DEVELOPMENT AND VALIDATION OF HEPTAPLEX POLYMERASE CHAIN REACTION ASSAY FOR THE DISCRIMINATORY DETECTION OF SELECTED MEATS IN FOOD PRODUCTS

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ABSTRACT

Food fraud is one of the most prevalent problems of the present day. It is important for consumers to ascertain the authenticity of the declared ingredients in food products. Beef, buffalo, goat, sheep, chicken, duck, and pork are the heavily consumed meats having enormous importance from nutritional, economic, and cultural/religious viewpoints and are often found to be mutually adulterated in raw and processed states. Available DNA-based approaches for species authentication are commonly based on long DNA markers that can be damaged during food processing, rendering the methods less reliable. The objective of the project is to develop a heptaplex Polymerase chain reaction (PCR) assay targeting short length amplicons for the detection and differentiation of bovine, buffalo, goat, sheep, chickens, ducks, and porcine materials in food chain simultaneously. Both conventional and real-time PCR systems were developed, and authentic target detection was ensured through sequencing and Restriction Fragment Length Polymorphism (RFLP) analysis. Mitochondrial cytochrome b (cytb) and NADH dehydrogenase subunit 5 (ND5) genes were targeted and seven different targets (73-263 bp length) each for pig (73 bp), cow (106 bp), buffalo (138 bp), chicken (161 bp), duck (203 bp), goat (236 bp) and sheep (263 bp) were amplified from raw as well as boiled, microwaved, and autoclaved meats under pure and admixed states. The specificity of the PCR assays was tested against seven target species and other related 19 non-targets. Specific PCR products were obtained only from the targeted cow, buffalo, goat, sheep, chicken, duck, and pig without any cross-species amplification. The use of universal eukaryotic primers eliminated any chance of false-negative detection. The assay was sensitive enough to detect 0.01-0.005 ng of DNA

from raw meat and 0.5% (w/w) adulterated meat under mixed and commercial matrices. The amplified PCR products from the targets showed more than 98% (98.5-100%) sequence similarity with specific target sequences in GenBank. The PCR products were digested by the restriction enzymes namely *Fat*I, *Bfa*I and *HPY1*88I that confirmed the authentic molecular fingerprints from the seven target species. The novel methods were applied to screen various commercially processed foods, namely meatballs, frankfurters, burgers and meat curries. A market survey revealed that 100% of beef meatballs were adulterated with buffalo along with total beef replacement in 20% of cases. Beef frankfurters and burgers were adulterated with buffalo in 84% cases. Beef curry samples contained 90% buffalo contamination. In contrast, porcine products were found to be 100% authentic and no porcine was detected in the halal branded foods. Finally, the developed TaqMan probe-based multiplex real-time PCR (mqPCR) systems successfully detected 0.006 ng DNA in raw state and 1% adulterated meat in mixed and commercial matrices. Screening of commercial products by mqPCR assays showed that 85% of beef burgers and 100% of beef frankfurters, meatballs and cocktails were adulterated with buffalo. Lamb products were also buffalo contaminated. Pork products contained chicken (50%). Given some advantageous features, including stringent specificity, exceptional stability and sensitivity, the developed approach could discriminatorily detect and quantify the target species even in severely processed specimens.

Keywords: Heptaplex PCR; Food products; Simultaneous detection; Short target; Adulteration.

PEMBANGUNAN DAN PENGESAHAN UJIAN TINDAK BALAS BERANTAI POLIMERASE HEPTAPLEX UNTUK PENGESANAN PEMBEZAAN DAGING-DAGING TERPILIH DALAM PRODUK MAKANAN

