14	0.17	0.17																															
Callosciurus finlaysonii	1.98	2.16	2.52	1.95	2.16	2.45	1.95	2.00	2.11	2.08	0.20	0.19	0.15	0.19	0.12																														
Callosciurus nigrovittatus	2.15	2.08	2.79	2.11	2.08	2.72	2.11	2.16	2.04	2.72	0.14	0.16	0.13	0.11	0.20	0.20																													
Callosciurus orestes	2.11	2.05	2.73	2.03	1.95	2.66	2.08	2.52	2.32	2.66	0.15	0.13	0.14	0.10	0.20	0.21	0.06																												
Oryctolagus cuniculus	0.38	0.38	0.34	0.40	0.41	0.34	0.35	0.34	0.35	0.34	2.91	3.10	2.23	3.04	3.04	2.86	3.13	3.08																											
Pentalagus furnessi	1.94	2.06	2.85	2.03	2.06	2.79	2.10	2.20	2.08	2.79	0.17	0.21	0.22	0.17	0.24	0.24	0.18	0.19	3.19																										
Bunolagus monticularis	2.20	2.54	2.80	2.34	2.39	2.73	2.52	2.60	2.41	2.73	0.21	0.18	0.23	0.19	0.23	0.27	0.21	0.19	3.03	0.14																									
Romerolagus diazi	2.07	2.19	2.99	2.16	2.19	2.93	2.23	2.80	2.11	2.93	0.24	0.20	0.18	0.20	0.18	0.19	0.19	0.19	3.23	0.16	0.21																								
Brachylagus idahoensis	2.12	2.07	2.71	2.06	1.97	2.20	2.11	2.11	1.93	2.19	0.21	0.23	0.24	0.25	0.24	0.24	0.27	0.25	3.09	0.17	0.17	0.14																							
Crocodylus porosus	2.03	1.99	2.11	2.07	2.03	2.02	2.12	2.12	2.07	2.04	2.96	2.90	3.35	2.84	2.96	3.09	3.22	3.23	2.25	3.41	3.45	3.24	3.18																						
Sus scrofa	0.44	0.41	0.43	0.48	0.48	0.41	0.43	0.43	0.46	0.43	3.15	3.27	2.79	3.21	3.26	3.04	3.09	3.03	0.40	3.26	3.20	3.00	3.20	1.91																					
Bos tauras	0.49	0.45	0.46	0.45	0.46	0.46	0.42	0.47	0.49	0.53	2.20	2.86	1.94	2.19	2.93	2.04	2.78	2.72	0.50	2.92	3.09	2.18	2.79	2.05	0.41																				
Gallus gallus	1.08	1.00	0.98	1.13	1.19	1.00	1.04	1.04	1.04	1.00	1.70	1.78	2.19	1.93	2.15	2.29	2.17	2.13	1.24	2.05	2.32	2.12	1.98	0.70	1.39	1.60																			
Maleagris gallapavo	1.38	1.25	1.29	1.40	1.38	1.18	1.28	1.32	1.19	1.26	1.95	2.03	2.55	2.00	2.50	2.62	2.48	2.43	1.47	2.55	2.80	2.61	2.47	0.62	1.75	1.72	0.20																		
Columba livia	1.34	1.34	1.38	1.58	1.55	1.41	1.47	1.36	1.12	1.25	2.02	1.93	2.19	2.06	2.20	2.28	2.38	2.25	1.21	1.95	2.43	2.03	2.06	0.75	1.45	1.80	0.30	0.34																	
Anas platyrhynchos	1.36	1.23	1.11	1.43	1.46	1.13	1.18	1.14	1.06	1.02	2.32	2.15	2.53	2.39	2.54	2.73	2.73	2.59	1.01	2.08	2.73	2.03	2.04	0.79	1.32	1.34	0.19	0.30	0.18																
Bubalus bubalis	0.47	0.44	0.46	0.47	0.44	0.46	0.42	0.47	0.53	0.53	1.81	1.94	1.75	1.79	1.98	1.79	1.95	1.92	0.46	1.88	2.05	1.77	1.90	2.05	0.36	0.14	1.24	1.73	1.44	1.23															
Ovis aries	0.46	0.46	0.49	0.46	0.49	0.51	0.44	0.46	0.53	0.53	1.90	2.03	1.70	1.89	1.97	1.84	1.94	2.15	0.45	2.15	2.65	2.00	2.16	2.20	0.38	0.18	1.48	1.84	1.81	1.44	0.16														
Capra hircus	0.42	0.39	0.45	0.42	0.45	0.43	0.40	0.39	0.44	0.39	2.23	2.02	1.99	2.00	2.07	1.89	2.52	2.47	0.32	2.72	2.79	2.11	2.72	2.85	0.31	0.26	1.20	1.41	1.32	1.13	0.22	0.15													
Cervus nippon yesoensis	0.46	0.40	0.45	0.47	0.43	0.39	0.42	0.45	0.50	0.48	2.12	2.00	1.75	2.11	2.04	2.11	2.33	2.29	0.45	1.95	2.48	2.08	2.24	2.60	0.34	0.16	1.38	1.57	1.57	1.30	0.13	0.18	0.23												
Felis catus	0.45	0.47	0.45	0.45	0.42	0.43	0.38	0.42	0.48	0.47	2.98	3.06	2.79	2.94	3.00	2.80	3.10	3.04	0.32	3.32	3.21	3.29	3.31	2.10	0.24	0.34	1.66	1.47	1.66	1.45	0.31	0.30	0.28	0.30											
Canis lupus familiaris	0.45	0.46	0.43	0.48	0.48	0.43	0.48	0.43	0.42	0.45	2.87	3.00	2.19	2.94	2.88	2.60	2.99	2.93	0.40	2.99	2.93	2.88	2.87	2.78	0.23	0.44	1.25	1.54	1.42	1.29	0.38	0.41	0.41	0.33	0.26										
Macaca fascicularis	0.48	0.49	0.48	0.52	0.47	0.47	0.50	0.52	0.50	0.50	2.23	2.98	2.07	2.78	2.92	2.20	2.91	2.85	0.48	3.04	2.98	2.99	3.18	1.88	0.44	0.53	1.39	1.89	1.46	1.58	0.49	0.60	0.51	0.44	0.46	0.42									
Equus asinus	0.43	0.42	0.40	0.43	0.46	0.38	0.37	0.39	0.39	0.34	2.79	2.99	2.71	2.93	2.87	2.65	3.03	2.98	0.28	3.26	3.15	3.13	3.25	1.95	0.24	0.31	1.27	1.29	1.31	1.13	0.33	0.29	0.17	0.35	0.20	0.28	0.45								
Cuora amboinensis	1.36	1.23	1.40	1.44	1.41	1.38	1.33	1.38	1.47	1.42	2.86	2.23	3.10	2.85	2.79	3.13	3.10	3.04	1.19	2.98	3.04	2.98	3.09	0.58	1.66	1.39	0.45	0.54	0.36	0.44	1.22	1.43	1.54	1.29	1.45	1.44	1.73	1.33							
Thunnus albacares	2.12	1.95	2.14	1.83	1.90	2.24	2.01	1.93	1.99	2.05	2.08	2.08	2.66	2.16	2.09	2.59	2.66	2.73	1.81	2.31	2.14	2.13	2.41	0.80	1.82	2.06	0.57	0.66	0.69	0.65	1.92	2.06	2.01	1.95	1.95	2.03	1.95	1.96	0.57						
Oncorhynchus nerka	2.08	1.97	2.16	1.87	1.83	2.23	1.95	2.06	2.17	2.08	2.53	2.68	2.90	2.67	2.85	3.01	2.73	2.74	1.72	2.97	3.02	3.28	2.90	0.73	1.80	1.91	0.54	0.54	0.55	0.54	1.81	2.05	1.88	2.06	1.88	1.96	2.11	1.77	0.53	0.34					
Capsicum annuum cultivar Jeju	3.13	3.04	3.27	3.18	3.19	3.09	3.19	3.26	3.26	3.13	2.97	2.82	3.22	2.96	2.60	2.81	3.06	3.11	3.51	2.82	2.82	2.43	2.69	1.82	3.28	3.08	2.76	2.47	2.58	2.56	3.16	3.39	3.39	3.13	4.33	4.44	3.33	3.34	2.37	2.67	3.19				
Cucumis sativus	3.03	2.98	3.19	3.14	3.14	2.99	3.14	3.18	3.18	3.03	3.20	3.07	3.34	3.19	2.86	2.97	3.27	3.31	3.45	3.07	3.07	2.60	2.97	1.55	3.21	3.14	2.63	2.04	2.34	2.23	3.12	3.45	3.44	3.19	4.41	4.51	3.25	3.40	2.14	2.59	2.97	0.04			
Allium cepa	3.08	2.83	3.13	3.04	3.04	2.94	3.04	3.03	3.13	2.82	3.34	3.18	3.47	3.26	2.93	3.20	3.34	3.39	3.32	3.22	3.14	2.82	3.18	1.81	3.33	2.84	2.41	2.35	2.64	2.37	2.93	3.20	3.19	2.89	3.38	4.23	3.24	3.08	2.11	2.69	3.04	0.11	0.13		
Triticum aestivum	3.19	2.94	3.09	3.14	3.15	2.88	3.04	3.08	3.14	2.81	2.96	2.81	3.14	2.96	2.59	2.70	3.05	3.11	3.40	2.81	2.81	2.30	2.68	2.25	3.41	3.13	2.98	2.81	2.76	2.45	3.13	3.36	3.39	3.14	4.21	4.29	3.46	3.21	2.44	2.87	3.24	0.06	0.08	0.12	0.00

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Table 4.7: Pairwise distances between 243-bp amplicon squirrel (Callosciurus notatus) and 45 numbers of target & non-target species

	C. notatus	*C. inornatus*	*C. prevostii*	*C. caniceps*	*C. erythraeus*	*C. finlaysonii*	*C. nigrovittatus*	*C. orestes*	*R.rattus*	*R. exulans*	*R. norvegicus*	*R. tanezumi*	*R. tiomanicus*	*R. leucopus*	*R. niobe*	*R. praetor*	*R. fuscipes*	*R. lutreolus*	*O. cuniculus*	*P. furnessi*	*B. monticularis*	*R. diazi*	*B. idahoensis*	*C. porosus*	*S. scrofa*	*B. tauras*	*G. gallus*	*M. gallapavo*	*C. livia*	*A. platyrhynchos*	*B. bubalis*	*O. aries*	*C. hircus*	*C.n yesoensis*	*F. catus*	*C.lfamiliaris*	*M. fascicularis*	*E. asinus*	*C. amboinensis*	*T. albacares*	*O. nerka*	*C. annuum*	*C. sativus*	*A.cepa*	*T. aestivum*
Callosciurus notatus	0.00																																												
Callosciurus inornatus	0.20																																												
Callosciurus prevostii	0.16	0.22																																											
Callosciurus caniceps	0.19	0.12	0.22																																										
Callosciurus erythraeus	0.15	0.16	0.15	0.16																																									
Callosciurus finlaysonii	0.14	0.14	0.16	0.16	0.08																																								
Callosciurus nigrovittatus	0.17	0.17	0.08	0.18	0.17	0.17																																							
Callosciurus orestes	0.18	0.22	0.11	0.21	0.19	0.18	0.11																																						
Rattus rattus	0.26	0.20	0.25	0.23	0.24	0.23	0.25	0.24																																					
Rattus exulans	0.24	0.20	0.21	0.24	0.23	0.21	0.22	0.22	0.08																																				
Rattus norvegicus	0.26	0.26	0.24	0.24	0.26	0.26	0.25	0.22	0.12	0.13																																			
Rattus tanezumi	0.25	0.20	0.27	0.22	0.25	0.25	0.23	0.25	0.04	0.08	0.14																																		
Rattus tiomanicus	0.25	0.22	0.22	0.23	0.24	0.23	0.24	0.23	0.04	0.05	0.12	0.04																																	
Rattus leucopus	0.27	0.25	0.21	0.24	0.22	0.24	0.24	0.23	0.11	0.10	0.13	0.12	0.10																																
Rattus niobe	0.27	0.24	0.25	0.24	0.24	0.25	0.26	0.23	0.11	0.09	0.10	0.11	0.10	0.06																															
Rattus praetor	0.28	0.23	0.24	0.24	0.24	0.25	0.23	0.24	0.11	0.10	0.11	0.11	0.10	0.06	0.03																														
Rattus fuscipes	0.27	0.22	0.25	0.22	0.25	0.23	0.24	0.27	0.13	0.12	0.14	0.14	0.13	0.11	0.08	0.09																													
Rattus lutreolus	0.27	0.24	0.22	0.23	0.24	0.24	0.24	0.25	0.12	0.09	0.14	0.12	0.10	0.06	0.06	0.06	0.07																												
Oryctolagus cuniculus	0.28	0.25	0.22	0.26	0.26	0.25	0.23	0.23	0.22	0.21	0.23	0.21	0.20	0.22	0.19	0.20	0.19	0.19																											
Pentalagus furnessi	0.28	0.25	0.25	0.26	0.26	0.23	0.27	0.24	0.22	0.20	0.25	0.20	0.19	0.24	0.21	0.22	0.20	0.21	0.12																										
Bunolagus monticularis	0.26	0.26	0.27	0.26	0.25	0.24	0.30	0.27	0.21	0.19	0.23	0.21	0.20	0.20	0.20	0.19	0.21	0.19	0.16	0.14																									
Romerolagus diazi	0.25	0.28	0.25	0.31	0.26	0.24	0.27	0.27	0.22	0.22	0.22	0.22	0.21	0.22	0.22	0.22	0.21	0.22	0.20	0.18	0.18																								
Brachylagus idahoensis	0.26	0.23	0.22	0.26	0.25	0.23	0.22	0.22	0.25	0.25	0.25	0.25	0.24	0.24	0.24	0.23	0.24	0.23	0.18	0.19	0.21	0.21																							
Crocodylus porosus	0.54	0.53	0.53	0.55	0.52	0.58	0.54	0.49	0.52	0.48	0.53	0.52	0.49	0.52	0.49	0.51	0.47	0.47	0.55	0.54	0.57	0.56	0.55																						
Sus scrofa	0.27	0.19	0.26	0.23	0.20	0.20	0.29	0.25	0.24	0.20	0.23	0.23	0.22	0.22	0.20	0.20	0.22	0.21	0.24	0.24	0.20	0.25	0.22	0.57																					
Bos tauras	0.25	0.26	0.25	0.28	0.28	0.25	0.28	0.27	0.20	0.18	0.19	0.19	0.18	0.21	0.17	0.18	0.18	0.16	0.24	0.24	0.23	0.25	0.23	0.58	0.19																				
Gallus gallus	0.38	0.37	0.37	0.37	0.35	0.35	0.36	0.30	0.34	0.33	0.30	0.33	0.31	0.35	0.31	0.33	0.34	0.33	0.34	0.34	0.35	0.37	0.31	0.44	0.35	0.32																			
Maleagris gallapavo	0.38	0.38	0.38	0.40	0.36	0.39	0.38	0.35	0.38	0.34	0.32	0.34	0.34	0.37	0.33	0.35	0.37	0.35	0.36	0.32	0.34	0.40	0.36	0.46	0.40	0.40	0.17																		
Columba livia	0.46	0.41	0.47	0.43	0.42	0.42	0.44	0.42	0.38	0.37	0.36	0.38	0.38	0.39	0.36	0.37	0.34	0.37	0.37	0.31	0.34	0.41	0.38	0.47	0.37	0.40	0.23	0.23																	
Anas platyrhynchos	0.38	0.37	0.36	0.38	0.38	0.38	0.35	0.33	0.42	0.40	0.36	0.40	0.41	0.43	0.37	0.39	0.38	0.39	0.36	0.35	0.38	0.43	0.37	0.46	0.42	0.37	0.19	0.24	0.28																
Bubalus bubalis	0.20	0.25	0.24	0.26	0.27	0.25	0.27	0.24	0.22	0.18	0.20	0.21	0.20	0.21	0.17	0.19	0.17	0.18	0.21	0.23	0.23	0.24	0.25	0.54	0.19	0.11	0.32	0.40	0.38	0.38															
Ovis aries	0.21	0.25	0.25	0.24	0.27	0.23	0.23	0.23	0.24	0.22	0.21	0.21	0.22	0.26	0.24	0.22	0.21	0.23	0.23	0.27	0.25	0.21	0.22	0.61	0.20	0.15	0.37	0.44	0.43	0.42	0.15														
Capra hircus	0.25	0.23	0.28	0.26	0.27	0.24	0.27	0.24	0.21	0.21	0.20	0.21	0.21	0.20	0.21	0.20	0.21	0.21	0.24	0.22	0.25	0.26	0.25	0.52	0.18	0.14	0.33	0.37	0.36	0.40	0.17	0.13													
Cervus nippon yesoensis	0.22	0.27	0.23	0.27	0.28	0.26	0.23	0.28	0.23	0.23	0.20	0.23	0.20	0.20	0.22	0.21	0.20	0.19	0.25	0.26	0.27	0.24	0.24	0.56	0.23	0.15	0.37	0.40	0.45	0.42	0.16	0.16	0.12												
Felis catus	0.24	0.21	0.24	0.25	0.25	0.25	0.25	0.24	0.23	0.22	0.22	0.23	0.23	0.21	0.23	0.22	0.26	0.23	0.24	0.22	0.22	0.25	0.25	0.60	0.22	0.21	0.35	0.40	0.42	0.36	0.22	0.26	0.20	0.23											
Canis lupus familiaris	0.29	0.32	0.24	0.28	0.29	0.28	0.26	0.22	0.27	0.27	0.23	0.27	0.26	0.27	0.27	0.26	0.31	0.27	0.29	0.30	0.31	0.33	0.29	0.61	0.26	0.27	0.34	0.41	0.41	0.38	0.29	0.23	0.25	0.25	0.26										
Macaca fascicularis	0.36	0.33	0.42	0.38	0.36	0.38	0.37	0.41	0.35	0.36	0.35	0.33	0.35	0.38	0.34	0.34	0.36	0.34	0.44	0.45	0.41	0.40	0.42	0.54	0.37	0.35	0.37	0.42	0.45	0.36	0.39	0.38	0.37	0.39	0.32	0.41									
Equus asinus	0.23	0.23	0.23	0.23	0.22	0.21	0.25	0.23	0.25	0.22	0.23	0.24	0.23	0.22	0.19	0.21	0.19	0.18	0.24	0.25	0.21	0.25	0.25	0.49	0.17	0.17	0.35	0.34	0.36	0.40	0.17	0.22	0.22	0.22	0.24	0.29	0.35								
Cuora amboinensis	0.42	0.40	0.35	0.41	0.37	0.41	0.36	0.36	0.37	0.39	0.36	0.37	0.37	0.33	0.35	0.34	0.36	0.35	0.40	0.42	0.43	0.42	0.41	0.54	0.36	0.39	0.36	0.38	0.40	0.37	0.39	0.43	0.37	0.40	0.40	0.37	0.40	0.38							
Thunnus albacares	0.36	0.30	0.36	0.32	0.36	0.30	0.35	0.32	0.32	0.32	0.30	0.33	0.31	0.34	0.31	0.32	0.31	0.33	0.26	0.32	0.36	0.39	0.31	0.52	0.35	0.34	0.30	0.36	0.34	0.30	0.34	0.33	0.31	0.36	0.39	0.40	0.39	0.34	0.34						
Oncorhynchus nerka	0.37	0.35	0.31	0.36	0.36	0.39	0.33	0.28	0.36	0.32	0.37	0.35	0.33	0.31	0.32	0.33	0.37	0.33	0.29	0.31	0.31	0.31	0.32	0.55	0.37	0.33	0.36	0.41	0.37	0.36	0.30	0.35	0.32	0.36	0.35	0.39	0.49	0.32	0.43	0.31					
Capsicum annuum cultivar Jeju	3.89	3.84	3.31	4.20	3.76	3.76	3.35	3.13	3.81	3.63	3.39	3.70	3.53	3.82	3.77	3.77	3.88	3.80	3.91	4.17	4.12	4.03	3.84	3.67	3.92	4.01	3.54	3.71	4.05	3.29	4.18	4.09	4.00	3.80	3.85	3.55	3.37	3.75	3.97	4.09	3.28				
Cucumis sativus	4.10	4.00	3.76	4.37	3.99	3.99	3.82	3.60	4.04	3.88	3.65	3.95	3.79	4.09	4.04	4.04	4.18	3.97	4.09	4.25	4.36	4.16	4.06	3.73	3.90	3.87	3.74	3.85	4.07	3.29	4.17	3.88	3.76	3.82	3.98	3.92	3.57	3.91	4.29	4.04	3.86	0.06			
Allium cepa	3.78	3.76	3.51	4.07	3.61	3.64	3.54	3.37	4.00	3.77	3.54	3.84	3.68	3.96	3.90	3.90	3.91	3.86	4.19	4.30	4.32	4.22	4.16	3.23	3.98	3.88	3.58	3.86	4.03	3.25	4.09	3.99	3.90	3.73	4.00	3.83	3.20	3.67	4.06	3.96	3.32	0.06	0.08		
Triticum aestivum	0.74	0.68	0.73	0.67	0.70	0.77	0.65	0.71	0.88	0.87	0.87	0.82	0.86	0.88	0.84	0.82	0.80	0.82	0.77	0.76	0.82	0.75	0.84	0.92	0.79	0.83	0.78	0.81	0.81	0.85	0.88	0.74	0.78	0.79	0.89	0.73	0.76	0.84	0.71	0.75	0.78	3.96	4.08	4.04	0.00