ABSTRAK

Penipuan makanan adalah salah satu masalah yang sering berlaku pada masa kini. Adalah penting bagi pengguna untuk memastikan keaslian ramuan yang dinyatakan dalam produk makanan. Daging lembu, kerbau, kambing, biri-biri, ayam, itik dan daging babi adalah antara daging yang sering dimakan kerana mempunyai kepentingan dari sudut pemakanan, ekonomi dan budaya / keagamaan dan sering berlaku campuraduk dalam bahan mentah mahupun produk terproses. Pendekatan yang selalu digunakan untuk pengesahan spesies berasaskan DNA biasanya menggunakan penanda DNA panjang yang boleh terlerai semasa pemprosesan makanan, menjadikan kaedah tersebut kurang dipercayai. Objektif projek ini adalah untuk membangunkan tindak balas berantai polimerase (PCR) heptaplex yang mensasarkan amplikon pendek untuk mengesan dan membezakan antara lembu, kerbau, kambing, biri-biri, ayam, itik, dan babi dalam rantaian makanan secara serentak. Kedua-dua sistem PCR konvensional dan masa nyata dibangunkan, dan pengesanan sasaran yang sah dapat dipastikan melalui penjujukan dan analisis Polimorfisma Panjang Jalur Terpotong (RFLP). Mitokondria sitokrom b (cytb) dan NADH dehydrogenase subunit 5 (ND5) telah menjadi gen sasaran dan tujuh saiz berbeza (73-263 pasangan bes (bp) panjang) masing-masing untuk babi (73 bp), lembu (106 bp), kerbau (138 bp), ayam (161 bp), itik (203 bp), kambing (236 bp) dan biri biri (263 bp) diamplifikasi daripada daging mentah serta daging yang direbus, daging yang dipanaskan menggunakan gelombang mikro dan daging yang diautoklaf dalam keadaan asal dan yang dicampur. Kekhususan ujian PCR diuji terhadap tujuh spesis sasaran dan 19 spesis bukan sasaran. Produk PCR yang khusus hanya diperoleh daripada lembu, kerbau, kambing, biri-biri, ayam, itik dan babi yang disasarkan tanpa sebarang

amplifikasi spesies silang. Penggunaan primer eukariotik yang universal menghapuskan kebarangkalian pengesanan negatif yang palsu. Ujian ini cukup sensitif untuk mengesan sehingga 0.01-0.005 ng DNA daripada daging mentah dan 0.5% (b/b) daging yang dicampuraduk menggunakan matriks campuran dan komersial. Produk PCR yang diamplifikasi daripada sasaran menunjukkan persamaan lebih daripada 98% (98.5-100%) dengan jujukan sasaran khusus di GenBank. Produk PCR kemudiannya dicernakan menggunakan enzim pembatasan iaitu FatI, BfaI dan HPY188I yang mengesahkan cap jari molekul yang asli bagi tujuh spesies sasaran. Kaedah novel digunakan untuk membuat saringan terhadap pelbagai makanan yang diproses secara komersial, seperti bakso, frankfurters, burger dan kari daging. Tinjauan pasaran mendapati 20% kes menunjukkan bahawa 100% bebola daging lembu dicampuraduk dengan daging kerbau. Frankfurters dan burger daging lembu telah dicampuraduk dengan kerbau dalam 84% kes. Sampel kari daging lembu mengandungi 90% daging kerbau. Walaubagaimanapun, 100% produk babi didapati asli dan tidak ada sebarang daging babi yang dikesan dalam makanan berjenama halal. Akhir sekali, sistem PCR masa nyata multiplex (mqPCR) menggunakan probe TaqMan yang dibangunkan berjaya mengesan 0.006 ng DNA dalam daging mentah dan 1% daging yang dicampuraduk dalam matrik campuran dan komersial. Saringan produk komersial dengan ujian mqPCR menunjukkan bahawa 85% burger daging lembu dan 100% frankfurter daging lembu, bebola daging dan koktail dicampuraduk dengan daging kerbau. Produk daging kambing juga dicampur dengan daging kerbau. Terdapat juga produk daging babi yang mengandungi daging ayam (50%). Memandangkan kelebihan ciri-cirinya, termasuk kekhususannya yang khusus, kestabilan dan kepekaan yang luar biasa, pendekatan yang dibangunkan dapat membezakan dan mengukur spesies sasaran secara jelas walaupun dalam sampel yang menjalani pemprosesan yang tinggi.

Kata kunci: Heptaplex PCR; Produk makanan; Pengesanan serentak; Sasaran pendek; campuraduk.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols:

µg	:	microgram
≥	:	greater than or equal to
%	:	percent
µL	:	microliter
µM	:	micromole
^{o}C	:	degree Celsius
$	:	dollar
pg	:	picogram

Abbreviations:

A260/A280	:	Ratio of absorbance at 260 and 280 nm
AIDS	:	Acquired immune deficiency syndrome
ASF	:	African swine fever
bp	:	Base pair
BLAST	:	Basic local alignment search tool
BSE	:	Bovine spongiform encephalopathy
COI	:	Cytochrome c oxidase subunit I
cytb	:	Cytochrome b
C_t	:	Threshold cycle
dH_20	:	Distilled water
D-loop	:	Displacement loop
DNA	:	Deoxyribonucleic acid

dsDNA	:	Double stranded-deoxyribonucleic acid
ELISA	:	Enzyme-linked immunosorbent assay
EMA		Economically motivated adulteration
EP-PCR	:	End-point polymerase chain reaction
FAO	:	Food and Agriculture Organization
FDA	:	Food and drug Administration
g	:	gram
h	:	hour
HEV	:	Hepatitis E virus
HIV	:	Human immunodeficiency virus
HPLC	:	High performance liquid chromatography
IDT	:	Integrated DNA technology
LOD	:	Limit of detection
MEGA7	:	Molecular evolutionary genetics analysis version 7
mg	:	milligram
$MgCl_2$	:	Magnesium chloride
min	:	minute
ml	:	milliliter
mM	:	milimolar
mPCR	:	Multiplex polymerase chain reaction
mqPCR	:	Multiplex real-time PCR
mtDNA	:	Mitochondrial deoxyribonucleic acid
NCBI	:	National center of biological information
ND5	:	NADH dehydrogenase subunit 5
ng	:	nanogram
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
s	:	second
ssPCR	:	Species specific PCR
T_a	:	Annealing temperature
T_m	:	Melting temperature
USDA	:	US department of Agriculture
w/w	:	weight/weight
IAC	:	Internal amplification control

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Red meats and poultry contribute significantly to the human intake of essential nutrients like proteins, fatty acids, trace elements and many vitamins. In recent years, food authenticity assessment, especially for meat and meat products, has been one of the most burning issues in the global food industry. Meats obtained from different animals differ in price and taste and consumers, nowadays, are concerned about the kind and quality of meat and meat products they purchase. From ancient times, meat has not been involved with adulteration as this has mostly been marketed and distributed readily as easily identifiable joints (Nakyinsige & Sazili, 2012). However, due to increased demand for commercial meat commodities, the popularity of ready-to-eat packaged food in the globalized market as well as available facilities to process meat into value-added products, counterfeiting, meat substitution and related fraud have become commonplace in both developing and developed countries (Cawthorn et al,, 2013). Meat products that are sold at a high price and undergo several processing steps, become a target of fake labelling and are often adulterated with meats of cheaper price or controversial species (Ballin et al,, 2009). A recent report reveals that food fraud causes a financial loss exceeding $40 billion per year to the food industry (B hme et al., 2019). Apart from economic loss, meat adulteration affects consumers from religious, medical (zoonotic threats and allergies), lifestyle (e.g., vegetarianism) and moral perspectives (Nešić et al, 2017). For instance, porcine consumption is strictly prohibited in Islam and Judaism (Nakyinsige et al., 2012; Soares et al, 2010), while the Hindus forbid any kind of beef and beef products (Hossain et al., 2017a). Vegetarians and vegans avoid all kinds of meat, and a certain group of people are allergic to selected meat species (Singh & Bhargava, 2019). Thus the assurance of religion and health compliant ready-made foodstuffs has

become an important issue. Detection of sensitive and economically important meat species in food products is, therefore, crucially important to protect consumers from food fraud and ensure discipline in the food business.

Enforcement of legislation has been practiced globally to ensure the authenticity of food products all along the supply chain (Hossain et al., 2019a). According to European Union food law, quality management and systematic controls are mandatory to maintain throughout the distribution chain, starting from the farm to the consumer's hand (Reg. EC 852/04, Reg. EC 853/04, Reg. EC 1379/13, Reg. EC 1420/13) (Di Pinto et al., 2015). Despite implementing stringent local and global food labelling regulations, the adulteration or misrepresentation of food products including meat products, to gain unethical financial profit, is rampant in society (Shears, 2010; Singh & Neelam, 2011). A recent report reveals worldwide incidences of mislabeling; 57% of processed meat products sold were found mislabeled in Italy, while up to 35% were observed in the United States (B hme et al., 2019). There are some reported incidences of objectionable or controversial species inclusion in commonly consumed food products. For instance, horse and pork meat in beef products (Walker et al,, 2013), horse meat for beef in UK and Ireland (Singh et al, 2014), monkey and dog meat in soup products (Rahman et al., 2014; Rashid et al., 2015), and cat meat in Indian curries in Britain (Ali et al., 2015a), pork and rat meat in lamb products (Ali et al., 2014a), dog and cat meat for chevon (Singh et al., 2014). These are of grave concern and highly alarming since most of these species carry potential zoonotic diseases; some are strictly restricted in several religions including Islam and Judaism.