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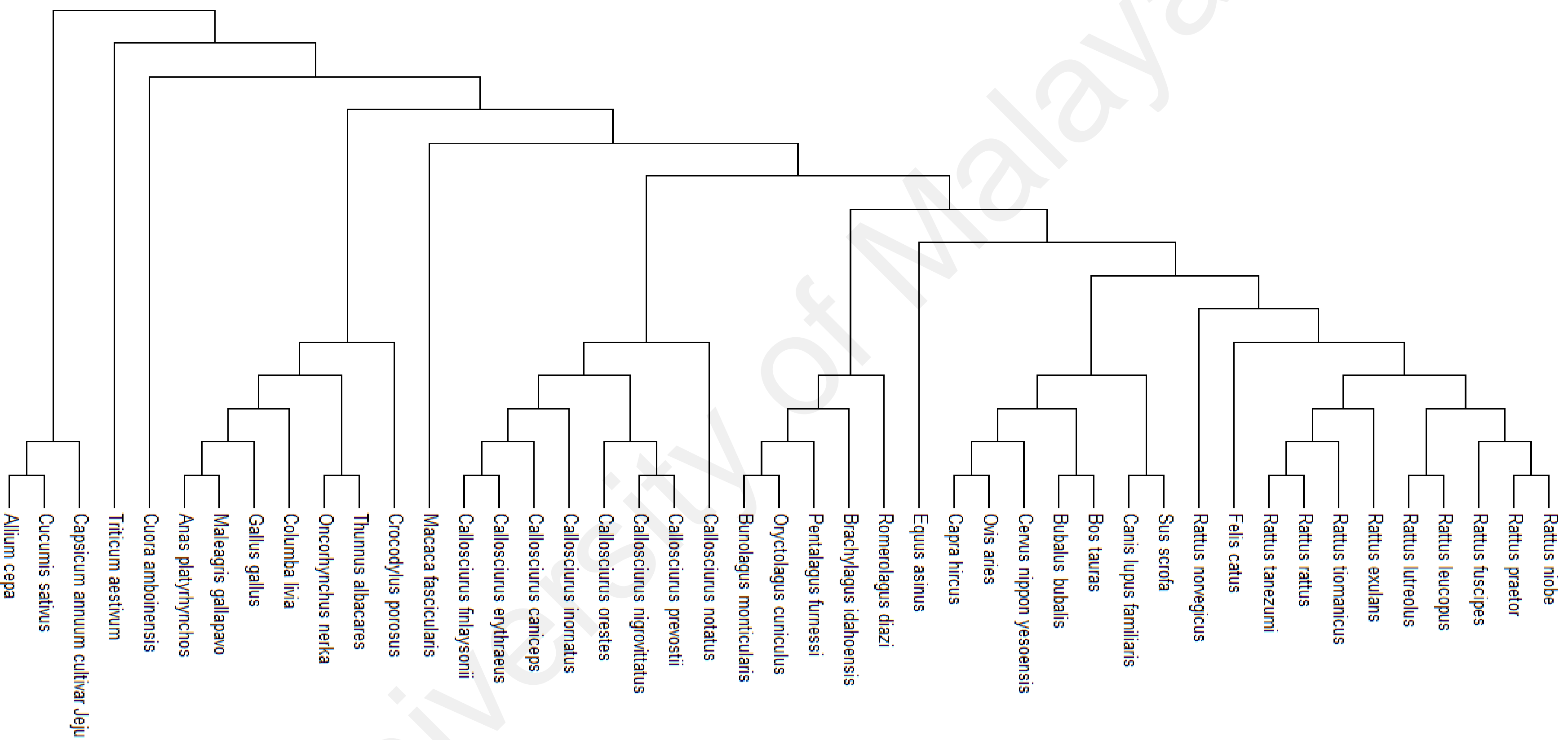

(a)

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Cucumis sativus
Capsicum annuum cultivar Jeju
Triticum aestivum
Allium cepa
Crocodylus porosus
Cuora amboinensis
Oncorhynchus nerka
Thunnus albacares
Meleagris gallopavo
Columba livia
Anas platyrhynchos
Gallus gallus
Bunolagus monticularis
Pentalagus furnessi
Callosciurus orestes
Callosciurus nigrovittatus
Romerolagus diazi
Brachylagus idahoensis
Callosciurus erythraeus
Callosciurus inornatus
Callosciurus caniceps
Callosciurus finlaysonii
Callosciurus prevostii
Callosciurus notatus
Rattus niobe voucher
Rattus exulans
Rattus tiomanicus
Rattus tanezumi
Rattus rattus
Rattus praetor
Rattus lutreolus
Rattus fuscipes
Rattus leucopus
Rattus novegicus
Oryctolagus cuniculus
Macaca fascicularis
Bubalus bubalis
Bos tauras
Ovis aries
Cervus nippon yesoensis
Equus asinus
Capra hircus
Felis catus
Canis lupus familiaris
Sus scrofa

(b)

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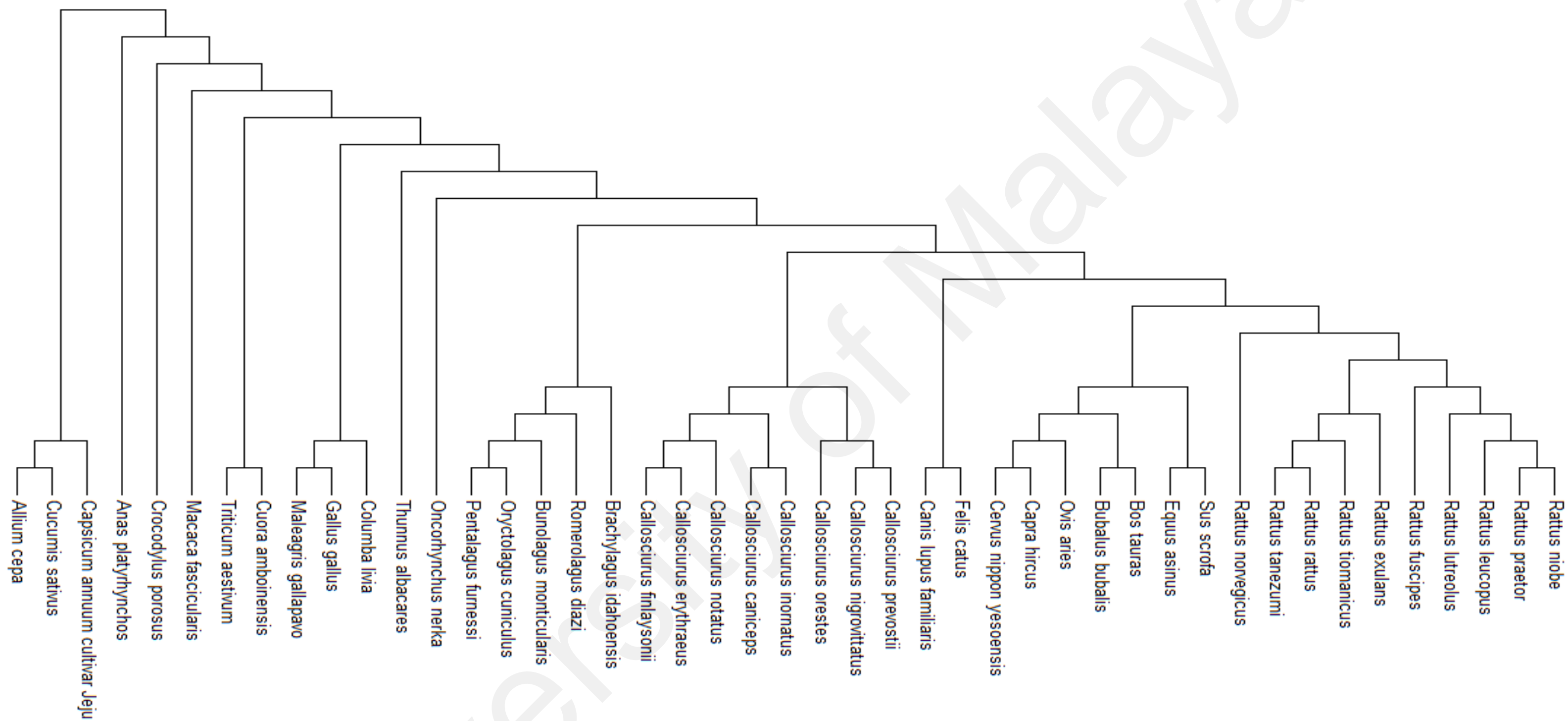

(C)

Figure 4.1: Phylogenetic Tree constructed using neighbor-joining method with 123-bp,108 bp and 243 bp regions of cytb/cob gene sequences of Oryctolagus cuniculus (a) Rattus rattus (b) and (c) Callosciurus notatus respectively and 45 number of other animal and plant species including target species.

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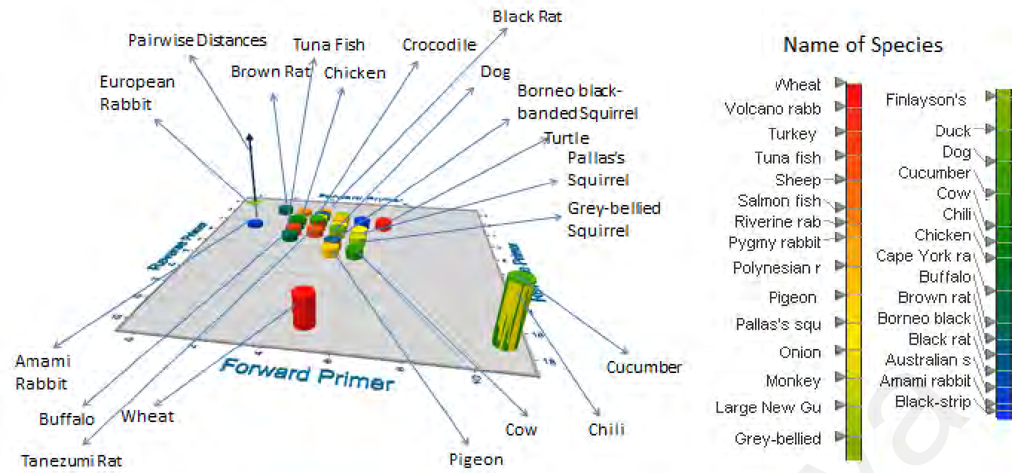
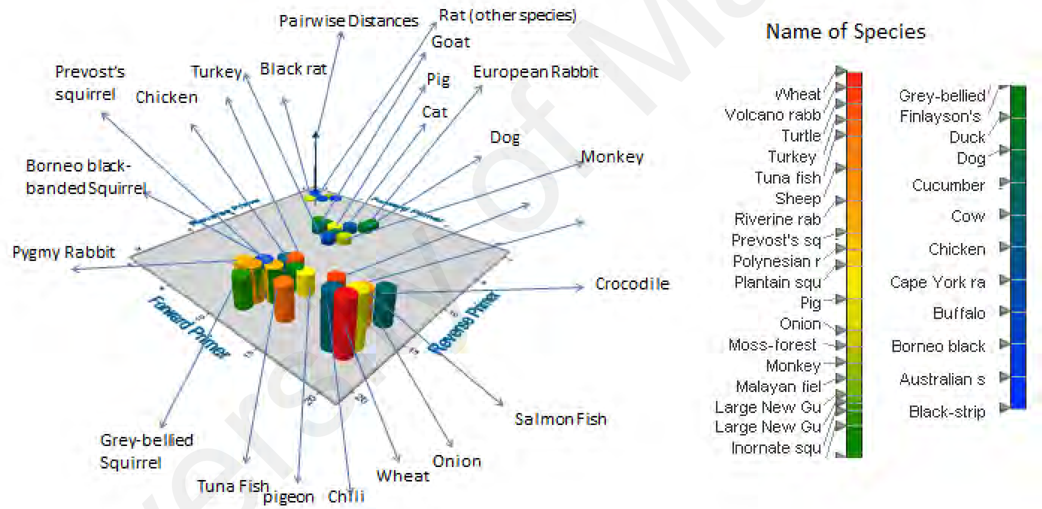


(a)



(b)

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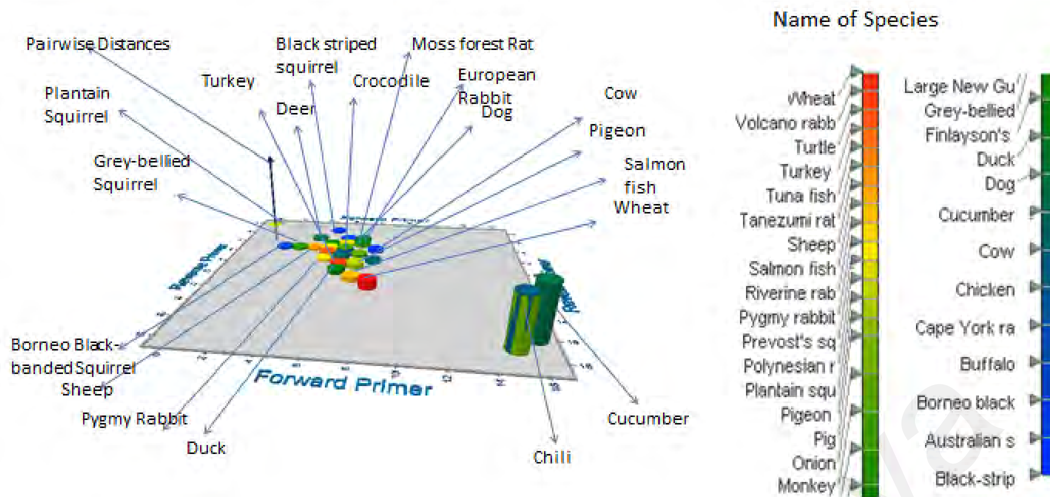

(c)

Figure 4.2: 3D plot showing the discrimination of European rabbit (a); black rat (b) and plantain squirrel (c) against 45 species based on the number of mismatches found in the primer binding regions and pair wise distances. Here, X and Y axis represent reverse primer and forward primer mismatches, respectively, between the targets ((European rabbit (a), black rat (b) and plantain squirrel (c)) and other potential non-target species and Z axis shows ir pair wise distances**.**

4.3 Simplex PCR Assay

4.3.1 Simplex PCR optimization:

To optimize the assays, the PCR reactions of three sets of primers were individually carried out on a gradient thermal cycler with total reaction volume of 25 µl containing appropriate quantity of all PCR components. The annealing temparatures of all set of primers were checked from 57 - 61°C in the gradient system to find out the optimum annealing temperature for successful PCR amplifications. Although some primer sets were successfully amplified at both 58, 59 and 60°C, but were properly amplified only at 58°C . Therefore, 58°C temperatures was the optimum annealing temperature for all the primer sets (Figure 4.3) as in multiplex PCR reaction all primer pairs have to be amplified in a single reaction condition.

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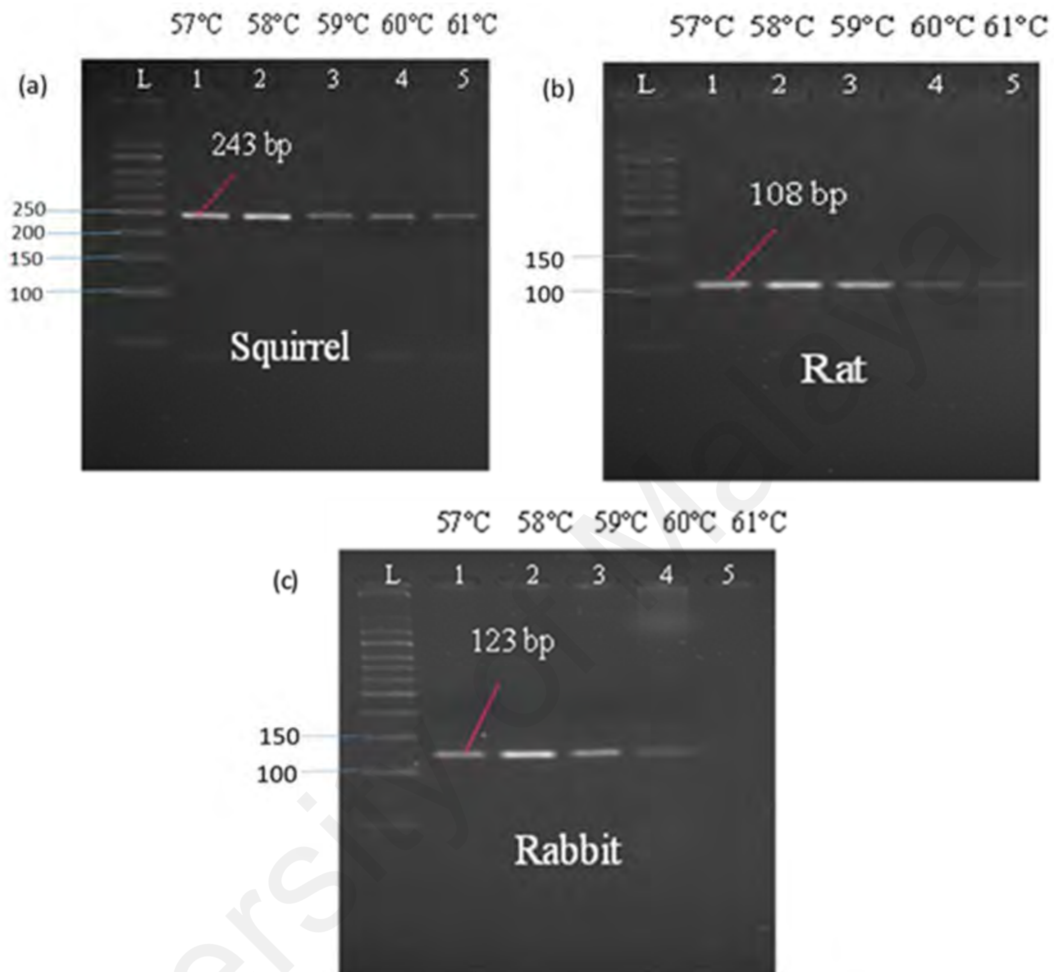

Figure 4.3: Optimization of annealing temperature of designed squirrel cytb (a), rat APT6(b) and rabbit cytb (c) primer sets. In the gel image, lane, L 50 bp DNA ladder, lane1-5, amplified PCR products for 57, 58, 59, 60 and 61 °C temperatures.

4.3.2 Simplex PCR Assay Specificity

The specificity of the primer is very important in developing a robust PCR assay since the primer that fully match the target species and mismatch the non-target species offer a high chance of having a highly specific PCR assay by eliminating the probability of non-target amplification (Wu, Hong, & Liu, 2009). After optimization of simplex

PCR, species specificity of the primers was cross-tested against one target and other 22 non-target of terrestrial and aquatic animal species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, cat, chinese frog, tuna, salmon) and 4 plant species (onion, cucumber, wheat, chili) which are commonly used in food matrices. The result showed that specific primer sets amplified only DNA of the target species but not any of the non-target species. While, universal eukaryotic primers amplified 141 bp sites from all species, reflecting the good quality of the extracted DNA and eliminating the possibility of any false- negative detection. This indicated a high specificity and fidelity of each set of designed primers for the target species (Figure 4.4, 4.5 and 4.6). All tests were repeated three times on three different days but the same outcomes were observed. The amplified PCR products were separated by running with agarose gel electrophoresis and detected in the gel documentation in presence of uv-light.