Beef, buffalo, chicken, duck, goat, sheep, and pork are the popular meats bearing nutritional, economic and cultural/religious importance having the top consumption rate in most corners of the globe. Given the growing demand for animal protein-based foods,

there have been frequently reported incidences of meat fraud (species substitution and mislabelling) involving these species. In many countries, buffalo meat is frequently counterfeited with beef and beef with buffalo (Chuah et al., 2016; Hossain et al., 2017a). Several incidences of beef substitution with relatively cheaper pork have been reported (Ha et al., 2017; Xu et al., 2013). Poultry meat is sometimes fraudulently mixed with red meat (Mane et al., 2009; Mehdizadeh et al., 2014). Given its high price, mutton is often adulterated with cheaper meats (e.g., lamb) (Li et al., 2019). Duck meat, being cheaper, is mixed into lamb or beef products; mutual adulteration occurs between lamb and beef (Qin et al., 2016). Pork has been detected in beef products (He et al., 2015). Moreover, there is a widespread use of porcine and its derivative (restricted for Muslims and Jewish) either as food or as a derivative of other products (Tasrip et al., 2019). Authenticating food products is, therefore, crucial to safeguard consumers from food fraud and ensure discipline in the food business. In particular, it is of great significance to identify species such as beef, buffalo, chicken, duck, goat, sheep and pork that are highly consumed and especially vulnerable to adulteration.

Halal foods are a new and better alternative for consumers looking for foods that are safer, healthier, and cleaner (Zulfakar et al., 2014). Because of the rapid spread of halal food markets in all parts of the globe, protecting the originality of halal branded food products, particularly meat and meat products, has become a global concern. The global halal food sector is expected to grow from over 1.25 trillion US dollars in 2016 to around 2.57 trillion US dollars in 2024 (Shahbandeh, 2019).

Consumers are willing to pay more for halal foods because of the unique manufacturing and supply chain requirements that make them vulnerable to adulteration. As a result, several countries such as Malaysia, Indonesia, Thailand, Brunei, Singapore, India, Australia, New Zealand, China, Turkey, and Brazil have formed halal certification

and regulatory organisations in order to promote and capture the tremendous potential of the global halal food markets (Ali et al., 2012c). Since more than half of the population in Malaysia is Muslim, halal issues for meat products are vital. Moreover, Malaysia is one of the largest halal food exporting countries and has been committed to building up a reliable halal hub through continuous surveillance. More than 10 integrated halal hubs in Malaysia have been established for monitoring and exporting halal goods to other countries. Malaysia, being the leading halal products exporting country in 2013, accounted for a total turnover of US$ 10 billion (HKTDC, 2014). Thus, the halal food industry has emerged as one of the vital contributors to the Malaysian economy. The ready-to-eat modern food products such as burgers, frankfurters, meatballs etc. are usually prepared from minced meat and identification of animal source in these foods is very troublesome given the complexity of the matrices. Therefore, following the development of an analytical method, assay validation is essential through screening of the real-world samples to be confident whether it is working in the practical field.

It is crucial to have reliable analytical techniques able to detect and ensure the ingredients in food products whether they match the attributes and features declared by the manufacturer or distributor. These could significantly contribute to overseeing the situations of species substitution in foods. To this-date, various analytical methods have been reported for detection of fraudulent adulteration in the food products. Traditionally, protein detection has been the most reliable technique to authenticate animal species and enzyme-linked immunosorbent assay (ELISA) gained huge popularity in the food industry given its simplicity and low cost (Zhao et al., 2019). Protein-based methods, on the other hand, are not always suitable because they are time-consuming, target-biomarkers are frequently modified, and thus are unable to distinguish species in highly processed foods such as thermally or chemically treated products, and are less sensitive (Lago et al., 2011). Furthermore, these approaches are unable to discriminate between

closely related species like cows and buffaloes. Unlike proteins, DNA offers more stability under harsh thermal and chemical treatments (Zhao et al., 2019). Moreover, DNA possesses some inherent features, including universal information content and excellent stability under high temperature, pressure, and chemical processing (Fernández-Tajes et al., 2010). Thus, more favourable DNA-based techniques are widely practiced in recent years in real-world applications for meat species detection. PCR techniques are popular among DNA-based methods because they can amplify target biomarkers from a single copy to easily measurable quantities, making them a highly sensitive, robust, and low-cost platform for identifying biological components. These techniques retain their efficiency and sensitivity even under DNA degradation in heat-treated samples.