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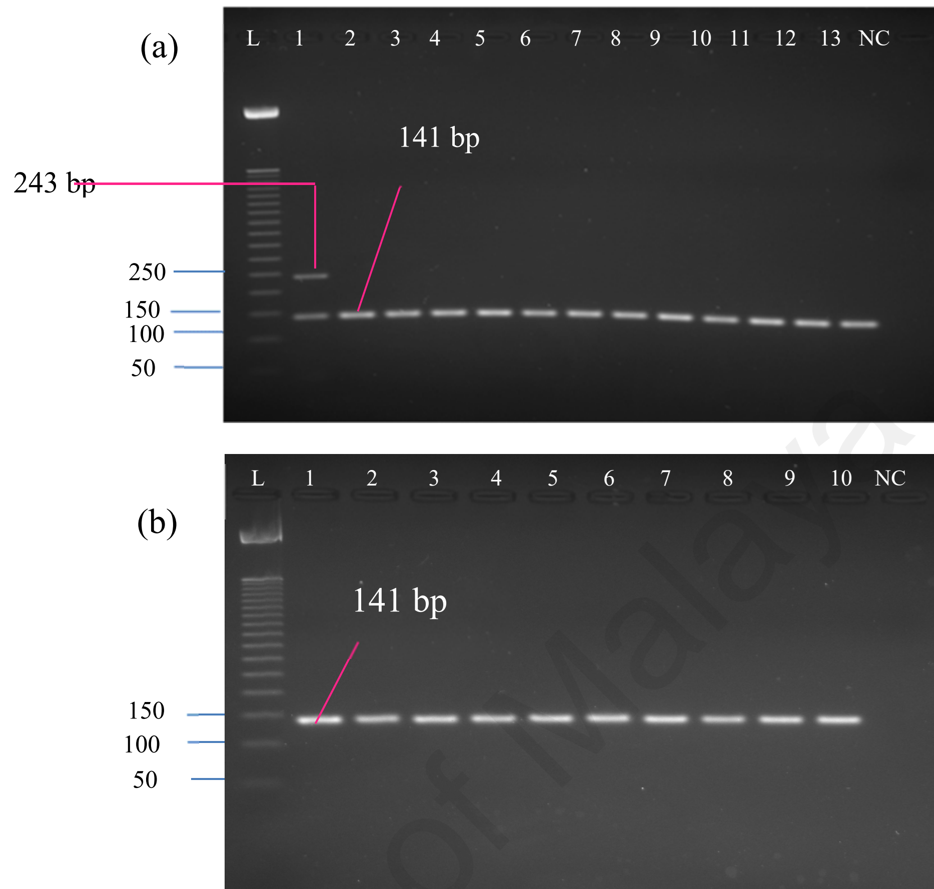

Figure 4.4: The specificity of the simplex PCR of squirrel cytb (243 bp) specific primer pair with DNA of different species. In the gel image, L DNA ladder, NC negative control, lane 1, PCR product of squirrel (243 bp) and endogenous control 141bp, Lanes 2-13(a) and lanes 1-10 (b) the products of 141 bp endogenous control for chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, rat, deer, rabbit, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili, respectively.

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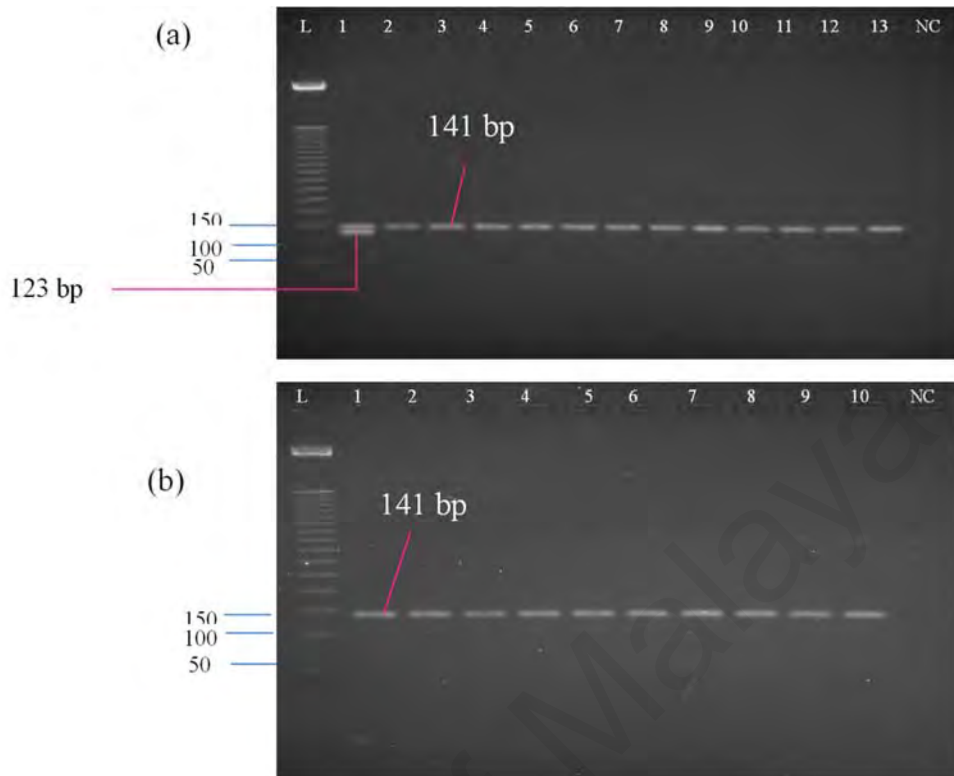

Figure 4.5: The specificity of the simplex PCR of rabbit cytb (123 bp) specific primer pair with DNA of different species. In the gel image, L DNA ladder, NC negative control, lane 1, PCR product of rabbit cytb (123 bp) and endogenous control 141bp, Lanes 2-13 (a) and lanes 1-10 (b) the products of 141 bp endogenous control for chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, squirrel, deer, rat, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili, respectively.

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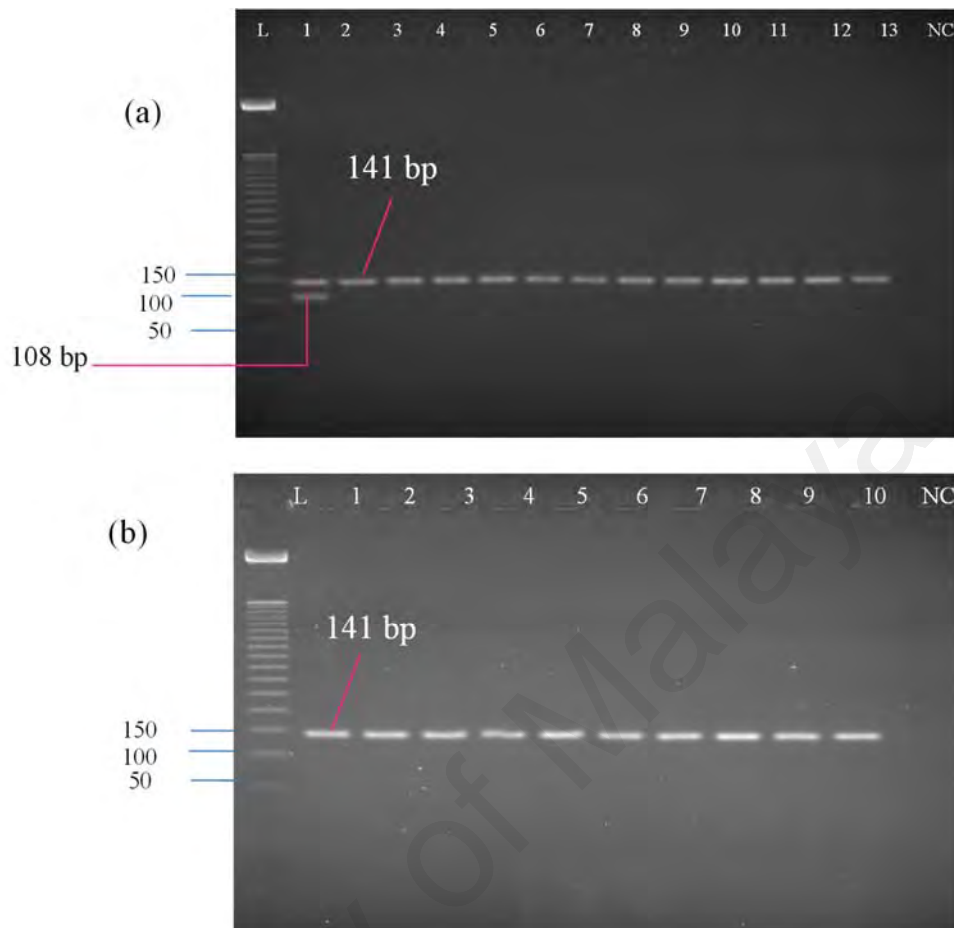

Figure 4.6: The specificity of the simplex PCR of rat ATP6 (108 bp)- specific primer pair with DNA of different species. In the gel image, L DNA ladder, NC negative control, lane 1, PCR product of rat ATP6 (108 bp) and endogenous control 141bp, Lanes 2-13(a) and lanes 1-10 (b) the products of 141 bp endogenous control for chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, squirrel, deer, rabbit, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili, respectively.

4.3.3 PCR product sequencing

PCR products of all targets were sequenced to authenticate originality of the amplified PCR products and their species relevance. Respective PCR products were cloned into a vector prior to sequencing because direct sequencing often fails to retrieve the first and last few sequences accurately (Silva, 2017). It is noteworthy here that the first 10-50 bp of the Sanger sequence fragments often contains a lot of noises that prohibit the retrieval of complete sequences when direct sequencing is performed. The obtained nucleotide sequences were aligned firstly, against GenBank

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(www.ncbi.nlm.nih.gov) sequences for testing any potential matching with other species and secondly, against specific gene sequence using the MEGA5 alignment tool to measure the degree of similarity. The amplified targets showed 99.18, 98.14 and 98.35 % sequence matching with European rabbits, black rats and plantain squirrels, respectively (Table 4.8). This value was within the acceptable limit because at least 98% sequence similarity is required for the accurate identification of species (Cawthorn, Steinman, & Hoffman, 2013).

Table 4.8: Sequencing result of the PCR products

Species	Target gene	Gene Bank accession ID	Similarity (%)
Squirrel	Cytb	AB499913.1	98.35
Rabbit	Cytb	NC_001913.1	99.18
Rat	ATP6	NC_012374.1	98.14

4.4 Multiplex PCR Assay:

4.4.1 Optimization of Multiplex PCR Assay

Initially, simplex PCR was optimized for each primer pair against the template DNA extracted from mucle tissue of each target species to ensure the specificity and ability for amplifying the target sites of the designed primers (Dalmasso et al., 2004). The step by step development of a multiplex PCR is demonstrated in Figure 4.7. As described in the methodology (section 3.5.1, simplex (lane 1-3) duplex (lane4-6) and triplex lane (7). PCR system was developed in an order way to ensure the clarity of the system (Ali et al., 2015a). The developed simplex, duplex, multiplex system amplified the target gene (cytb and ATP6) sites of fragment-size 243 and 123 and 108 bp for squirrel, rabbit and rat, respectively (Figure 4.7), reflecting full consistency with the simplex PCR system**.**

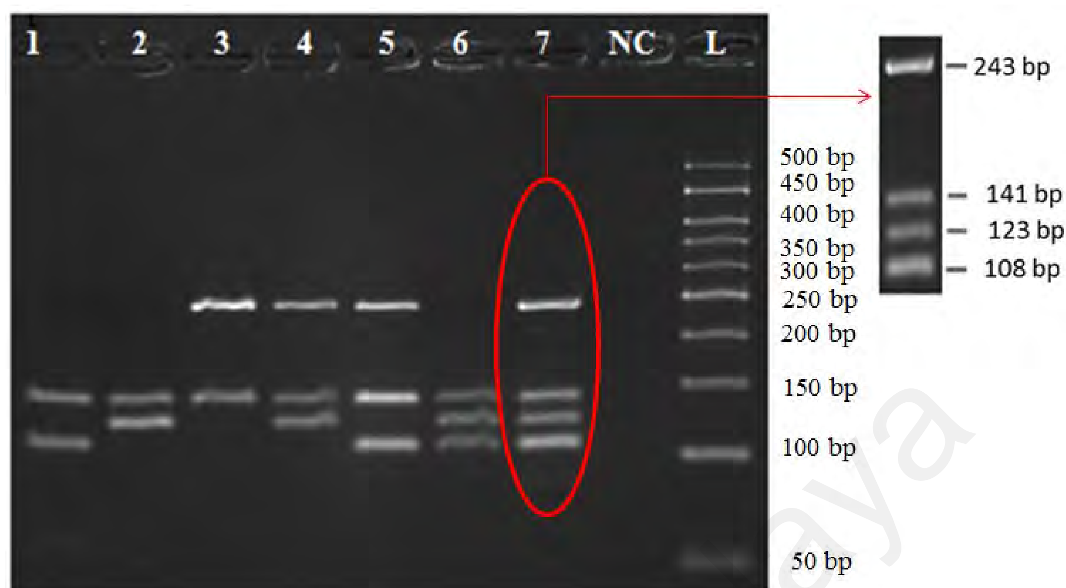

Figure 4.7: Optimization of multiplex PCR. Lane 1-3: Simplex PCR for rat (108 bp); rabbit (123 bp) and squirrel (243bp) along with internal amplification control (IAC) (141 bp). Lane 4-6: Duplex PCR for squirrel (243 bp) & rabbit (123 bp); squirrel (243 bp) & rat (108 bp); and rabbit (123 bp) & rat (108 bp) along with IAC (141 bp). Lane 7: Multiplex PCR for rat (108 bp), rabbit (123 bp) and squirrel (243 bp) along with IAC. Lane NC: Negative control (NC) and Lane L: DNA ladder.

4.4.2 Multiplex PCR Assay Specificity:

The specificity of the developed multiplex PCR assay was screened against three targets (rabbit, rat and squirrel) and other 22 non-targets of terrestrial and aquatic species and 4plant species (Section3.5.2); wherein the developed multiplex PCR system yielded PCR products only from the rabbit, rat and squirrel targets and no products from non-targets (Figure 4.8). The figure clearly showed that when DNA of three targets were added in a single reaction tube, three targets species were amplified simultaneously 243,141,123 and 108 bp product from that tube (Figure 4.8, lane 4). When DNA of single targets species was added, the assay amplified only the added species (In Figure 4.8, lane 1 - 3 for squirrel, rabbit, rat and respectively). However, no cross-amplified products were observed from non-target species without IAC (lane 5-26), indicating that developed multiplex PCR system was also highly specific like simplex PCR.

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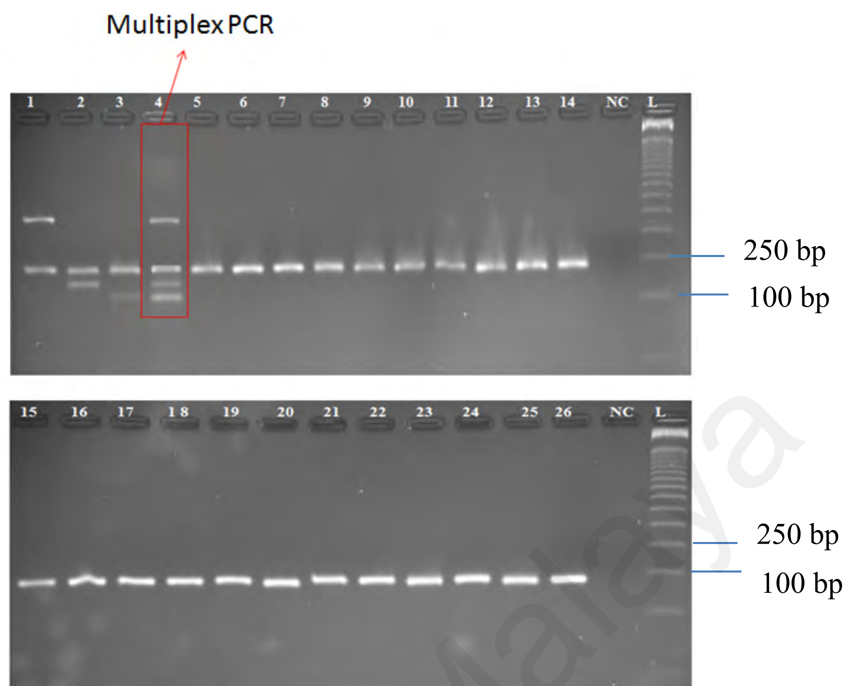

Figure 4.8: Multiplex PCR assay specificity for the rat, rabbit and squirrel specific primers against 22 non-target species (lanes 5-26). Lanes 1- 3: PCR products from squirrel (243 bp), rabbit (123 bp) and rat (108 bp), respectively, along with IAC (141 bp). Lane 4: mPCR products from squirrel, rabbit and rat along with IAC (141 bp). Lanes 5-26 for the amplification products of 141 bp endogenous control from non-target species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili) and no amplification products were found from the 3 primer sets target species due to highly specificity. Lane L: DNA ladder and Lane NC: Negative template control.

4.4.3 Limit of Detection of Multiplex PCR Assay under Raw State:

Extracted DNA of target- species (rabbit, rat and squirrel) was 10 fold serially diluted from 10 ng/ul to 1, 0.1, 0.01, 0.001.and 0.0001 ng/ul used as a template to determine the multiplex PCR sensitivity since I have found spectroscopic determination of nucleic acid concentration is more reliable at higher concentration. The gel electrophoresis produced four bands corresponding to two cytb and one ATP6 and one 18sRNA genes of rabbit, rat and squirrel species from as low as 0.01ng DNA template (Figure 4.9). The band of lane 5 in Figure 4.9 (0.001ng) was visualized although the band intensity was very low. Thus 0.001 ng of source DNA was defined as the absolute LOD of the developed multiplex system.

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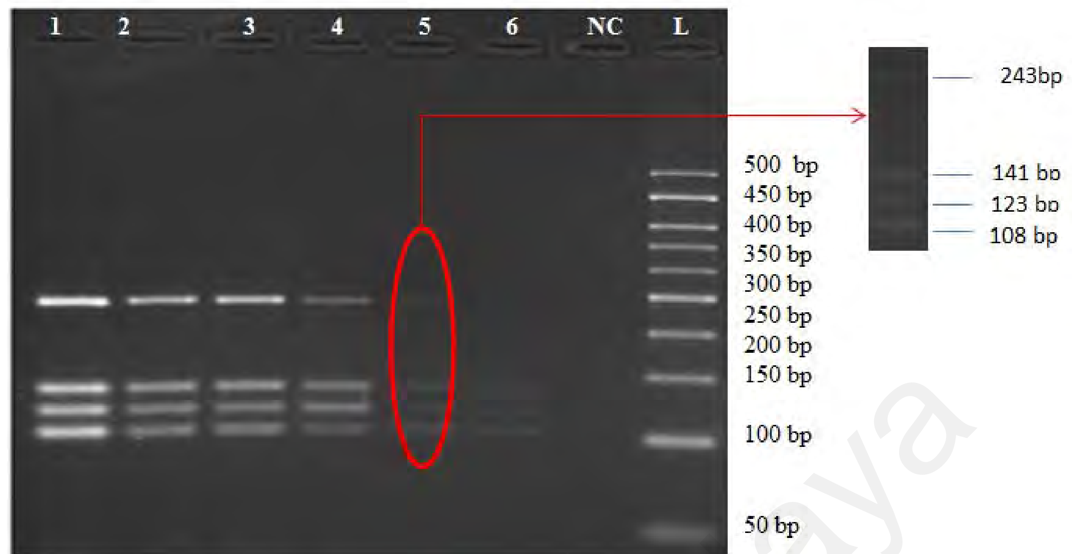

Figure 4.9: Sensitivity test of multiplex PCR using 10 - 0.0001 ng of DNA template from each target species (rat, rabbit and squirrel) in a common reaction mixture. Lane 1-6: 10, 1, 0.1, 0.01, 0.001, 0.0001 ng DNA of each species. Lane NC: negative control and Lane L: DNA ladder.

4.4.4 Target DNA Stability Test using the Multiplex PCR Assay

To evaluate the detection efficiency of the developed multiplex PCR, various heat treated meat samples were analyzed. For this purpose, rabbit, rat and squirrel meat were subjected to three different thermal treatment processes, namely boiling autoclaving and microwave cooking. The method of the cooking was described in earlier literatures (Ali et al., 2015c) and in section 3.5.4. The developed multiplex PCR system successfully identified all target species rabbit, rat and squirrel under all thermal processing conditions, including extensive autoclaving for (121°C at 15-psi for 2.5h) and extensive microwaving at 700 watt for 30 min (Figure 4.10),

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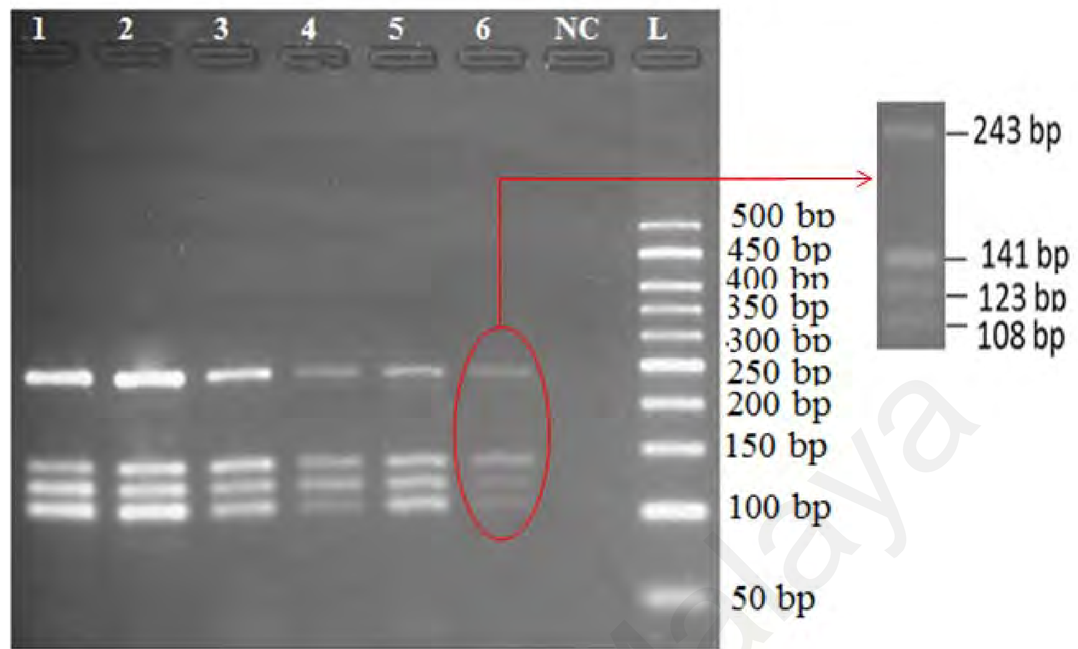

Figure 4.10: Stability test of multiplex PCR. Lane 1: boiling at 1000C for 90 minutes. Lane 2-4: 30 minutes microwave cooking at 500 watt, 600 watt and 700 watt respectively. Lane 5-6: autoclaved (1210C,15 Ibs) for 20 minutes and 2.5 hours respectively. Lane NC: negative control and Lane L: DNA ladder.

4.4.5 Sensitivity test of Multiplex PCR assay

4.4.5.1 Sensitivity test of multiplex PCR assay using DNA from raw meatball:

Meat ball is one of the popular meat products all over the world. Deliberatly contaminated model meatballs of each target species were prepared and auotoclaved at 121°C for 2.5h under 15-psi pressure to simulate extensive cooking effect.in laboratory. The chicken model meatball was deliberately adulterated with 0.1% of squirrel, rat and rabbit separately and showed the band for squirrel (243 bp), for rabbit (123 bp) and rat (108 bp) in the lane 1, lane 2 and lane 3 of the Figure 4.11.respectively. On the other hand, the chicken meatball was spiked with 1%, 0.5% and 0.1% menace meat of three target species and reflected the PCR band of three target species in the Figure 4.11, lane 4, lane 5 and lane 6 of the Figure 4.11 for 1%, 0.5% and 0.1% spiking respectively. In this experiment, the PCR product was in carried out in the 2.5% (w/v) agarose gel in gel electrophoresis to visualized the clear band of the multiplex PCR product. Generally,

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the PCR product was carried out in the 2% (w/v) agarose gel for pure meat sample. In the admixed condition, the gel concentration was 2.5% because I found the more concentration showed the clear band than the lower concentration. The 0.1% adulterated autoclaved meatballs also positively amplified the DNA of squirrel, rabbit and rat meat. Thus the relative LOD of the assay was 0.1%. The commercial meatball of different branded were purchased from different selling outlets across Malaysia on three different dates. In case of commercial meat ball products, no target species was amplified, reflected that commercial meatball was not contaminated with rabbit, rat and squirrel in Malaysia (Table 4.9).

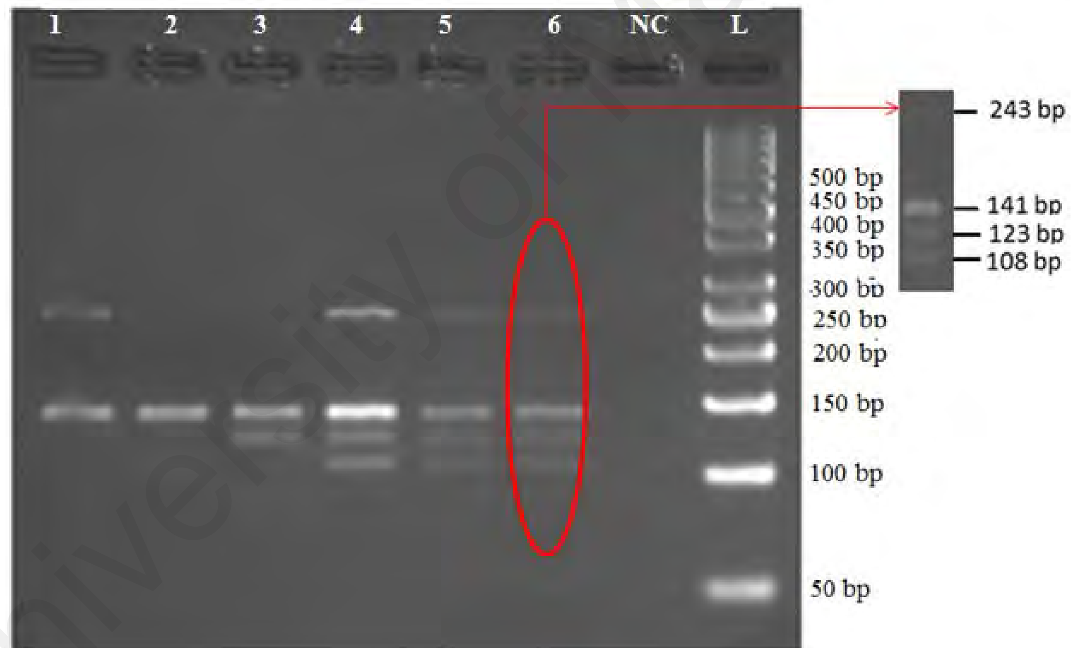

Figure 4.11: Specificity of multiplex PCR under food matrices. Lane 1-3: Simplex PCR for chicken meatball contaminated with 0.1% minced meat of squirrel, rat and rabbit, respectively and lane 4-6: multiplex PCR for chicken meatball contaminated with 1%, 0.5% and 0.1% minced meat of squirrel, rat and rabbit, respectively. Lane NC: Negative control. Lane L: DNA ladder. Please note that IAC (141) was amplified from all samples.

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Table 4.9: Analysis of admixed of commercial meatball products with rat, rabbit and squirrel meat specific PCR assay

Meat products	Contamination label (%)	Number of samples	Rabbit DNA detection	Rat DNA detection	Squirrel DNA detection	Detection accuracy (%)
Model meatball						
Pure chicken meatball	0	9	0/9	0/9	0/9	100
Rabbit-spiked chicken meatball	1	9	9/9	0/9	0/09	100
	0.5	9	9/9	0/9	0/9	100
	0.1	9	9/9	0/9	0/9	100
Rat-spiked chicken meatball	1	9	0/09	9/9	0/09	100
	0.5	9	0/09	9/9	0/09	100
	0.1	9	0/09	9/9	0/09	100
Squirrel-spiked chicken meatball	1	9	0/09	0/09	9/9	100
	0.5	9	0/09	0/09	9/9	100
	0.1	9	0/09	0/09	9/9	100
Commercial chicken meatball						
Ramly	-	9	0/9	0/9	0/9	NA
Tesco	-	9	0/9	0/9	0/9	NA
Ayamas	-	9	0/9	0/9	0/9	NA
Farm's best	-	9	0/9	0/9	0/9	NA
Ayamas breaded	-	9	0/9	0/9	0/9	NA
Figo Foods	-	9	0/9	0/9	0/9	NA
ARMIYA	-	9	0/9	0/9	0/9	NA
Hip chick Farms	-	9	0/9	0/9	0/9	NA

Note: NA, Not Applicable

4.4.5.2 Sensitivity test of multiplex PCR assay using DNA from frankfurter meat

Frankfurter is another widely consumed meat product all over the world. Deliberately contaminated model frankfurters were prepared in laboratory as described in section 3.5.9. The different branded commercial frankfuter were purched from different outlets in Malaysia on three different dates. The model chicken and beef frankfuter were deliberately adulterated with 1%,0.5% and 0.1% of raw meat of three target species (rabbit, rat and squirrel). The 0.1% spiked frankfuter were autoclaved at 121°C for 2.5 h under 15-psi and 45-psi pressure, respectively to simulate extensive cooking effect. The experimental finding of frankfuter is given in Figure 4.12 and analytical data are presented in Table 4.10. The model frankfuter adulterated with 1%,

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0.5% and 0.1% of target species, amplified all the tree targets. The Figure 4.12. Lane 1-3 and Lane 5-7 represented the three-target species. The 0.1% adulterated autoclaved chicken and beef frankfuter also positively amplified the rabbit, rat and squirrel (lane4 and. lane 8 in Figure 4.12). In case of commercial frankfuter, no DNA of target species was amplified without IAC, reflected the accuracy of the multiplex PCR assay.

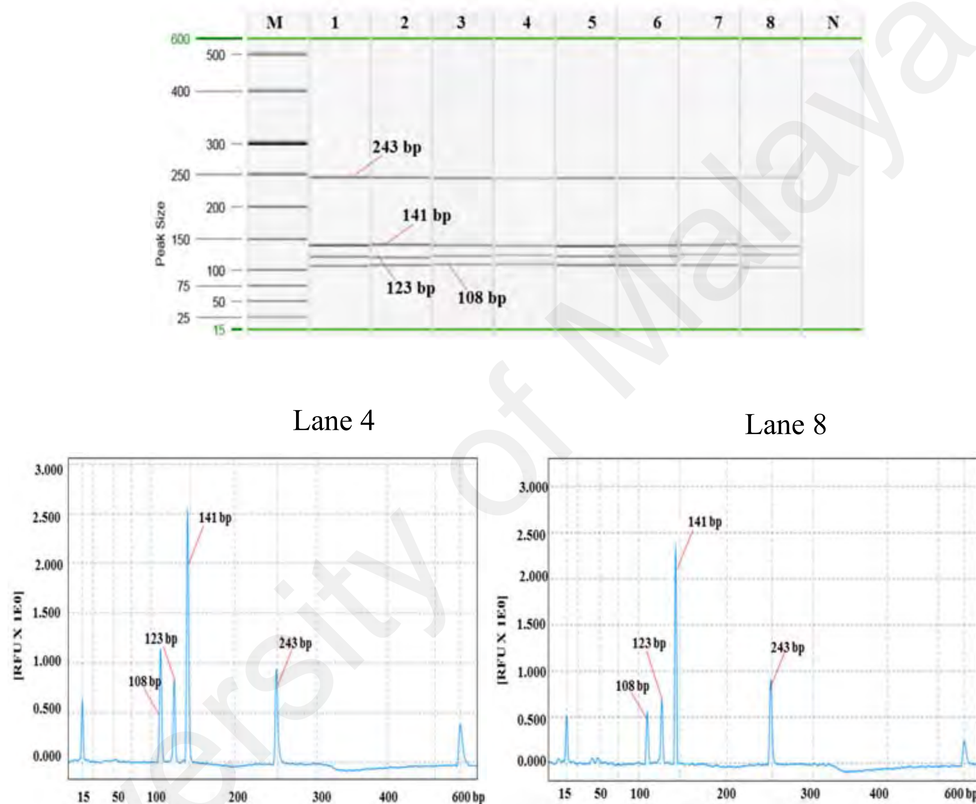

Figure 4.12: Gel image and the electropherograms of multiplex PCR for the detection of squirrel, rat and rabbit in deliberately adulterated model beef and chicken frankfurters under raw and processed states. In the gel image, M, Ladder; Lane 1−3, multiplex PCR of beef frankfurter and Lanes 5−7 multiplex PCR of chicken frankfurter spiked with 1%, 0.5%, and 0.1% meat from each of squirrel, rabbit and rat species under raw state. Lanes 4 and 8 multiplex PCR of heat-treated (autoclaved at 121°C und 15 psi for 2.5 h) 0.1% squirrel, rabbit and rat meat adulterated beef, and chicken frankfurters, respectively; Lane N, negative control. The corresponding electroferograms of Lane 4 and 8 are as shown.

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Table 4.10: Analysis of admixed of commercial frankfuter products with rat, rabbit and squirrel meat specific PCR assay.

Items	Contamination label (%)	Number of samples	Rabbit DNA detection	Rat DNA detection	Squirrel DNA detection	IAC	Detection accuracy (%)
Meat products							
Pure CFF	0	9	0/9	0/9	0/9	9/9	100
Rabbit-spiked ACFF	1	9	9/9	0/9	0/9	9/9	100
	0.5	9	9/9	0/9	0/9	9/9	100
	0.1	9	9/9	0/9	0/9	9/9	100
Rat-spiked CFF	1	9	0/9	9/9	0/9	9/9	100
	0.5	9	0/9	9/9	0/9	9/9	100
	0.1	9	0/9	9/9	0/9	9/9	100
Squirrel-spiked CFF	1	9	0/9	0/9	9/9	9/9	100
	0.5	9	0/9	0/9	9/9	9/9	100
	0.1	9	0/9	0/9	9/9	9/9	100
Pure BFF	0	9	0/9	0/9	0/9	9/9	100
Rabbit-spiked BFF	1	9	9/9	0/9	0/9	9/9	100
	0.5	9	9/9	0/9	0/9	9/9	100
	0.1	9	9/9	0/9	0/9	9/9	100
Rat-spiked BFF	1	9	0/9	9/9	0/9	9/9	100
	0.5	9	0/9	9/9	0/9	9/9	100
	0.1	9	0/9	9/9	0/9	9/9	100
Squirrel-spiked BFF	1	9	0/9	0/9	9/9	9/9	100
	0.5	9	0/9	0/9	9/9	9/9	100
	0.1	9	0/9	0/9	9/9	9/9	100
Commercial CFF-							
Ramly	-	9	0/9	0/9	0/9	9/9	NA
Tesco	-	9	0/9	0/9	0/9	9/9	NA
Ayamas	-	9	0/9	0/9	0/9	9/9	NA
Prima	-	9	0/9	0/9	0/9	9/9	NA
Commercial BFF-							
Ramly	-	9	0/9	0/9	0/9	9/9	NA
Figo Foods	-	9	0/9	0/9	0/9	9/9	NA
SAUDI Gold	-	9	0/9	0/9	0/9	9/9	NA
Farm's best	-	9	0/9	0/9	0/9	9/9	NA

Notes: NA, Not Applicable; CFF, Chicken Frankfurter; BFF, Beef Frankfurter; IAC, Internal Amplification Control.

4.5 PCR Product Authentication by RFLP Analysis:

4.5.1 Authentication of Rabbit, Rat and Squirrel PCR Products of Raw Meat by RFLP Analysis

To authenticate the PCR products of rabbit, rat and squirrel, the PCR product were digested with restriction enzymes which was cited in section 3.6.1. The clear bands

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from each target PCR product were found after treatment of restriction enzyme. In this analysis, each PCR product was digested separately with an selected restriction enzyme to study its individual restriction fragment (Table 3.10, Figure 4.13). Both rabbit (123bp) and squirrel (243bp) PCR product were digested by BtsCI restriction enzyme and two fragments were generated from each target (115 and 8 bp for Rabbit ((lan 6) and 176 and 67 bp for squirrel (lane 2) in Figure 4.13). Whereas the PCR product of rat (108 bp) were digested with BtsIMutI which produced two fragment of PCR product (64 and 44 bp (lane 4) in Figure 4.13). Moreover 8 bp fragment was not visualized because the gel instrument can detect 15bp fragment size. On the other hand, the multiplex PCR product were digested with the two enzyme in a single PCR tube and total seven fragment were visualized after gel electrophoresis (Figure 4.13, Lane 8).

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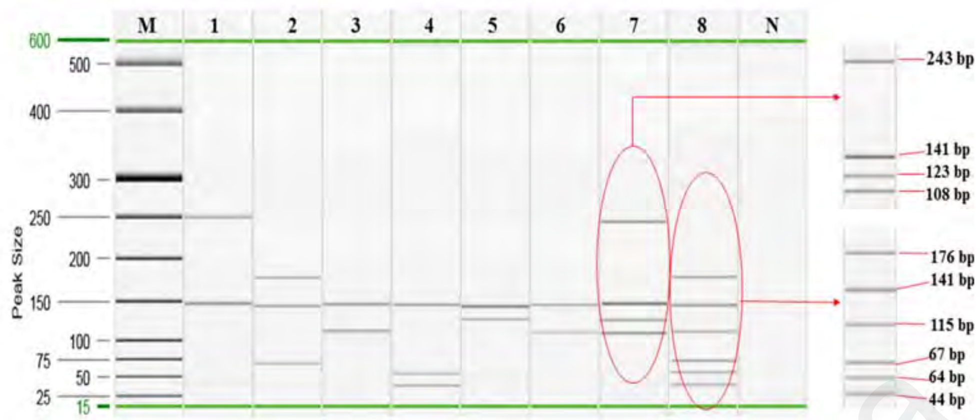
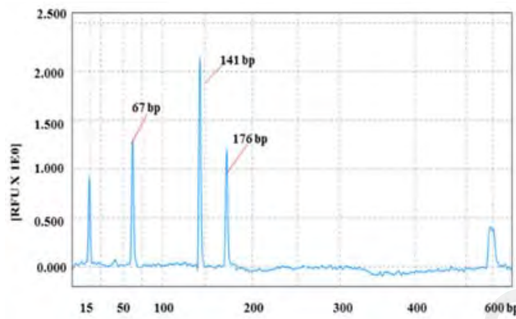
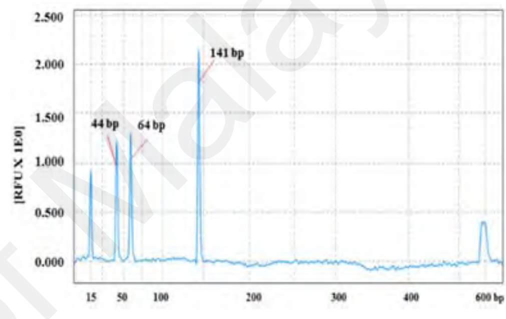


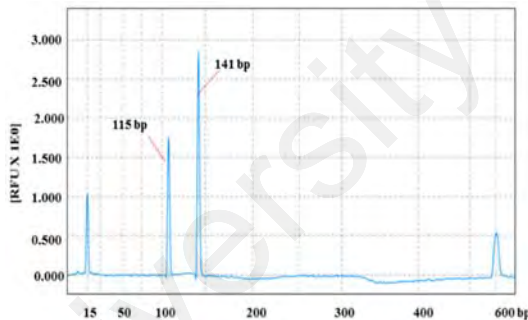
Lane 2



Lane 4



Lane 6



Lane 8

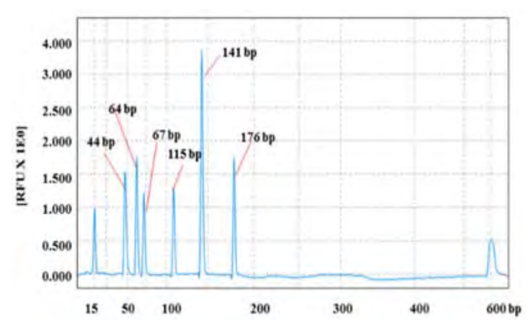


Figure 4.13: RFLP analysis of duplex (lanes 1, 3, 5) and multiplex PCR (lane 7, 8) products before (Lanes 1, 3, 5, and 7) and after (Lanes 2, 4, 6 and 8) restriction digestion. In the gel images: lane M, Ladder; lanes 1-8: endogenous control (141 bp); lanes N, negative template control; lanes 1 and 2, squirrel; lanes 3 and 4, rat; lanes 5 and 6, rabbit; lanes 7 and 8, multiplex PCR of squirrel, rabbit and , rat. The corresponding electropherograms are shown with labels.

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4.5.2 Authentication of Multiplex PCR Products of Frankfuter by RFLP Analysis

After optimization of multiplex PCR RFLP assay, it was also evaluated the validity and stability of the developed PCR RFPL assay was evaluated. In this study, dummy beef and chicken frankfuter were prepared and deliberately adulterated with target species (rabbit, rat and squirrel). The multiplex PCR products derived from raw, boiled and autoclaved meat product were digested with their selected restriction enzyme. After restriction digestion, the generated of total seven fragment were clearly showed in the gel electrophoresis, showed their stability and validity in raw, boiled and autoclave condition (Figure 4.14).