Mitochondrial genes are usually targeted for the design of species-specific primers used in species authentication. Mitochondrial DNA (mtDNA) offers an extra advantage over nuclear DNA since mt-genes are present in multiple numbers in every cell, maternally inherited, protected by the membrane and extremely conserved in nature and, there is no chance for sequence ambiguities (Murugaiah et al., 2009). They ensure the possibility of obtaining the desired PCR results even under the circumstances of serious DNA breakdown through extreme processing treatments (Mane et al., 2012a). Compared to the nuclear sequence, they also offer the discrimination of closely related animal species in admixture (Gupta et al., 2011). Mitochondrial cytb and ND5 genes are most commonly used for this purpose since they met required criteria such as suitable target length, high level of intra-species conserved regions within the species and interspecies polymorphism, in addition to sequence database availability for most animals and plants (Mohamad et al., 2013). Additionally, some other mitochondrial-genes namely, 16S rRNA, 12S rRNA, ATPase6/ATPase8 and D-loop are also used depending on assay design and the target species (Mohamad et al., 2013).

Numerous PCR approaches have been developed for the detection of different animal species in raw and processed food products. They include species-specific PCR (Davy et al., 2015; Karabasanavar et al., 2014), restriction fragment length polymorphism (RFLP) PCR (Meganathan et al., 2009), multiplex PCR (Ahamad et al., 2017), quantitative PCR (qPCR, known as real-time PCR) (Kesmen et al., 2013), random amplified polymorphic DNA (RAPD)-PCR (Saez et al., 2004), DNA barcoding (Liu et al., 2013), and PCR product sequencing (Lo et al., 2006).

Multiplex PCR (mPCR) is highly promising because they offer the identification of multiple targets in a single assay platform and thus, several species identification and differentiation can be done at a reduced cost and time (Ali et al., 2014). Several mPCR techniques have been documented for species detection in food products. Some examples are triplex PCR for beef, lamb, and duck (Qin et al., 2016), tetraplex for bovine, sheep, goat and fish (Safdar & Junejo, 2015), pentaplex for pig, dog, monkey cat, and rat (Ali et al., 2015b), and hexaplex PCR for chicken, beef, pork, lamb/mutton, horsemeat and ostrich meat (Kitpipit et al., 2014).

The PCR-RFLP is particularly desirable in this endeavor since, in addition to amplification of specific targets, it offers PCR products authentication by restrictive digestion of the amplified products using one or more restriction enzymes (RE) (Rashid et al.,2015). Using the sequence variation that occurs within a defined region of DNA, the differentiation among species of close relations is possible using a PCR-RFLP assay with appropriate REs. These techniques have been successful in discriminating the closely related species like sheep-goat and cow-buffalo (Girish et al., 2005), cattle-yak (Chen et al., 2010), swine-wild boar (Fajardo et al., 2008), beef, buffalo and pork (Hossain et al., 2016), as well as rabbit, rat and squirrel (Ali et al., 2018).

Although conventional PCR is a simple and low-cost technique, due to its inability to provide quantitative information regarding the target analyte in the specimens, researchers are now more interested in fully automated real-time PCR (qPCR) which is reliable, rapid and highly sensitive. In addition to detection, they provide real-time quantification of analyte targets, obviating the need for a time-consuming post-PCR analysis step such as electrophoresis in traditional PCR (Cheng et al., 2014). In particular, qPCR enables direct monitoring of PCR products generation throughout each amplification cycle and can measure at the exponential phase of the reaction, where the reaction does not need to be completed. Because fluorescent molecules are applied to collect real-time data, the intensity of the fluorescent dye and the quantity of PCR products are highly correlated (Fajardo et al., 2010). Two general categories of fluorescent chemistries namely double-stranded (ds) DNA-intercalating dyes such as SYBR Green (Asing et al., 2016) or Eva Green (Safdar & Abasıyanık, 2013) and probe-based chemistry such as TaqMan (Ahamad et al., 2019) or Molecular Beacon (Hadjinicolaou et al., 2009) probes are available for the qPCR systems. The TaqMan probe-based technique has received wider acceptance given its enhanced specificity and reliability. Herein, both the primers and probes find their complementary sites in the template DNA thus providing the opportunity of double checking that gives extra specificity and authenticity (Hossain et al., 2017a). Moreover, the TaqMan probe technique is especially advantageous in developing mqPCR systems, because upon labelling of specific probes with several reporter dyes, it allows unambiguous identification of amplified targets by using single or multiple primer sets in a single reaction tube (Arya et al., 2005).

Simplex PCR tests require many independent assays involving individual set of species-specific biomarkers to detect multiple targets in a given sample. Thus, they cause an increase in reagent quantity consumption and sample material requirements thereby

incurring additional cost, labor and time. Using multiplex PCR, on the other hand, maximizes the use of limited starting material and lowers reagent costs to amplify several target sequences at a time. However, most of the reported PCR assays involved long-length amplicons that are susceptible to break down during food processing treatments making the assays less trustworthy and often inconclusive (Rashid et al., 2015). In this regard, multiplex (heptaplex) PCR technique involving short DNA targets to authenticate animal species in food products would be beneficial. The aim of the study was to detect and quantify seven animals namely cow, buffalo, chicken, duck, goat, sheep, and pig that are widely consumed globally and are especially vulnerable to mutual adulteration as evidenced by lots of related meat fraud reports mentioned above in this Section. It is notable that, the adulteration incidence of religiously sensitive pig and cow in different food items has been a serious issue to a large group of people worldwide. Considering the facts, it is of great significance to identify, in particular, the selected species (cow, buffalo, chicken, duck, goat, sheep and pig) in raw meat and processed meat products. The incidences of mislabelling, cross-contamination and species substitution were investigated in commercial meat products sold in Malaysia.

1.2 Project Rationale

Beef, buffalo, goat, sheep, chicken, duck, and pork are commonly consumed as raw and processed products worldwide. These are the most popular meats having top consumption rate globally and they bear nutritional, economic and cultural/religious importance. There are religious, cultural, and geographical restrictions and preferences over the consumption of these meats. In recent years, there have been several social outcries over their adulteration/substitution and consumption (Girish et al., 2013). For instance, beef has evolved as the preferred meat, and it is heavily consumed in almost all parts of the world. Buffalo has a massive turnover in India, for domestic consumption as

well as for exports. On the other hand, cow is prohibited from being slaughtered, consumed, and exported in the same country because of restriction in Hinduism (Girish et al., 2013). Religious sensitivity regarding meat consumption may also provoke social unrest. A man in India was brutally killed and radical Hindus beat his family members based on false information about keeping beef in his refrigerator (Matthew, 2015). The above incident reflects that a meat scandal can also destroy social harmony. Buffalo is preferred over beef by the Egyptians and some Europeans because of the cultural preference and concern about the zoonotic disease like bovine spongiform encephalopathy (BSE) (Sakaridis et al., 2013). Pork has gained increased popularity in most Western countries despite its complete unacceptability to the Muslim community and Jewish people. Poultry meat has little or no detrimental effects on health and is acceptable to all religions and cultures. The goat meat and sheep meat, which have no religious taboos, are popular in certain regions and associated with some religious festivities.

Meat fraud might be threatening to public health as some animal species could carry multiple diseases that can be transmitted to humans and bring a regional emergency. Bovine and porcine materials have been found to be associated with the fatal neurodegenerative disease BSE and dioxin induced disorders (Bottero & Dalmasso, 2011). Porcine contamination can spread swine influenza (Bottero & Dalmasso, 2011). Buffalopox, a common zoonotic disease, is transmitted by buffalo and cattle (Gurav et al., 2011). Moreover, consumption of animal originated foods also creates concerns among consumers due to the prevalence of scrapie in goat and sheep (McIntyre et al., 2011), avian influenza in poultry (Stevens et al., 2013) that resulted in meat consumption reductions (Safdar & Junejo, 2016). Food allergies to meats such as beef, chicken, duck and mutton are often observed in a certain group of people (Bhat et al., 2015). Thus, the social, religious, health and business interests in beef, buffalo, goat, sheep, chicken, duck,

and pork are huge. Until now, no study is available detecting these seven commonly consumed species that are highly vulnerable to adulteration, in a single platform. Therefore, there is a clear room for the innovation of a reliable, cost-effective traceable system for their discriminatory authentication in food products so that the consumers can be protected from food fraud and a fair, sustainable food business could be established.