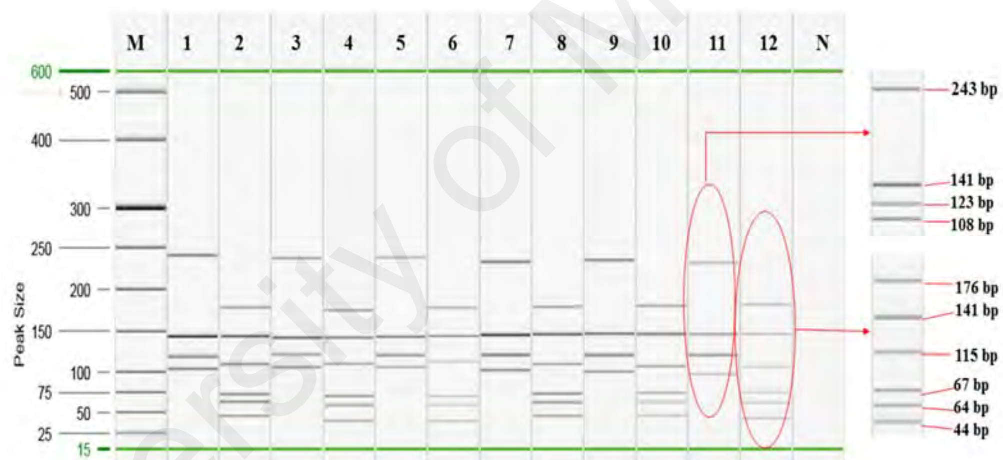

Figure 4.14: PCR-RFLP analysis of multiplex PCR products using capillary electrophoresis from deliberately adulterated raw (lanes 1, 2, 7, 8), boiled (lanes 3, 4, 9, 10) and autoclaved (lanes 5, 6, 11, 12) beef (Lanes 1−6) and chicken (Lanes 7−12) frankfurters. In gel image, Lanes 1 and 2, squirrel, rabbit and rat meat adulterated raw beef frankfurter before and after digestion, respectively; Lanes 3 and 4, squirrel, rat and rabbit meat-adulterated boiled (98 °C for 90 min) beef frankfurter before and after digestion, respectively; Lanes 5 and 6, squirrel, rat and rabbit meat-adulterated autoclaved (121° C and 15 psi pressure for 2.5 h) beef frankfurter before and after digestion, respectively; Lanes 7 and 8, squirrel, rat and rabbit adulterated raw chicken frankfurter before and after digestion, respectively; Lanes 9 and 10, squirrel, rat and rabbit meat -adulterated boiled (98 °C for 90 min) chicken frankfurter before and after digestion, respectively; Lanes 11 and 12, squirrel, rat and rabbit meat adulterated autoclaved (121°C and 15 psi pressure for 2.5 h) chicken frankfurter before and after digestion, respectively. In the capillary gel electrophoresis images: Lanes 1-12: endogenous control (141 bp); Lane N, negative template control.

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4.6 Real –Time PCR Assay

4.6.1 Development of Multiplex qPCR Model

The species specific primers and probes were carefully evaluated for mismatch and melting temperature (Tm) because in a multiplex PCR system multiple primers and probes interact with several templates at the same and very closely related temperature (Cheng et al., 2014). In this assay, squirrel, rat, rabbit and cat specific primers and probes had very closely spaced Tm (58 ± 1 and 69 ± 1 °C) that ensured proper annealing of the primers and probes with their respective templates at a selective PCR condition.(Cheng et al., 2014)The Tm values of the developed primers were 58.8 -60.3 °C and so all the primers were annealed at 58 °C but about 11 °C higher Tm of the probes allowed preferential primers' annealing before binding of the probes and it was necessity for TqMan chemistry (Arya & Iqbal, 2005).

The assay allowed the discrimination of four different amplicon in the same reaction tube through four different florescent reporter dyes tagged with the probes (Table 3.13). Initially simplex qPCR system for each target species was optimized and then sequentially duplex, triplex, tetraplex and finally multiplex qPCR was optimized. The Ct values of the multiplex qPCR assay were Ct = 17.16 ± 0.05, 17.62 ± 0.07, 17.14 ± 0.04 and 16.92 ± 0.04 and they were very close to the corresponding Ct values of simplex qPCR for squirrel (Ct = 17.06 ± 0.05), rat (Ct = 17.46 ± 0.06), rabbit (Ct = 17.1 ± 0.03) and cat (Ct = 16.86 ± 0.06), respectively. Thus, the findings of the simplex and multiplex qPCR systems were very consistent, mutually validating each other.

4.6.2 Specificity Evaluation of the Multiplex qPCR System

The species specificity of the multiplex qPCR system was critically evaluated because it is the foundation pillar of any PCR systems. Following optimization, specificity was tested by cross-challenging the primers and probes against 22 non-target

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species on three different days in triplicates. In order to avoid false negative detection, IAC was used as universal internal target to ensure that good quality DNA templates were present in all tubes (Figure 4.15). On the other hand, a blank or negative template control was made with everything except the replacement of template with equal volume of nuclease free deionized water to eliminate the chances of any false positive amplification. The obtained amplification profile clearly demonstrated that the species-specific amplification curves and background fluorescence were realized only for the relevant species in a 40 cycle PCR, confirming that no cross-amplifications took place in the multiplex qPCR system (Figure 4.15). While the amplification signal (Ct values) of the multiplex qPCR assay for squirrel, rat rabbit and cat were 17.143 ± 0.04, 17.48 ± 0.03,17.196 ± 0.05 and 16.8 ± 0.09, respectively, only IAC signal (Ct = 15.24 -18.78) was obtained for the other 22 non-target species (Table 4.11).

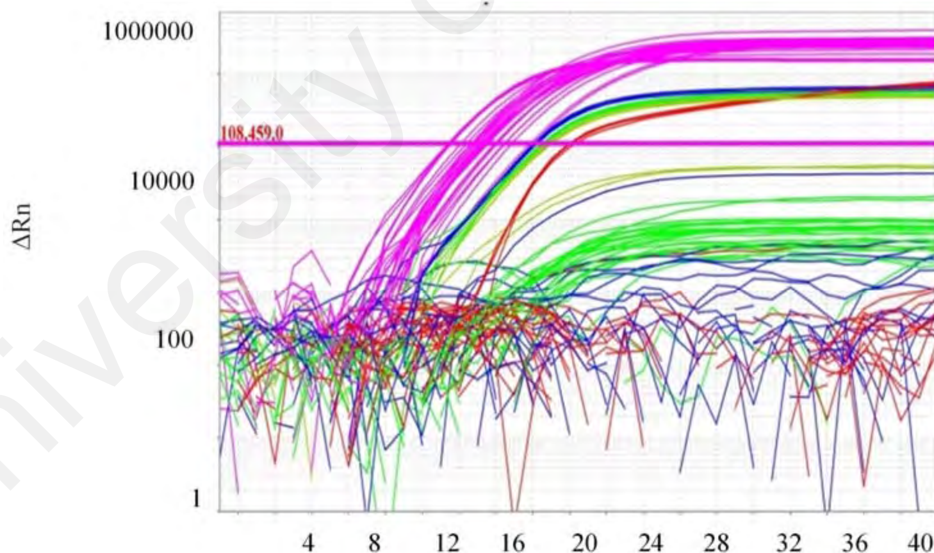

Figure 4.15: Multiplex qPCR amplification plot for squirrel (red), rat (lime), rabbit (blue)and cat (bright green) species along with the endogenous control for eukaryotes (pink) against 22 species (below the threshold cycle).

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Table 4.11: Specificity/Cross-Reactivity Test of Multiplex qPCR and Endogenous System

	multiplex real-time PCR system		endogenous PCR system	
animal species tested	**increase of fluorescence signal**	**mean Ct value**	**increase of fluorescence signal**	**mean Ct value**
Squirrel	+	17.14±0.04	+	15.44±0.13
Rat	+	17.48±0.03	+	16.11±0.09
Rabbit	+	17.19±0.05	+	16.87±0.1
Cat	+	16.8±0.09	+	15.79±0.12
Chicken	-	-	+	15.24±0.06
Goat	-	-	+	16.58±0.13
Cow	-	-	+	18.7±0.16
Buffalo	-	-	+	16.21±0.09
Sheep	-	-	+	15.52±0.15
Pigeon	-	-	+	17.42±0.14
Duck	-	-	+	15.84±0.13
Pig	-	-	+	16.22±0.13
Turkey	-	-	+	15.36±0.14
Quail	-	-	+	15.71±0.11
Monkey	-	-	+	16.24±0.11
Donkey	-	-	+	15.48±0.16
Salmon	-	-	+	18.78±0.16
Tuna	-	-	+	17.6±0.06
Frog	-	-	+	16.24±0.12
Crocodile	-	-	+	16.63±0.08
Turtle	-	-	+	16.18±0.07
Deer	-	-	+	15.59±0.08
Dog	-	-	+	15.9±0.11
Onion	-	-	+	17.24±0.06
Chili	-	-	+	17.48±0.13
Wheat	-	-	+	17.79±0.13
Cucumber	-	-	+	16.68±0.14

4.6.3 Limit of Detection

The limit of detection (LOD) of an assay determines the minimum amount of target analytes that could be detected in an adulterated food stuff. So, it was ascertained by analyzing serially diluted DNA extracts mixed into equal proportion in a fixed amount of genomic DNA and subsequent PCR amplification. In this case, 10-fold serially diluted mixed genomic DNA was used and so the concentration of the diluted DNA sample of each target species was 30, 3, 0.3, 0.03, 0.003 ng/µl. When the mixtures were run in a PCR machine, the amplification curve reflected the corresponding Ct values that varied from higher to lower concentrations of each DNA sample. The resulted Ct

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values and the corresponding relative standard deviations (RSD) for all diluted DNA sample were listed in Table 4.12 . It was found that the assay could detect and quantify the 0.003 ng/µl of DNA from each target species under mixed states with RSD values 0.07 - 0.56. Recently, Camma, Domenico, & Monaco, (2012). reported an LOD of 0.02 pg and 0.80 pg of template DNA in a qPCR assay for turkey, chicken, beef, pork and sheep meat in complex food matrices. On the other hand, Cheng et al. (2014) etected 0.15 ng/µl DNA from blood curd samples of duck, pig and chicken. Similarly, 0.32 ng of DNA was determined by Koppel et al. (2008) from boiled and raw sausages as well as fresh meat of beef, pork, chicken and turkey. These studies clearly revealed that LOD may vary from species to species and depends on many factors such as degree of decomposition, sample age, processing conditions and background matrices. The LOD of the developed multiplex qPCR assay was 0.003 ng DNA and so it reflected very high level of sensitivity to detect and quantify the aforesaid cat, rabbit, rat and squirrel species under complexed matrices and processed conditions.

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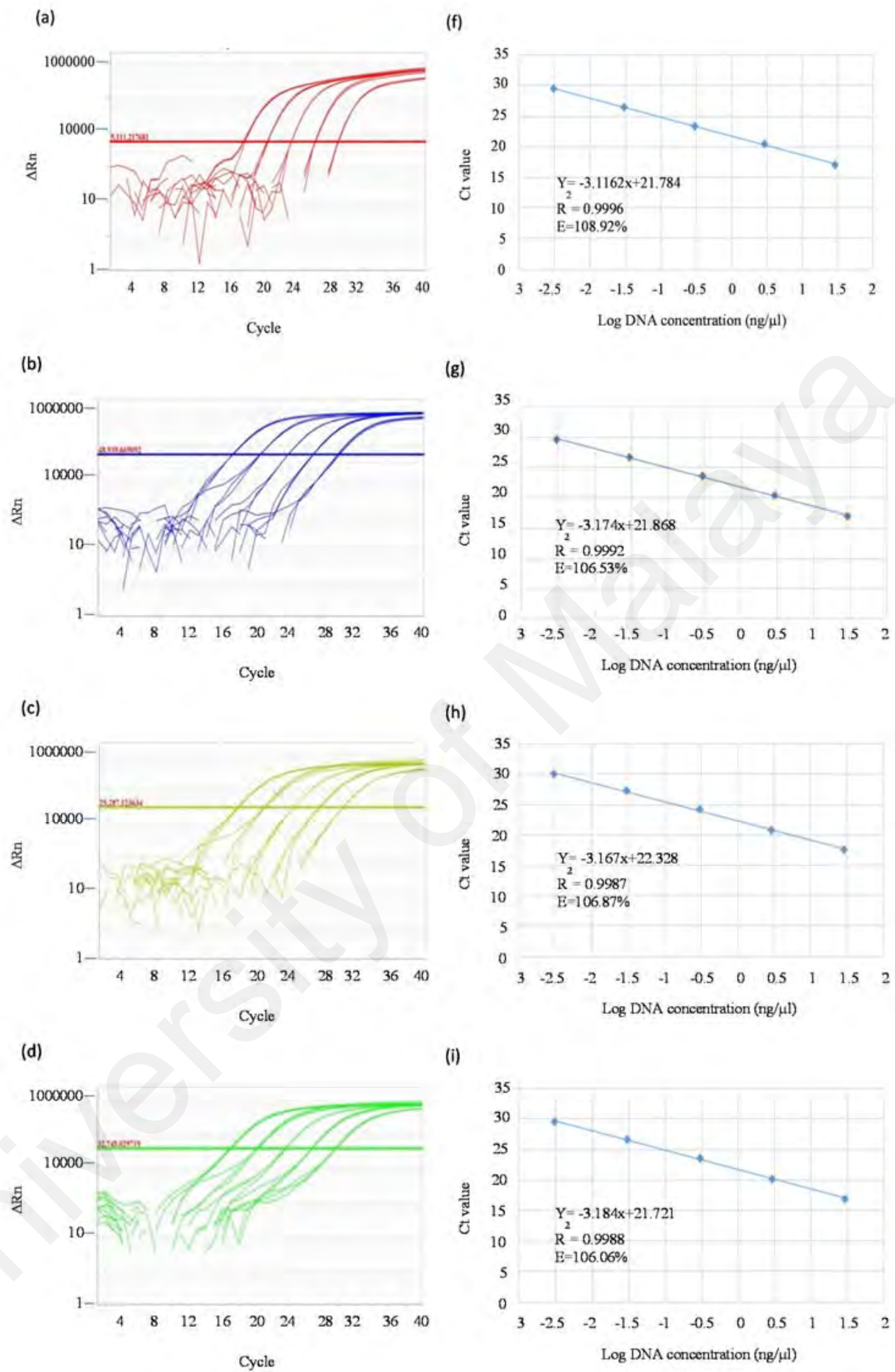

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Figure 4.16: Amplification plots (a-e) and standard curves (f-j) of multiplex qPCR products obtained from 10-fold serially diluted mixed DNA of four target species. Amplification plots and standard curves for squirrel (a and f), for rabbit (b and g), and for rat (c and h), for cat (d and i) specific qPCR system, respectively.

Table 4.12: Ct value of each target species obtained from the amplification plot with a 10- fold serially Diluted DNA of Each Target Species

	Squirrel				Rat				Rabbit				Cat			
DNA Concentration (ng)	Ct value	mean Ct value	SD	RSD	Ct value	mean Ct value	SD	RSD	Ct value	mean Ct value	SD	RSD	Ct value	mean Ct value	SD	RSD
30	17.113				17.69				17.055				16.956			
	17.007	17.081	0.064	0.38	17.513	17.59	0.09	0.52	17.045		0.03	0.18	16.88		0.053	0.32
	17.125				17.567				17.102	17.067			16.983	16.939		
3	20.459				20.808				20.322				20.163			
	20.496	20.466	0.026	0.13	20.618	20.751	0.116	0.56	20.492		0.085	0.42	20.078		0.113	0.56
	20.445				20.829				20.428	20.414			20.302	20.181		
0.3	23.434				24.179				23.715				23.552			
	23.455	23.457	0.024	0.1	24.177	24.188	0.017	0.07	23.719		0.011	0.05	23.535		0.033	0.14
	23.482				24.209				23.736	23.723			23.6	23.562		
0.03	26.417				27.264				26.683				26.766			
	26.483	26.46	0.037	0.14	27.258	27.309	0.083	0.31	26.695		0.044	0.17	26.675		0.048	0.18
	26.48				27.406				26.766	26.714			26.747	26.729		
0.003	29.563				30.124				29.742				29.658			
	29.681	29.618	0.059	0.2	30.131	30.1	0.047	0.16	29.865		0.126	0.42	29.465		0.137	0.46
	29.611				30.046				29.613	29.74			29.393	29.538		

Note: *SD*, standard deviation; *RSD*, relative standard deviation

4.6.4 Target Quantification and qPCR Efficiency

Each of the target species DNA was quantified from standard curve and for this purpose 30 ng/µl DNA extracted from equal meat mixture (1:1:1:1) of each target species was 10-fold serially diluted to get 3, 0.3, 0.03, 0.003 ng of total DNA in the reaction mixture. Multiplex qPCR was performed using each of these diluted DNA sample and five different standard curves were constructed for squirrel, rat, rabbit, cat and IAC by plotting the Ct value against the logarithmic concentration of DNA (Figure 4.16). A good linear regression was found for all of the standard curves as reflected by the respective regression coefficient (R^2), 0.9996, 0.9987, 0.9992, 0.9988 and 0.9988 for squirrel, rat, rabbit, cat and IAC, respectively. The corresponding slope of each standard curve were -3.1162, -3.1671, -3.174, -3.184 and -3.2204, respectively. Thus the calculated PCR efficiency was 109.36%, 106.87%, 106.53%, 106.06% and 104.4% for squirrel, rat, rabbit, cat and IAC, respectively.

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Table 4.13: Mean Ct values and Inter Day RSD of Different Model Meat Products

			Mean Ct value				
Products	**spike level (%)**	**species**	**day 1**	**day 2**	**day 3**	**SD**	**RSD (%)**
Chicken-burger	10	squirrel	18.437	18.614	18.782	0.172	0.93
		rat	19.141	19.275	19.026	0.124	0.65
		rabbit	18.747	18.698	18.631	0.058	0.31
		cat	18.632	18.5	18.589	0.067	0.36
	1	squirrel	21.715	21.637	21.841	0.102	0.47
		rat	22.383	22.589	22.145	0.222	0.99
		rabbit	21.73	21.814	21.767	0.042	0.19
		cat	21.72	21.952	21.714	0.135	0.62
	0.1	squirrel	24.936	24.795	24.647	0.144	0.58
		rat	25.537	25.159	25.492	0.206	0.81
		rabbit	24.946	25.204	25.175	0.141	0.56
		cat	25.138	24.912	25.016	0.133	0.45
Chicken-frankfuter	10	squirrel	18.639	18.821	18.487	0.167	0.9
		rat	19.044	19.129	19.178	0.067	0.35
		rabbit	18.671	18.784	18.553	0.115	0.62
		cat	18.606	18.418	18.624	0.144	0.62
	1	squirrel	21.748	21.967	21.693	0.144	0.66
		rat	22.129	22.583	22.315	0.228	1.02
		rabbit	21.706	21.752	21.983	0.148	0.68
		cat	21.857	21.946	21.592	0.184	0.84
	0.1	squirrel	24.975	24.645	24.512	0.238	0.96
		rat	25.608	25.73	25.046	0.364	1.43
		rabbit	24.876	24.695	24.783	0.09	0.37
		cat	24.869	24.513	24.988	0.247	1
Beef-burger	10	squirrel	18.627	18.784	18.393	0.196	1.06
		rat	19.331	19.174	19.014	0.158	0.83
		rabbit	18.485	18.63	18.498	0.08	0.43
		cat	18.458	18.557	18.519	0.049	0.27
	1	squirrel	21.762	21.295	21.932	0.329	1.52
		rat	22.182	22.198	22.216	0.017	0.08
		rabbit	21.73	21.585	21.618	0.307	1.4
		cat	21.756	21.642	21.759	0.075	0.35
	0.1	squirrel	24.813	24.958	24.441	0.266	1.08
		rat	25.148	25.83	25.52	0.341	1.34
		rabbit	24.998	24.817	24.793	0.112	0.45
		cat	24.673	24.975	24.751	0.156	0.63
Beef-frankfuter	10	squirrel	18.686	18.493	18.932	0.22	1.18
		rat	19.149	19.387	19.152	0.136	0.71
		rabbit	18.74	18.661	18.485	0.13	0.7
		cat	18.514	18.482	18.414	0.051	0.28
	1	squirrel	21.874	21.632	21.325	0.275	1.27
		rat	22.359	22.198	22.269	0.08	0.36
		rabbit	21.549	21.746	21.619	0.099	0.46
		cat	21.98	21.462	21.518	0.284	1.31
	0.1	squirrel	24.764	25.01	24.912	0.123	0.5
		rat	25.791	25.371	25.448	0.223	0.88
		rabbit	24.812	24.549	24.992	0.222	0.9
		cat	24.751	24.938	24.563	0.187	0.76

***Note*:** *SD,* standard deviation; *RSD,* relative standard deviation.

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4.6.5 Sensitivity and Validity of the multiplex qPCR Assay using Commercial Meats

To evaluate the sensitivity of the multiplex qPCR assay under complex matrices, two types of model meat products were made following Ali et al. 2013. The chicken and beef burger and frankfurter were spiked with 10%, 1 and 0.1% of squirrel, rat, rabbit and cat meat. The multiplex qPCR assay was performed using the as extracted DNA from adulterated meat products (beef and chicken burgers and frankfurters). The Ct value for the lowest detectable quantity (0.1) were 24.441 ± 0.266 to 25.83 ± 0.341 for all the four target species (Table 4.13), but the IAC constantly yielded a mean Ct between 14.534 ± 0.13 and15.35 ± 0.19 for all level of adulteration, revealing the endogenous target did not change significantly with a variation in adulteration level because all adulterants were from eukaryotic origins. The analysis results revealed that the target recoveries were 91.667 - 122.333% for 10% to 0.1% spiked levels with systematic error between -8.333 to 22.333 and RSD 3.08 - 20.57% (Table 4.14). Thus, the maximum recovery was 122.333% for 1 % spiked beef frankfurter but the minimum was 91.667% for 0.1% spiked chicken burger. On the other hand, the maximum and minimum RSD were found in beef burger and chicken burger containing 0.1% adulteration, respectively. A graph was generated by plotting the recovered values (y axis) (Figure 4.17) against the reference (actual) values (x-axis) for each target and it provided a very high correlation coefficient ($R^2 = 0.9999$) (Figure 4.17), confirming that the experimental values were fairly close to the actual values (Rojas et al., 2010).

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Table 4.14: Reproducibility and Recovery of Target Species in Model Meat Products

			Content of target determine (%)						
Products	**spike level (%)**	**species**	**day 1**	**day 2**	**day 3**	**Mean**	**RSD (%)**	**recovery (%)**	**systematic error (%)**
Chicken burger	10	squirrel	11.548	10.132	8.95	10.21	12.74	102.1	2.1
		rat	10.146	9.204	11.03	10.126	9.02	101.266	1.266
		rabbit	9.692	10.044	10.546	10.094	4.25	100.94	0.94
		cat	9.336	10.271	9.631	9.746	4.9	97.46	-2.54
	1	squirrel	1.025	1.085	0.934	1.0146	7.49	101.466	1.466
		rat	0.961	0.827	1.142	0.976	16.19	97.666	-2.334
		rabbit	1.106	1.04	1.076	1.074	3.08	107.4	7.4
		cat	1.001	0.846	1.005	0.950	9.54	95.066	-4.934
	0.1	squirrel	0.095	0.105	0.117	0.105	10.42	105.667	5.667
		rat	0.097	0.128	0.1	0.108	15.78	108.333	8.333
		rabbit	0.106	0.088	0.09	0.094	10.42	94.667	-5.333
		cat	0.084	0.099	0.092	0.091	8.19	91.667	-8.333
Chicken frankfuter	10	squirrel	9.947	8.695	11.129	9.923	12.27	99.236	-0.764
		rat	10.887	10.235	9.876	10.332	4.96	103.326	3.326
		rabbit	10.243	9.434	11.162	10.279	8.41	102.796	2.796
		cat	9.513	10.899	9.39	9.934	8.44	99.34	-0.66
	1	squirrel	1	0.851	1.041	0.964	10.37	96.4	-3.6
		rat	1.156	0.831	1.009	0.998	16.3	99.866	-0.134
		rabbit	1.125	1.088	0.92	1.044	10.46	104.066	4.066
		cat	0.906	0.85	1.098	0.951	13.67	95.133	-4.867
	0.1	squirrel	0.092	0.118	0.13	0.113	17.14	113.333	13.333
		rat	0.092	0.084	0.12	0.098	19.16	98.666	-1.334
		rabbit	0.112	0.128	0.12	0.12	6.67	120	20
		cat	0.103	0.133	0.094	0.11	18.56	110	10
Beef burger	10	squirrel	10.036	8.936	11.93	10.3	14.7	103.006	3.006
		rat	8.837	9.905	11.127	9.956	11.51	99.563	-0.437
		rabbit	11.728	10.553	11.617	11.299	5.74	112.993	12.993
		cat	10.588	9.856	10.131	10.191	3.63	101.916	1.916
	1	squirrel	0.99	1.206	0.873	1.023	16.51	102.3	2.3
		rat	1.112	1.099	1.085	1.098	1.23	109.866	9.866
		rabbit	1.106	1.229	1.2	1.178	5.46	117.833	17.833
		cat	0.975	1.059	0.973	1.002	4.9	100.233	0.233
	0.1	squirrel	0.104	0.093	0.137	0.111	20.57	111.333	11.333
		rat	0.129	0.113	0.098	0.113	13.68	113.333	13.333
		rabbit	0.103	0.117	0.119	0.113	7.71	113	13
		cat	0.118	0.095	0.112	0.108	11.01	108.333	8.333
Beef frankfuter	10	squirrel	9.607	11.08	8.011	9.566	16.05	95.66	-4.34
		rat	10.087	8.484	10.065	9.545	9.63	95.453	-4.547
		rabbit	9.741	10.318	11.728	10.595	9.65	105.956	5.956
		cat	10.168	10.406	10.93	10.501	3.71	105.013	5.013
	1	squirrel	0.911	1.089	1.367	1.122	20.48	122.333	22.333
		rat	0.978	1.099	1.044	1.04	5.82	104.033	4.033
		rabbit	1.261	1.093	1.199	1.184	7.17	118.433	18.433
		cat	0.829	1.206	1.158	1.064	19.28	106.433	6.433
	0.1	squirrel	0.108	0.09	0.097	0.098	9.23	98.333	-1.667
		rat	0.081	0.109	0.103	0.097	15.09	97.667	-2.333
		rabbit	0.117	0.142	0.103	0.120	16.37	120.667	20.667
		cat	0.112	0.098	0.128	0.112	13.32	112.667	12.667

Note: RSD, relative standard deviation

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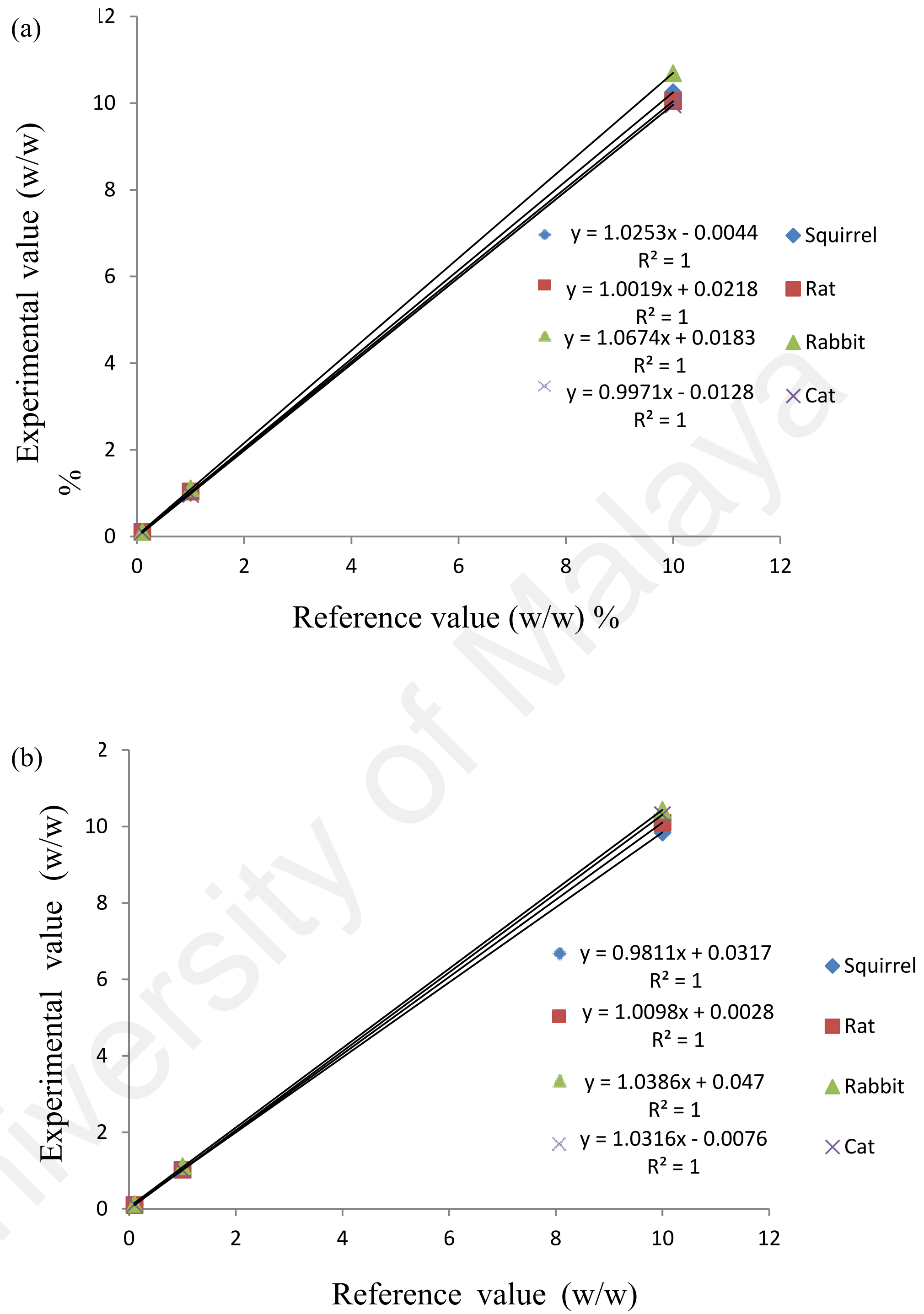

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Figure 4.17: Relationship between the experimental and reference values of the multiplex qPCR system

4.6.6 Residual Analysis

Residuals reflects the difference between the measured and practical values and allows the estimation of outliers and potential experimental errors (Ali, et al., 2012a). The resulted graph obtained from residuals and fitted recovery values of the variables for both burger and frankfuter of four target species (squirrel, rat and rabbit, and cat), the Figure 4.18 showed a random distribution of all variables and they were within +2.0 to -2.0 from the zero line for all meat products.

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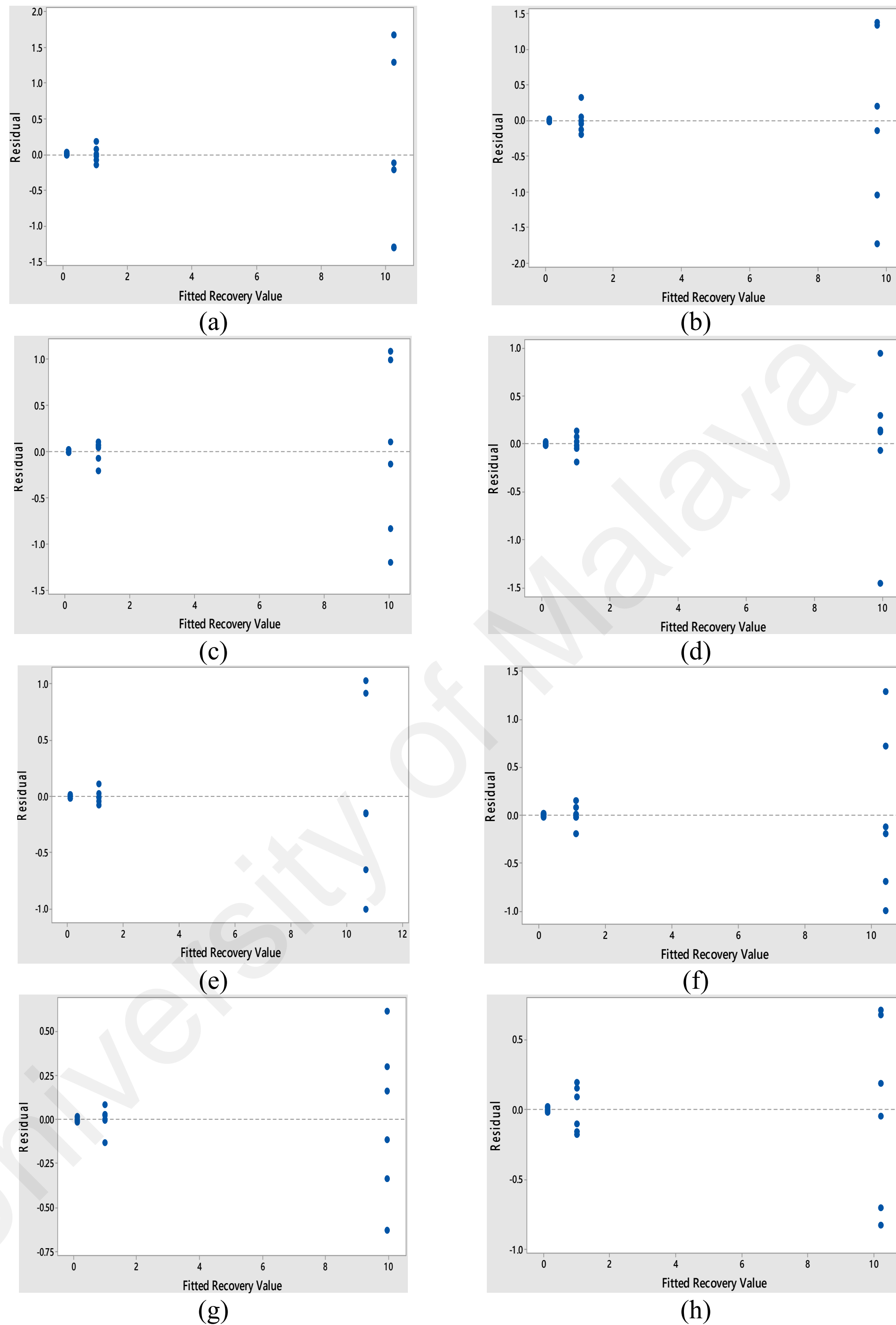

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Figure 4.18: Graph of residual versus fitted recovery values of the multiplex qPCR assay of model burger and frankfuter adulterated with 0.1,1 and 10% of target species. Figure (a-d) represent the squirrel, rat, rabbit and cat in burger respectively whereas and frankfuter (e-h) represented the squirrel, rat, rabbit and cat in frankfuter, respectively.

4.6.7 Commercial Burger and Frankfuter Analysis

The commercial food product such as burger, meatball, frankfurter, hotdog, nugget etc. are very popular all over the world. These types of products are highly processed that results in total or partial annihilation or modification of morphological features and other physical properties. Therefore, manufacturers can easily mix an unexpected and lower priced meat in the final products for profit making purposes. To prevent or monitor these types of undesirable incidences, the accurate screening of commercial meat products can play a great role and build public confidence on health, religious, social and cultural perspectives. In this work, two types of commonly used commercial products, namely, burger and frankfurter were evaluated using the developed multiplex qPCR system. A total of 72 burgers (36 beef and 36 chickens) and 72 frankfurters (36 beef and 36 chickens) were purchased from different Malaysian outlets and were tested (Table 4.15). The experimental results revealed that no target species (cat, rabbit, rat, and squirrel) were present in the burger and frankfurter products but only IAC was amplified, reflecting the 100% accuracy of the qPCR assay. The meat of cat, rat, rabbit and squirrel species was not mixed with commercial burger and frankfuter because this might be due to the tight monitoring of halal products in Malaysia by Government agencies because all model products were positively detected (Table 4.15).

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Table 4.15: Screening of Model and Commercial Meat Products Using the Developed multiplex qPCR Assaya

	Adulteration	Detected Species				
Sample	Spiked level of target species (%)	Squirrel	Rat	Rabbit	Cat	PCR accuracy (%)
Chicken burger	10	3/3	3/3	3/3	3/3	100
	1	3/3	3/3	3/3	3/3	100
	0.1	3/3	3/3	3/3	3/3	100
Chicken frankfuter	10	3/3	3/3	3/3	3/3	100
	1	3/3	3/3	3/3	3/3	100
	0.1	3/3	3/3	3/3	3/3	100
Beef burger	10	3/3	3/3	3/3	3/3	100
	1	3/3	3/3	3/3	3/3	100
	0.1	3/3	3/3	3/3	3/3	100
Beef frankfuter	10	3/3	3/3	3/3	3/3	100
	1	3/3	3/3	3/3	3/3	100
	0.1	3/3	3/3	3/3	3/3	100
commercial chicken burger						
ramly		0/9	0/9	0/9	0/9	100
tesco		0/9	0/9	0/9	0/9	100
ayamus		0/9	0/9	0/9	0/9	100
prima		0/9	0/9	0/9	0/9	100
commercial chicken frankfuter						
ramly		0/9	0/9	0/9	0/9	100
tesco		0/9	0/9	0/9	0/9	100
ayamus		0/9	0/9	0/9	0/9	100
prima		0/9	0/9	0/9	0/9	100
commercial beef burger						
ramly		0/9	0/9	0/9	0/9	100
tesco		0/9	0/9	0/9	0/9	100
ayamus		0/9	0/9	0/9	0/9	100
prima		0/9	0/9	0/9	0/9	100
Commercial beef frankfuter						
ramly		0/9	0/9	0/9	0/9	100
tesco		0/9	0/9	0/9	0/9	100
ayamus		0/9	0/9	0/9	0/9	100
prima		0/9	0/9	0/9	0/9	100

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CHAPTER 5: DISCUSSION

5.1 DNA Extraction

The yield of extracted total genomic DNA depends on quantity and quality of starting materials, state of samples (raw, processed, heat or chemical treated etc.), extracted kit and protocol (Martin-Laurent et al., 2001). To get good quality DNA, I used three different types of commercial DNA extraction kit for the extraction of total DNA from three different sample such as pure meat, meat products (burger, meatball and frankfuter) and plant species. Because specific type of kit was designed for specific sample depending on the presence of proteins, ingredients etc. Furthermore, commercial DNA extraction kits offered higher yield of DNA than the conventional liquid-liquid extraction techniques due to the present of aqueous and organic phases of this system. Moreover, commercial kits were safer for handling and there is minimal chance of damage of DNA during extraction (Martin-Laurent et al., 2001).

The Genomic DNA mini kit was designed for the purification of total DNA, including mitochondrial DNA and genomic DNA from different animal tissues. To shorten the cell lysis time, tthe kit was combined with micropestle which facilitated the disintegration of homogenized tissue specimens' efficiency. Protenase K and lysis buffer were used to perform cell lysis and degredation of protein to eliminate contamination of proteins. The use of chaotropic salt enhanced the stability DNA binding to the spin column glass fiber matrix, Effective wash buffer was used to remove any contamination followed by DNA was eluted using low salt containing TE buffer which facilitated the stabilization of storage DNA.

The concentrated of the extracted DNA was determined based on the absorbance reading at 260 nm and its purity was evaluated based on the ratio of absorbance at 260 nm and 280 nm. This is because 260 nm is the absorbance maxima of nucleic acids and

that at 280 nm reflects the absorbance maxima of proteins. Finally, the A260/A280 ratio provides the DNA purity indication with respect to the protein contamination.

I found the highest DNA yield in raw meat (150-250 ng/ul) and lowest in severely microwaved (700 w) samples (35-69 ng/ul) (Table 4.1). This might be due to the higher degree of denaturation and degradation of the DNA under extensive heat treatment (Ali et al., 2016). Similarly, second lowest DNA yield was obtained from the autoclaved samples (50-69 ng/ul for raw meat and 42-65 ng/ul for meat products), as prolonged heat and pressure are applied under autoclaved condition. The DNA concentration from the boiled treated samples were found relatively higher (80- 120 ng/ul for raw meat and 50-70 ng/ul for meat products) than those of the microwaved and autoclaved treated samples, this might be less due to the degradation and denaturation under relatively mild heat treatment. On the other hand, the purity and yield of DNA was comparatively higher in all pure meat samples (raw, boiled and autoclaved) than those of the meat products (raw, boiled and autoclaved); this might be due to the presence of higher amount of fat and food ingredients including salt, spices, vegetables and other food additives in the commercial meat products. The absorbance ratio at A260/A280 was between 1.7 and 2.0 for all extracted DNA. This ensured that good quality DNA was extracted from all samples and it was suitable for PCR amplification (Nejad, Tafvizi, Ebrahimi, & Hosseni, 2014).