1.3 Problem Statements

Meat products that are of high price and undergo several processing steps become the target of fake labelling and are often adulterated with meats of cheaper price or of controversial species (Ballin et al., 2009), affecting consumers' trust, economic, health and religious interests. The morphological, protein and lipid-based methods for animal material authentication are not reliable because of the modification of the analyte biomarkers during food processing treatments. Consequently, DNA-based PCR methods have been evolved as the method of choice. Although several simplex and multiplex PCR schemes have been proposed for species detection in food products individually or parallelly, these methods are mostly based on long DNA markers which often break down during food processing. In particular, the decomposed specimens might encounter false-negative detection due to target breakdown and hence long-amplicon target PCR assays are less trustworthy and often inconclusive. Furthermore, most of the reported assays are not validated under admixed and processed food products. Cow, buffalo, goat, sheep, chicken, duck, and pig species are highly vulnerable to adulteration in the food chain. Most of them pose zoonotic threats, possess religious sensitivity and economic interests and thus, their adulteration creates increasing concerns among consumers. The discriminatory identification and determination of these species simultaneously would certainly contribute to boosting consumers' trust, safeguarding public health, religious practices, and, above all, running fair business, thereby establishing discipline in the food

industry. Therefore, a reliable and cost-saving short targeted (73− 263 bp) heptaplex assay for confirmed and simultaneous detection of bovine, buffalo, caprine, ovine, chicken, duck and porcine materials in raw meat and processed meat products would be beneficial.

1.4 Study Objectives

Based on the above problem statements, the following objectives have been set for the present research:

1.4.1 General Objective

The overall objective of the present study is to develop and validate a heptaplex PCR assay for the discriminatory detection and quantification of bovine, buffalo, caprine, ovine, chicken, duck and porcine materials in order to authenticate them in food products.

1.4.2 Specific Objectives

1. To develop primers and probes for each target species (cow, buffalo, goat, sheep, chicken, duck and pig) to amplify shorter PCR products and check the specificity of the developed primers against non-target species by bioinformatics software.

2. To optimize and validate heptaplex PCR assays for the differential detection of cow, buffalo, goat, sheep, chicken, duck and pig species as well as to develop and validate PCR-RFLP assays for the discriminatory authentication of PCR products under various raw, admixed and processed food samples as well as the screening of commercial meat products.

3. To develop and validate multiplex real-time PCR systems for both detection and quantification of target species (cow, buffalo, goat, sheep, chicken, duck and pig) under raw, admixed and processed samples as well as the screening of commercial meat products.

1.5 Scopes of the Research

Beef, buffalo, goat, sheep, poultry and pork are popular meats having an enormous importance from health, cultures, religions, and businesses viewpoint. This study primarily focuses on developing and validating a reliable, robust and sensitive analytical technique for confirmed detection of seven highly consumed animal species (cow, buffalo, goat, sheep, chicken, duck and pig) in raw meat and processed meat products in a single reaction.

1.5.1 Design of Biomarkers and Evaluation of the Biomarkers' Specificity

Recently, DNA-based techniques have been widely used for species authentication. However, successful detection of species using PCR assays crucially depends on the appropriate design and development of acceptable primers. The mitochondrial DNAs (mt-DNA) are more focused on the nuclear ones for authentication studies. DNA biomarkers using mtDNA have proven high efficiency given their ubiquitous presence in multiple copies in all cells having intraspecies conserved and interspecies polymorphic nature (Rashid et al., 2015). This study targeted the cytb and ND5 genes of mitochondrial origin to design cow, buffalo, goat, sheep, chicken, duck, and pig primers. Recently, researchers have paid increased interest in using short amplicon length biomarkers given their extra-ordinary stability against harsh food processing treatments which make them still traceable in the specimens subjected to high temperature and pressure (Rashid et al., 2015). Due to the extensive sensitivity and stability of the shorter amplicon DNA target, it has vast application in forensic analysis, biochip and biosensor development. In this study, short-length targets were used which offered stringent stability even under decomposition of samples. I have designed a total of seven sets of primers, one for each of the target species, with amplicon sizes of 73-263 bp. Thus, the present study developed a short amplicon targeting heptaplex PCR assay

for the discriminatory authentication of bovine, buffalo, caprine, ovine, chicken, duck, and porcine materials in the food chain.