5.2 Development of Biomarker

The motivation of adulteration comes from a company's interest in making a growing profit by selling a cheaper item in the name of its expensive counterparts. It incurs a serious risk especially when an animal material is involved. According to the US Department of Agriculture, about 75% of the recently emerging infectious disease affecting humans are the diseases of the animal origins (USDA,2015). Certain animals such as rat, cat and squirrel are also sensitive social and religious issues. Overall food

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falsification is a crime under the food and drug laws in most countries and its prevention is a long-cherished hope. In this regard, authentication technology plays a key role by verifying the food ingredients prior to the enforcement of regulatory lows. The key purpose is not to punish the violators but to prevent the practices at its origin for the greater societal and health benefits. The adulteration of rabbit meat with cat, rat and squirrel in many cases are reported, especially when societal issues are dominant such as in Malaysia. However, it is a matter of economic cheating and also it involves certain degree of health risk and socio-culture outburst depending on the place and availity (Girish et al., 2013; Karabasanavar, Singh, Umapathi, Girish, et al., 2011; Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013). Considering the needs, I developed here species-specific primers targeting the interspecies hyper variable and intra-species conserved regions of cytb and ATP6 genes of cat, rabbit, squirrel and rat. The mitochondrial DNSa (mtDNA) are more focused over the nuclear ones (nDNA) for authentication studies because of its maternal origins, extra protection by mitochondrial membrane and abundance in multiple copies (Girish et al., 2004; Zha, Xing, & Yang, 2010). Additionally, all targets were kept within 243 bp in length since short-targets are thermodinamicaaly more stable over the longer (Ali et al., 2016). Biomarker targets within this range (108 - 243 bp) were suitable for efficient amplification and suitable under extreme food processing conditions. Overall, this ensured better efficiency and accuracy of the assay to detect targets even in degraded samples (Ali et al., 2016). The success of an mPCR assay mainly depends on primer specificity and melting temperature (Tm). This is because all primers must anneal to their respective binding regions under the same set of PCR condition. In the design of species-specific primer, the oligonucleotide mismatch calculation plays critical roles since the efficiency of a PCR assay may reduce or amplification reaction may fail due to the presence of a presence of a critical mismatch in the primer binding site. In the present study, the

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developed primer sets contained 100% matching with specific gene target and 6 - 37 nucleotide (14.63 - 84.77%) mismatching with other related or non-target species, reflecting there is no probability of cross reaction even with closely related species during PCR assays. Because the presence of single mismatch at the primer binding position might be effective to failure the PCR amplification. Furthermore, identical Tm (58°C (57 - 59 °C)) of all primers confirms that all primers would anneal only with the target template and there is very little or no possibility to anneal with any others non-target species. The pairwise distances between the amplicon of each target species and the respective gene of the target and non-target species were analyzed using neighbor-joining method; while the zero distance was found for the exact species, the distances among the 10 rat species were 0 - 0.12 (Table 4.6), among the 8 squirrel species were 0 - 0.20 (Table 4.7), and among the 5 rabbit species were 0 - 0.35 (Table 4.5). The genetic distances among the targets and other non-targets were significantly higher (0.2 - 4.10), suggesting very little or no probability of cross-target amplification. Moreover, the analysis of phylogenetic tree (Figure 4.1) based on genome sequence demonstrated similer findings, supporting the result of other in silico tests. In addition, the 3D plot was created from the data of mismatch of primer pairs and positive distance, which also support the adequate genetic distance among the targets and non-targets species (Figure 4.2). Thus, bioinformatics studies ensured that there were no or very little chance for amplifying a cross- species target (Ali et al., 2014a). To confirm the theoretical finding, PCR experiments were carried out against 22 non-target species.

5.3 PCR Assay Optimization

Optimization of the PCR reaction is a vital step to get successful PCR Products. I optimized simplex PCR assay first and then duplex, triplex, and finally multiplex. Varies component the reaction was optimized step by step. First thing considered was the reaction volume; the higher reaction volume causes higher cost, but very low

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volume might be insufficient for the amplification of primers, particularly for the multiplex PCR assay. Therefore, we optimized in 25 ul reaction volume which was cost effective but sufficient for a multiplex PCR reaction. Buffer concentration is also important in PCR reaction. The cation of buffer neutralizes the negative charged of the phosphate group of DNA template which decrease the electrorepulsive forces of between the DNA stands. As a result, primer can come into contact with DNA strands easily that facilitate the annealing between them. By following the supplier instruction, we used 1x buffer concentration for successful reaction. Magnesium chloride plays a critical role for success PCR amplification.

Mg^{2+} is said to be a cofactor of the polymerase enzyme because it forms soluble complexes with deoxnucleotide triphosphate (dNTPs) to prepare a recognizable substrate for polymerase. Therefore, Mg^{2+} may affect DNA polymerase activity activity and fidelity, specificity of PCR, denaturation temperature of both template and PCR product DNA strand, annealing of primer and formation of primer dimer. Excess Mg^{2+} leads to nonspecific implication due to nonspecific primer annealing, while inadequate magnesium result in decreased the yield of the expected amplified product. Thus, for optimum activity, polymerase enzyme requires sufficient free magnesium other than that of bound with dNTP and template DNA (Markoulatos, Siafakas, & Moncany, 2002). Several experiments were repeated by changing the $MgCl_2$ concentration and finally optimized $MgCl_2$ which are 2.5, 3.5 and 4.0 mM for simplex, duplex and multiplex reaction, respectively. On the other hand, two different concentrations (0.2 mM and 0.3 mM dNTP for simplex and multiplex, respectively) of the dNTP (dATP, dCTP, dGTP, and dTTP) were used to optimize the simplex to multiplex reactions. Because, concentration of free Mg^{2+} is affected by the amount of dNTPs. Hence Mg^{2+} binds with dNTPs. DNA polymerase fidelity reduce due to the imbalance amount of four dNTPs (Kunz & Kohalmi, 1991), whereas, excess dNTPs may result in inhibition

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of amplification due to increase error rate of polymerase (Kramer & Coen, 2001) . Another important parameter determined experimentally was annealing temperature (Ta). The highest annealing temperature is favorable because it increased specificity by reducing non-specific binding of primer (Ali, Hashim, Mustafa, & Man, 2012c; Wu et al., 2009). Tm of all primers should be same in multiplex PCR assay because all primers are amplied in a single reaction tube with same conditions. Although Tm values of the developed three sets of primers were different (59.8 - 61.6°C) but all primers sets were able to amplify at same temperature (58°C), resulting the favorable for the development of mPCR assay (Figure 4.3) After optimization the simplex PCR, duplex and multiplex PCR were optimized step by step to eliminate the possibility of forming any unwanted primer dimer or multi primers (Figure 4.7) (Ali, et al., 2015c). The novel mPCR system clearly amplified target products squirrel, rat and rabbit. The well separated simplex, duplex and multiplex PCR products were clearly visualized in the gel image along with the electropherograms (Figure 4.7) for all of the three targets.

5.4 Assay Specificity

To obtain a highly specific assay, design of primers with adequate species-specific fingerprints is a must. Since primers regions play critical roles in PCR amplification (Rychlik et al., 1990; Wu et al., 2009), they were critically evaluated for potential nucleotide mismatches and melting temperatures (*Tm)* that play vital roles in primer annealing, especially in multiplex PCR that requires quite identical *Tm* for all primers to be annealed with respective templates under the same set of PCR conditions. In this study, three primers sets were designed targeting cytb for squirrel and rabbit and ATP6 for rat having *Tm* about 59.8 - 61.6°C for all primers (Table 3.2). The designed primer sets were aligned against 45 potential species, including 5, 10 and 8 closely related species of the *Oryctolagus, Rattus* and *Callosciurus* genus, respectively, using ClustalW and MEGA5 multiple alignment software. The results reflected 100% sequence

matching only with the respective targets but multiple mismatches ((6-37 nt) (14.63-84.77%) against the non-target species; these made the likelihood of cross-species amplification quite impossible (Table 4.2- 4.5). However, the close species of *Oryctolagus*, *Rattus* and *Calloscious* genus contained 5 - 9, 2 - 3 and 6 - 13 bp mismatches, respectively. The pairwise distances between the amplicon of each target species and the respective gene of the target and non-target species were analyzed using neighbor-joining method; while the zero distance was found for the exact species, the distances among the 10 rat species were 0 - 0.12 (Table 4.6), among the 8 squirrel species were 0 - 0.20 (Table 4.7), and among the 5 rabbit species were 0 - 0.35 (Table 4.5). The genetic distances among the targets and other non-targets were significantly higher (0.2 - 4.10), suggesting very little or no probability of cross-target amplification. When phylogenetic tree was constructed using the amplicon sequences, all rabbit, rat and squirrel species clustered in their respective domains. When 3 D plots were constructed, huge dissimilarities were observed among the targets and 45 non-target species that were cross-tested by PCR (Figure 4.2). The above outcomes collectively reflected a very high unlikelihood that any cross-species would be detected by the developed primers. Only 2/3 bp mismatches and very close pairwise distance among the rat species also suggested that rat primers were universal for all rat species.

The multiplex PCR assay was optimized step by step from simplex, duplex and multiplex formats (Figure 4.7) and in each case, the species-specific primers amplified 108, 123 and 243 bp fragments from rat, rabbit and squirrel DNA templates, respectively. The specificity of the multiplex system was carried out against the target and 22 different non-target species (chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, cat, chinese frog, tuna, salmon species, wheat, cucumber, onion, chili) using 20 ng of DNA extracted from all of the tested samples (Figure 4.8) but no cross-species amplification was detected. Blind tests

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were also performed but no inconsistencies were observed. A cross specificity of the designed primers was also examined in chicken meatballs having complex matrices (Figure 4.11). Meat products might contain various types of chemicals such as the Maillard reaction products, milk proteins, glycogen, fat, collagen, folic acids, and iron, which might be co-purified with the target DNA and inhibit the PCR (Wilson et al., 1997). However, the use of both the negative and positive controls in all of the PCR assays eliminated any possibility of false positive or negative target detection. The presence of amplifiable DNA in non-target tubes was confirmed through the amplification of IAC that amplified 141-bp PCR products from the 18Sr RNA gene all eukaryotic species through a set of universal eukaryotic primers (Rojas et al., 2010)

5.5 PCR Product Sequencing Analysis

Although a properly designed andoptimzed species-specfic PCR assys are often conclusive to assign specific species (Ali et al., 2015c; Karabasanavar, Singh, Kumar, & Shebannavar, 2014) but authentication of PCR products by sequence analysis greatly increase the reliability of the PCR assay. Moreover, PCR products indicate only the presence or absence of the species, but PCR products sequencing result properly confirm whether the accurate species are detected (Bevan, Rapley, & Walker, 1992). The PCR products obtained in this research were cloned prior to sequencing because they were very short-length and direct sequencing could not derive the full-length sequence of the products. The PCR product sequencing result showed that all PCR products were 99.18, 98.14 and 98.35 % sequence matching with European rabbits, black rats and plantain squirrels, respectively this similarity was within the acceptable limit because at least 98% sequence similarity is required for the potential species identification (Cawthorn et al., 2013). Previosly, Cawthorn et al. (2013) reported that 99% sequencing similarity for three tested samples (one ‘blesbok biltong’ and two

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‘kudu biltong’). On the other hand, Bevan et al. (1992) and Natoek- Wisniewskaer al (2013) found 97.78% sequene similarity for bone specific PCR products, whereas ovine specific prodcts showed more than 94% similarity with ovis species and incase of porcine products it was more than 99% similar. Hsieh et al., (2005) also found a sequence similarity of 98-100% for various samples. Thus, little variation in sequence similarity is a common phenomenon.

5.6 Multiplex PCR Assay

5.6.1 Lower Limit of Detection

The lower limit of detection (LOD) of an assay is a critical aspect that helps in the determination of marginal-level targets in adulterated foodstuffs. LOD values for several types of animal species, such as beef (Mane, Mendiratta, & Tiwari, 2012) (Rodriguez et al., 2003), chicken, turkey (Mane, Mendiratta, & Tiwari, 2009), goat (Karabasanavar, Singh, Umapathi, Girish, et al., 2011), lamb and pork (Rodríguez et al., 2004b), deer and wild boar (Mutalib et al., 2012) have been defined for simplex PCR for food authenticity purposes. Recently, Ali, et al. (2015a) found variable LOD for cat, rat, dog and monkey species in a multiplex platform. However, rabbit, rat and squirrel species are relatively new in food chains and so their LODs have not been defined under various food matrices. Thus, this study addressed this research gap by determining the LOD by two different ways. , the concentration of the extracted DNA was measured by UV-VIS spectrophotometer at a relatively high concentration (100 ng.µl^{-1}) (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK), and then various concentrations (10, 1, 0.1, 0.01, 0.001, 0.0001 ng) were made by dilution in nuclease-free water because inaccuracies and inconsistencies have been observed in spectrophotometric readings when low concentrations are used (Ali et al., 2015c) . A 10-fold serial dilution method has been used by several studies to determine the PCR sensitivity for porcine, mutton (Karabasanavar et al., 2011). Previously, Karabasanavar et al. (2011) detected the 0.001

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ng of DNA for mutton, whereas Che Man et al. (2012) detected 0.001 ng of DNA for pig. On the other hand, in case of multiplex PCR, Cheng et al. (2014) found the 0.15 ng of DNA for duck, pig and chicken and Dai et al. (2015) detected 0.001 ng of DNA for pork, beef, chicken and mutton. Thus the limit of detection (LOD) of my developed multiplex PCR was not less than the previously developed method.The amplified PCR product was found from as low as 0.001 ng DNA template extracted from pure meat of all target species under multiplex format (Figure 4.9).

5.6.2 Stability analysis

Target stability is a must for the validation of any analytical tests, especially for forensic samples, wherein less stable analytes are often decomposed, resulting in false negative identifications. Reasonably, scientists have put significant effort into the development of short-length DNA targets, which are thermodynamically more stable than the longer targets and hence survive under extreme stresses that often break down longer DNA markers (Ali et al., 2015a). Previously, various thermally treated samples have been used to benchmark target biomarker stability in many forensic investigations (Arslan et al., 2006; Haunshi et al., 2009; Ilhak & Arslan, 2007). However, there is no scientific evidence about the stability of rabbit and squirrel biomarkers in food forensic detection.

Here, I confirmed the stability of the targets through three different thermal treatment approaches, namely, boiling, microwave cooking and autoclaving. Boiling simulates traditional cooking, in which meat is cooked in boiling water at 100 °C for a fixed amount of time (Ali et al., 2015a), and over the years, steam cooking or boiling have increased in popularity over pan frying for improved health benefits. In contrast, microwave cooking is a modern technique that heats and cooks food through exposure to electromagnetic radiation in the microwave spectrum (Ali et al., 2015a). On the other hand, autoclaving is the most appropriate method to simulate steaming and

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canning-based meat processing because it cooks at a high temperature (121°C) under pressurized conditions to kill any potential microbes in the samples and extreme autoclaving (2.5 h at 121 °C and 45 psi) has been used as a benchmark for target DNA stability in several studies (Ali et al., 2012; Yang et al., 2005).

In this study, target PCR products were obtained from all thermally processed samples (Figure 4.10) ; wherein, rat, rabbit and squirrel meats were boiled at 100 °C for 150 min but no adverse effects on the amplification cycle were found (Figure 4.10). Previously, (Ali et al., 2012) detected a 109-bp porcine target after boiling for 2.5 h. Haunshi et al., (2009), Karabasanavar, et al., (2011), and Mane et al., (2012) also identified target species after autoclaving various types of domestic meat at 121 °C for 15-30 min. Here, I autoclaved rat, rabbit and squirrel meat at 121 °C under 15 psi for 150 min (extensive treatment) and obtained targeted PCR products from all treated samples (Figure 4.10) . Finally, extreme microwave cooking was done at 500, 600 and 700 W for 30 min, and clear bands for the desired products (108, 123 and 243 bp) were realized (Figure 4.10); but PCR products of all targets at 700 W were turned into smeared, reflecting DNA breakdown at 700 W (Ali et al., 2015b). However, when meat samples were cooked in a microwave at 700 W for 30 min, they turned into ashes that were no longer suitable for consumption (data not shown); this demonstrated that use of 700W is not used in any cooking practices. Arslan et al. (2006) also could not amplify the target product from pan-fried beef meat at 190 °C for 80 min. In this current study, target amplification from ash-like specimens clearly indicated that this method could be used to detect the rat, rabbit and squirrel target from any highly decomposed specimens, which are frequently found in forensic samples.

5.6.3 Sensitivity test under admix (meatball)

Secondly, since meat adulteration is normally done in processed meat products, wherein, there is a possibility of DNA breakdown, the base adulterated chicken

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meatballs containing 1%, 0.5%, and 0.1% rat, rabbit and squirrel ground meat were made in triplicates (Ali et al., 2015c) to simulate a real form of meat adulteration in commercial meat products (Figure 4.11). The meatballs of 0.1% spiked meat from the three species were also autoclaved at 121 °C and 15 psi for 2.5 h to simulate an extensive cooking practice that are known to breakdown DNA. Clear PCR products from rat, rabbit and squirrel contaminated meatballs demonstrated that the assay was suitable for adulteration detection having 0.1% contamination in highly processed foods (Ali et al., 2015c) . From a practical point of view and also based on published reports, it is clear that meat products that are adulterated by less than 0.1% do not yield remarkable profits for the manufacturers and so the study was not done at below 0.1% adulteration (Ali et al., 2012). In a published report (Ali et al., 2015c), 0.1% LOD was accepted for porcine, canine, feline, monkey, and rat meat in mixed food matrices. Safdar & Junejo, (2015) also found 0.1% LOD for ovine, caprine, fish, and bovine material in a multiplexPCR assay for heat-treated (133 °C at 300 kPa for 20 min) mixed meat samples. In another report, Amaral et al. (2014) detected 0.1% and 0.01% rabbit meat in commercial and raw meat products, respectively using simplex PCR. A Lee et al. (2016) also found 0.1% of pork in a beef-meet mixture. Similarly, Dai et al. (2015) found 0.05% in admix condition for pork, beef, chicken and mutton. Thus, the LOD in admixed condition obtained in this study was below those of previously reported assays.

However, they did not check the assay validity in heat treated samples and also in multiplex PCR platform. However, Ali et al. (2015c) scientifically proven that the stability and sensitivity of a PCR system under extremely processed atmosphere largely depends on the amplicon sizes; longer targets break down before the shorter ones. Therefore, the short amplicon length (108-243 bp) of this novel PCR assay offered better reliability and very low LOD compared to those of the published reports.

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To test the suitability of the developed multiplex PCR assay, 9 sets of chicken meatballs having 1, 0.5 and 0.1% adulteration from the each of rabbit, rat and squirrel (3 x 9 x 3 =81) were made in the laboratory by spiking 1%, 0.5 % and 0.1% of ground meat of the target species in chicken meatballs. In each case, 100% pure chicken meatballs were used as negative control of the target species but positive IAC for all eukaryotes. All meatball products were autoclaved at 121 °C under 15 psi for 2.5 h to check the targets stability under complex matrices in processed conditions (Ali, Asing, et al., 2015b; Arslan et al., 2006). Rat, rabbit and squirrel targets were amplified from all adulterated samples but IAC (141bp) was obtained from all samples because all samples contained eukaryotic elements.

Finally, a total of 72 (8X9) meatballs of 8 different halal brands were screened but no rat, rabbit and squirrel adulteration were detected (Table 4.9). All experiments were carried out in triplicate to confirm the reproducibility of the results. Malaysia is leading country of halal food exports and has been committed to develop a halal hub industry; so, the absence of rat, rabbit and squirrel meat in Malaysian food products was quite encouraging.

5.6.4 Sensitivity under complex food matrices (Frankfurter)

Frankfuter is one of the popular food items whose morphological aspects of identifications are greatly disrupted through processing treatments (Ali, et al., 2017; Rahman et al., 2014). To simulate this form of adulteration, the rabbit, rat and squirrel specific multiplex PCR assay was evaluated under chicken and beef frankfurter matrices; a very popular food item consumed all over the world (Ali et al., 2015c). The chicken and beef frankfurters were made in the laboratory having 1%, 0.5% and 0.1% adulteration from minced and deboned meat from each of the squirrel, rat and rabbit species. Moreover, frankfurters of 0.1% adulteration were further autoclaved at 121°C and 15 psi for 2.5 h that is known to breakdown target DNA (Ali et al., 2016). However,

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rabbit (123 bp), rat (108 bp), squirrel (243 bp) specific PCR products were obtained from both treated and untreated samples having 1%, 0.5%, and 0.1% adulterations but only IAC (141 bp) that was the signature of eukaryotic DNA was amplified from both pure and contaminated chicken and beef frankfurters (Figure 4.12), reflecting the sensitivity and discriminatory attributes of the developed PCR assay even at 0.1% contamination. The developed multiplex PCR assay was tested for the screening of commercially available 36 beef and 36 chicken frankfurters of four different brands (9x4=36) procured from Malaysian outlets (Table 4.10). All of the experiments were carried out in triplicate by three independent analysts on the different dates to confirm the reproducibility of the results. The excremental and theoretical specificity, stability and sensitivity of the developed assay indicated that it was a reliable and rapid technique for the authentication of rabbit, rat and squirrel adulteration in the food chain. Malaysia also committed to developed a halal hub industry and to being a competitive partner in the global halal food business, so the absence of target meat in Malaysian was quite encouraging. The screening result reflected that beef and chicken frankfurters in Malaysia did not have any rabbit, rat and squirrel adulteration. Rationally rabbit adulteration in chicken and beef products is unlikely because rabbit meat is sold at higher prices than chicken and beef. On the other hand, rat and squirrel are not permissible in Malaysian markets, especially in halal food items. However, squirrels are sold as exotic dishes in certain places in Malaysia such as Jalan Pudu in Kuala Lumpur; but definitely the price is higher than those of the regular dishes.

5.7 RFLP analysis

5.7.1 RFLP Analysis

Verifying the authenticity of the amplified PCR products definitely increases assay reliability (Yang et al., 2005) and it could be done through PCR-PFLP, PCR product sequencing and probe hybridization techniques (Maede, 2006). Probe hybridization is a

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laborious method and it needs high quality DNA (Mafra, Ferreira, & Oliveira, 2008) which is highly unlikely in case of processed foods treated under extensive heat, pressure or chemicals. On the other hand, DNA sequencing procedure require expensive laboratory set up and highly skilled human capital (Girish et al., 2004; Mafra et al., 2008) and so it is not suitable for routine food screening program (Albers, Jensen, Bælum, & Jacobsen, 2013). However, PCR-RFLP technique is quite simple and could be done in ordinary laboratories (Park et al., 2007), offering a great analytical support when a fraudulent substitution or any unintentional contaminations are done (Sharma et al., 2008). In this method, a profile of signature nucleotide fragments is created by restriction digestion with one or two endonucleases followed by separation in gel or capillary electrophoresis (Ballin et al., 2009). In this study, at first each target was digested separately with an appropriate restriction enzyme to define its individual restriction patterns to eliminate any ambiguities that may arise from the multiplex PCR products. Both squirrel (243 bp) and rabbit (123 bp) products ware digested by *Bts*CI, which generated two fragments for each of the targets (176 & 67 bp for squirrel and 115 & 8 bp for rabbit); but rat PCR product was cleaved by *Bts*I*Mut*I that also yielded two fragments (64 & 44 bp) (Figure 4.13 and Table 3.10).

Finally, the multiplex PCR products were subjected to restriction enzyme digestion with the two enzymes in a single tube, and this collectively generated molecular fingerprints of total six fragments (8, 44, 64, 67, 115, 176 bp) plus IAC (141 bp) (Figure 4.13). The finding was consistent with RFLP profile of simplex PCR products, indicating that the developed multiplex PCR also amplified the same target region as simplex PCR assay. The sizes of the digested fragments were the same as the sizes obtained from the theoretical RFLP analysis using NEB cutter software (Table 3.10). Thus, experimental results were supported the theoretical RFLP analysis, indicating that developed PCR systems amplified exact target sites

Subsequently the optimized multiplex PCR-RFLP assay tuned for the chicken and beef frankfurter analyses under raw, boiled and autoclaved condition. PCR products were obtained from lab made frankfurters (beef and chicken frankfuter) having deliberate adulterations of target meat items (rabbit, rat and squirrel) were digested and their restriction patterns were observed. Expected PCR and RFLP fragments were clearly visualized upon electrophoresis (Figure 4.14), reflecting that variations in food processing treatments could not affect the stability of the optimized assay.

5.7.2 Analysis of Commercial Frankfurters

The developed mPCR-RFLP assay was evaluated for the screening of commercial meat product. The optimized multiplex PCR-RFLP assay was tuned for the chicken and beef frankfurter analyses under raw, boiled and autoclaved condition. PCR products were obtained from lab made frankfurters (beef and chicken frankfuter) having deliberate adulterations of target meat items (rabbit, rat and squirrel) were digested and their restriction patterns were observed. Expected PCR and RFLP fragments were clearly visualized upon electrophoresis (Figure 4.14), reflecting that variations in food processing treatments could not affect the stability of the optimized assay. Thus, the developed multiplex PCR assay is highly stable, reliable and very sensitive tool for the identification and differentiation of rabbit, rat and squirrel materials in raw, boiled and autoclaved condition.

Previously, Haider et al. (2012) reported a PCR-RFLP assay with a 710bp amplicon that was amplified using common primer pairs for the cow, chicken, turkey, sheep, pig, buffalo, camel and donkey. Girish et al. (2005) also documented a PCR-RFLP assay with 456 bp amplicon length for the detection of goat, sheep, cattle and buffalo. Recently, Kumar et al. (2014) proposed a RFLP pattern with a 609 bp target to discriminate cattle, buffalo, goat, sheep and pig. In addition, Erwanto et al. (2012) demonstrated a PCR-RFLP technique for a 359 bp product. On the other hand, Verkaar

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et al. (2002) introduced a PCR-RFLP assay for the identification and discrimination of bovine species with 271, 651, 604 and 822 bp products containing four set of primers. A PCR-RFLP assay with universal primer pair of 360 bp amplicon size was used for the detection of ten common meat species (cow, buffalo, pig, deer, chicken, goat, duck, turkey, rabbit and ostrich). However, such long targets (271 – 822 bp) are more prone to break down and thus would definitely lose their applicability for the analysis of highly processed foods. In contrast, developed mPCR-RFLP showed its reliability and sensitivity under raw, boiled (98 °C for 90 minutes), and autoclave (121 °C and 15 psi pressure for 2.5 h) atmosphere for differential identification of rabbit, rat and squirrel in deliberately adulterated frankfurters.

5.8 RT-PCR Assay

5.8.1 Multiplex Real time PCR System

Design of specific primers and probes were the key step in the development of mqPCR system for cat, rabbit, rat and squirrel species detection because it was necessary to ensure that all the primers and probes must have the same or very close related melting temperature (Tm) so that they can anneal to their specific partner sites in template DNA under the same set of PCR conditions. (Cheng et al., 2014). The Tms of five primer sets were (57.8 - 60.9 °C) which annealed to the primer binding sites at 60 °C and Tms of the probes (69.9 - 70.70 °C) were 8-10 °C higher than that of the primers to facilitate the preferencial binding of the probes prior to the annealing of the primer to template (Arya et al., 2005). The multiplex amplicon was discriminated in the same reaction tube through five different florescent reporter dyes ROX and TAO/3IAbRQSp; HEX and ZEN/3IABkFQ; Cy5 and TAO/3IAbRQSp; and TAMN and TAO/3IAbRQSp for squirrel, rat, rabbit and cat, respectively (Table 3.12). The Ct values of the multiplex qPCR assay were Ct = 17.16 ± 0.05, 17.62 ± 0.07, 17.14 ± 0.04 and 16.92 ± 0.04 that were very close to the respective Ct values of the simplex qPCR for squirrel (Ct = 17.06

± 0.05), rat (Ct = 17.46 ± 0.06), rabbit (Ct = 17.1 ±0 .03) and cat (Ct = 16.86 ± 0.06), respectively., effectively confirming that there was not any significant variation of Ct values when the platforms were changed from single to multiplex. The use of endogenous system in the qPCR assay eliminated any false negative detection as well as helped in accurate quantification of target. It also indicated whether there is any effect of inhibitors and reagents in the reaction mixture (Rojas et al., 2011b). Moreover, the presence of endogenous control was mandatory, particularly for the analysis of extremely processed food sample since the extracted DNA might be of low quantity and degreded. Fothermore, the endogenous system plays a key role to a verify qPCR assay if any amplification variation was occurred with species specific biomerkers due to the variation in template DNA concentration, purity of extracted DNA, degredation of DNA and the presence of PCR inhibitors (Soares et al., 2013). Therefore, factual error between the unknown sample and standards can be eliminated by the comparison of endogenous system and species–specific assay signal generated from samples (Rojas et al., 2010).

5.8.2 Specificity of the Multiplex Real-time PCR System

NCBI BLAST analysis results demonstrated that the designed primer pairs and probes had completely identical sequence with target species and sufficient mismatch with the other species. Aligment of primer sets and probes with target and non-target species (commonly used in the meat products) using MEGA5 software showed 100% sequence similarity with the target species and multiple nucleotide mismatches with other related or non-target species. From the in silico specificity analysis, it can be concluded that there were no or very little possibility for amplifying the non-target species in a practical PCR experiments. Because the existence of a single mismatch at the primer annealing position may reduce the PCR efficiency or causes false or no amplification (Wu et al., 2009). Finally, the practical specificity of the mqPCR system

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was conducted with 10 ng of DNA extracted from fresh muscle tissue of the four target species (cat, rabbit, rat and squirrel) and 22 non-target species (chicken, cow, goat, pig, turkey, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, cat, chinese frog, tuna, salmon, onion, cucumber, wheat, chili) on three different day in triplicates. The amplification profile clearly showed that the mqPCR system amplified only four target species (cat, rabbit, rat and squirrel) with the Ct values of The Ct values of the multiplex qPCR assay were 17.143 ± 0.04, 17.48 ± 0.03,17.196 ± 0.05 and 16.8 ± 0.09for squirrel, rat rabbit and cat, respectively and only background florescence were provided from non-target species within 40 cycles, confirming the absence of any cross-amplifications. On the other hand, this study used the endogenous PCR system (eukaryotic 18S RNA) to eliminate any false negative amplification. The endogenous system amplified eukaryotic target from all target and non-target species with the Ct values between 15.24 and 18.78 (Table 4.11) reflected that good quality DNA template was present in all tubes. Thus, the developed mqPCR system effectively amplified only target species and no cross-amplifications were observed, reflecting the high specificity of the technique.

5.8.3 Limit of detection and Efficiency of the Multiplex Quantitative PCR system

Tenfold serially diluted genomic DNA (10 to 0.001 ng) from each of the target species (cat, rabbit, rat and squirrel) were used to determine the LOD of the assay and amplification plots clearly demonstrated that the system amplified up to 0.001 ng DNA with detectable fluorescence signal for all targets , suggesting the assay could detect and quantify minimum 0.003 ng target DNA (Figure 4.16). The RSDs were calculated for the Ct values of closely distances three replicate of all diluted DNA that were less than 1.0 for all DNA samples.

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Table 4.13, indicated that minimum variation between the replicates were present in the developed mqpCR assay. Previously, (Cheng et al., 2014) Cheng et al. reported an mPCR system for the identification of duck, pig and chicken wherein the LOD was 0.15 ng DNA for each species. On the other hand, it was 0.32 ng DNA for beef, pork, chicken and turkey as documented by Koppel et al. (2008). Recently, Fang, & Zhang. (2016) also detected lower than 1 pg of DNA by TaqMan real-time PCR for murine compound. Thus, LOD might vary from species to species and sample to sample but 0.003 ng detectable limit of the present assay made it highly sensitive for adulteration authentication. For quantatiive detection standard curves of all target species were generated by ploting the Ct values against the logarithmic value of each DNA concentration. The standatrd curve of squirrel, rabbit, rat and cat were constructed from five-point dilutions (30-0.003ng) and the quantification of 0.003 ng DNA was sufficient to detect any commercial frauding for profit making purposes. In fact, a good linear regression was found in the standard curves for all measurements, wherein the regression were found in the standard curves for all measurement, wherein the regression coefficient (R^2) was 0.9996, 0.9987, 0.9992, 0.9988 and 0.9988 for squirrel, rat, rabbit, cat and IAC, respectively, and the corresponding slopes were were -3.1162, -3.1671, -3.174, -3.184 and -3.2204, respectively. The PCR efficiency were found to be 109.36%, 106.87%, 106.53%, 106.06% and 104.4% for squirrel, rat, rabbit, cat and IAC, respectively. These values were within the recommended values (91-122%) (Ali et al., 2012) and thus, the generated standard curves and mqPCR system were suitable for the quantitative determination of the target species contribution from mixed meat samples. The findings were supported by Cheng et al. (2014) in which the mqPCR efficiencies were 104.38, 91.75 and 97.46% for chicken duck and pig species, respectively. Similarly, Iwobi et al. (2015) found the efficiencies of their mqPCR system for beef and pork at 101.1% and 91.6%, respectively.

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5.8.4 Sensitivity and validity of the mqPCR Assay under Ternary and Commercial Matrices

Sensitivity of the PCR system is a key factor for the authentication of processed food products. Deliberately adulterated model tertiary meat admixture of cat, rabbit, rat and squirrel (10, 1 and 0.1%) were prepared (section 3.7.6) to evaluate the sensitivity of the mqPCR method. All the species were detected until 0.1% adulteration in the tertiary admixes with Ct values of 24.44 1 ± 0.266 to 25.83 ± 0.341for all the four targets species but the endogenous system constantly yielded a mean Ct between 14.534 ± 0.13 and15.35 ± 0.19 for all level of adulterations, reflecting that the endogenous target did not change significantly with a variation in adulteration because all adulterants were eukaryotic. These clearly demonstrated that the developed mqPCR system was very sensitive, specific and robust and can reliable detect all the four targets from 0.1% contaminated specimens. Cheng et al., (2014) developed an mqPCR system for the detection of pig, chicken and duck with the sensitivity of 1% for all target species in ternary mixture. Recently, the sensitivity of the mqPCR in binary admixtyre was found to be 0.5% spiked level of pork in beef background. The same sensitivity (0.5%) also found by Dooly et al., (2004) in a Taq-Man real-time PCR assays for the detection of beef, pork, turkey, chicken and lamb. More recently, Fang, & Zhang (2016) established a qPCR assay for the detection of murine species with sensitibity of 0.1% murine adulteration in meat admixtures. The mqPCR system was further validated for the analysis of processed meat products (frankfuter and burger). The analysis results (Table 4.14) of the four-target species revealed that the target recoveries from 10% to 0.1% spiked level were 91.667 - 122.333% along with a systematic error between -8.333 to 22.333 and RSD 3.08 - 20.57%. Thus, the maximum recovery was 122.333% for 1 % spiked beef frankfurter but the minimum was 91.667% for 0.1% spiked chicken burger. On the other hand, the maximum and minimum RSD were found in beef burger

containing 0.1% adulteration chicken burger containing 1% adulteration, respectively. When a graph was generated by plotting the recovered values (y-axis) against the reference (actual) values (x-axis) for each target, a very high correlation coefficient (R^2 = 0.9999) was attained (Figure 4.17), confirming that the experimental values were fairly close to their actual values. Druml et al., (2015) found 40.9 % systematic error and 12.9 % RSD for 2% adulteration. Thus, the systematic error between-8.333 to 22.333 % of this assay was within the acceptable limits of the published reports.

5.8.5 Residual Analysis

Residuals reflects the difference between the measured value and practical value and allows the estimation of experimental errors. (Ali et al., 2012b). The resulted graph obtained from residuals and fitted recovery values of the variables for both burger and frankfuter of four target species (squirrel, rat and rabbit, and cat), (Figure 4.18) showed the random distribution of all variables were very low (within +2.0 to -2.0 from zero line) for all meat products. This distribution of residuals indicated a good precision and accuracy of the developed multiplex qPCR system for the measurement of 0.1 -10 % adulteration of the four-target species in meat products.

5.8.6 Analysis of Commercial Meat Products by mqPCR

The commercial food products such as burger, meatball, frankfurter, hotdog, nugget etc. are very popular all over the world. These types of products are highly processed that results in total or partial annihilation or modification of morphological features and other physical properties. Therefore, manufacturers can easily mix an unexpected and lower priced meat in the final products for profit making purposes. To prevent or monitor these types of undesirable incidences, the accurate screening of commercial meat products can play a great role and build public confidence on health, religious, social and cultural perspectives. In this work, two types of commonly used commercial products, namely, burger and frankfurter were evaluated using the developed multiplex

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qPCR system. A total of 72 burgers (36 beef and 36 chickens) and 72 frankfurters (36 beef and 36 chickens) were purchased from different Malaysian outlets and were tested (Table 4.15). The experimental results revealed that no target species (cat, rabbit, rat, and squirrel) were present in the burger and frankfurter products but only IAC was amplified, reflecting 100% accuracy of the qPCR assay. The meat of cat, rat, rabbit and squirrel species was not mixed with commercial burger and frankfuter because this might be due to the tight monitoring of halal products in Malaysia by Government agencies.

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CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATION

6.1 Conclusion

To make sure food products are safe for consumption, knowing the accurate ingredients of the food products is a must. It is very objectionable to adulterate foods with animal materials that might have a negative impact on public health, religious and cultural issues. Although some authentication techniques are available for the detection of cat, rabbit, rat, and squirrel species in separate assays, no method has been documented for the simultaneous detection, discrimination and quantification of cat, rabbit, rat and squirrel species in food products. Therefore, the objectives of the study were briefly described:

6.1.1 Species Specific Biomarker Development

The developed biomarkers of cat, rabbit, rat and squirrel species were tested to evaluate its specificity with in-silico analysis and it showed the 100% matching with target species (cat, rabbit, rat and squirrel) and 6-37 nucleotides (14.63- 84.77%) mismatching with non-target species, eliminating the probability of any non-target DNA amplification. Furthermore, these biomarkers were also tested for specificity against 22 different species of terrestrial and aquatic origins including meat providing animals, fish and plant species commonly used in different food formulation to demonstrate its potentially to detect target materials with 100% accuracy. Thus, the cross-PCR assays confirmed that the developed primers were solely specific for the cat, rabbit, rat and squirrel species.

6.1.2 Assay performance and Validation

The developed conventional multiplex PCR assay simultaneously amplified the DNA of rabbit, rat and squirrel meat in the form of raw, boiled, autoclaved and microwaved cooked meat under pure and mixed matrices. In the raw meat state, the detection limit in multiplex PCR assay was 0.001 ng DNA and 0.1% target meat in the admixed

condition. 141 bp IAC was used throughout the assay to eliminate the chance of any false negative detection. To authenticate the multiplex PCR assay, the PCR products was were treated with the restriction enzymes, namely BtsCI, BtsIMutI. After treatment of the restriction enzymes, the authentic restriction fragments were found, reflected the accuracy of the developed multiplex PCR assay. PCR-RFLP assay was also applied in the chicken and beef frankfurters which were deliberately adulterated with target species and clear restriction fragments were found. On the other hand, PCR product were sequenced and confirmed the authenticity of the developed multiplex PCR. I also described introduced here a quantitative quantative quantitative multiplex real time PCR assay with Taq Man Probes to detect and quantify the cat, rabbit, rat and squirrel materials simultaneously. To develop the mqPCR assay, short length DNA was selected (108 bp, 123,161 1nd 172 bp for rat, rabbit, squirrel and cat respectively) because short length amplicons offer more stability over longer one for thermodynamic reasons. Additionally, the use of IAC effectively eliminated any false negative results, enhancing the assay reliability. Ultimately the designed primers, probes, shorter-size of amplicon and IAC targets provided extraordinary specificity, stability and reliability of the developed mqPCR system. This assay also detected and quantified 0.003 ng of DNA of the target species in pure and o.1% of DNA in admixed states.

6.1.3 Assay performance under commercial product:

Nowadays the commercial meat products such as Meatball, Frankfuter and Burger are widely consumed in all over the world. Since these commercial meat products were highly processed, physical attributes of meat are significantly modified and identification cannot be made without analytical supports. The developed method was used for the screening of cat, rabbit, rat and squirrel materials in various commercial food products such as meatball, burger, and frankfurters. To authenticate the meat product, commercially available 36 beef and 36 chicken frankfurters, meatball and

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burger of four different brands (9 x 4 = 36) were procured from Malaysian outlets and the developed method was applied on the that commercial product. No target DNA was amplified from the meat product, reflected the meat product was not mixed or contaminated with any other of the cat, rabbit, rat and squirrel materials. Malaysia is leading country of halal food exports and has been committed to develop a halal hub industry to monitor food products; so the absence of cat, rat, rabbit and squirrel meat in Malaysian food products was quite encouraging.

6.2 Recommendation for the Future Work

The developed methods are highly promising tool for determination of the specific DNA target in raw, admix and heat-treated state. DNA based species specific detection techniques can lead to distinguish permissible and non-permissible food materials to protect the human health, religious faith and secure fair trade.

However, due to the time constrain I did not go through side by side real run analysis of our and described PCR assay. In the future work, short length DNA from same gene or different gene and more target species can be added for better understanding of gene nature and effect of different primers for species authentication. Generally human food products are composed of different component and especially meat product with multiple species background. In this research, the developed method was used in the screening test of meatball, frankfuter and burger which are collected from Malaysian outlets. This method should be applied in out of Malaysia specially where adulteration percentages is comparatively higher. Furthermore, this method should be applied on another meat product such as hot dog, nugget, readymade curry, etc. Additionally, this method can be applied on feed stuff. In future a multiplex PCR assay can be designed by the more species which is halal or haram such as pork, horse and dog or chicken, beef, turkey, duck, buffalo including my target species (cat, rabbit, rat and squirrel).

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When a multiplex PCR assay will be designed in such a way that more number of species can be determined, both time and cost will be saved.

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