The specificity of the designed primers plays a vital role in a successful PCR assay. Primers significantly matching the target species and thus revealing huge mismatches with the non-targets, contribute to an increased possibility of a highly specific PCR assay and eliminate non-target amplification chances (Murugaiah et al., 2009). Therefore, the evaluation of the primers' specificity by using a well-known system is crucially important to avoid any ambiguity. The designed primers were checked for theoretical specificity among the closely related and the distant species using the online Basic Local Alignment Search Tool (BLAST) in the NCBI database (http://blast. ncbi.nlm.nih.gov/Blast.cgi). Finally, the performance of the developed biomarkers was verified, and absolute species-specificity was confirmed by a practical PCR assay through a cross-amplification reaction using DNA templates from the target and 25 non-targett species.

1.5.2 Development of Multiplex PCR Assay

Favorable DNA-based techniques are widely practiced in recent years in real-world applications for meat species detection. PCR approaches are greatly promising since they offer amplification of target biomarkers from a single copy to easily detectable quantities and thus offer a highly specific, sensitive and reliable platform for detecting biological ingredients even if DNA is degraded under heat treatments. Multiplex PCR (mPCR) is highly promising for simultaneous identification and differentiation of animal species at a reduced cost and time and have been widely used for food products (Ali et al., 2014a). Species-specific PCR assay, although often provides conclusive results, may be proved unsuitable to be considered a definitive analytical tool due to certain limitations (Yang et al., 2005). However, the PCR-restriction fragment length polymorphism (PCR-RFLP) technique is free from the shortcomings and has attracted worldwide researchers (Hashim

& Al-Shuhaib, 2019). It authenticates the PCR product amplified from a selected gene fragment by generating species-specific fragments through digestion with restriction enzymes (Pereira et al., 2008). In this study, sequencing of the PCR products was done, and the PCR products of pig, cow, buffalo, chicken, duck, goat, and sheep were digested with three restriction endonucleases. However, the conventional PCR assay provides only qualitative information and cannot be used for quantitative detection to measure the amount of adulterant originally present in each specimen. In contrast, the real-time multiplex PCR assay, in addition to detection, also offers quantification opportunities of many analyte targets in real-time with more precision and accuracy (Asing et al., 2016). Therefore, this research designed and evaluated different specific oligonucleotide biomarkers, developed and validated simplex and multiplex conventional PCR, PCR-RFLP and TaqMan probe real-time PCR assays for detection, differentiation and quantification analysis of bovine, buffalo, chicken, duck, goat, sheep, and porcine DNA in the food chain.

1.5.3 Assay Validation and Applicability Test under Food Products

Checking the validity of the developed authentication tool is very important because the reliability and acceptability of the assay greatly depend on the validity performance. Hence, the assay was validated in terms of specificity, sensitivity, and stability under various conditions. The initial performance of the developed multiplex systems was evaluated using the raw DNA extracted from target species and various other non-target species. Since extreme heat or other processing treatments usually result in DNA degradation, assay validation in terms of stability is essential for heat-treated samples prior to applying the technique to analyze commercially processed food products (Qin et al., 2016). Therefore, the assay validation was performed under different cooking conditions, like boiling, microwave cooking, and autoclaving to simulate the food

processing treatments to test the stability of the developed multiplex system. Next, the assay sensitivity and efficacy were evaluated by performing it under admixture of the target meats. Notably, PCR sensitivity and efficiency may often be reduced under complex food matrices and processed meat products because of various spices and additives that may interfere with the binding of primers at specific sites (Di Pinto et al., 2005). Compared to raw meat, adulteration in processed meat products could be more competently manipulated and thus, these products are susceptible targets for such fraud (Soares et al., 2013). Therefore, the developed assay was finally validated under various laboratory made model and commercially available popular food products including meatball, burger and frankfurter which are highly consumed worldwide. Thus, the novel assay might be used by regulatory authorities for animal species authentication even under degradation of DNA in processed samples.

1.6 Thesis Organization

This thesis consists of six chapters: introduction, literature review, materials and methods, results, discussion, conclusions and future recommendations. The contents of each chapter are described below:

Chapter 1 (Introduction): This chapter describes, in short, the general background of the study, project rationale, problem statement, objectives and scope of the present research. It also briefly presents the importance of the present research, with a short discussion on the limitation of the previous works and also the innovative aspects of the present study overcoming the shortcomings of the previous works.

Chapter 2 (Literature Review): This chapter critically presents in detail, the significance of food authentication, the global prevalence of food fraud along with its multifarious impacts, global consumption and production of some commonly consumed

meats, currently available species detection techniques and their applications and validation of PCR methods.

Chapter 3 (Materials and Methods): All the methodologies, materials and protocols as well as bioinformatics tools adopted in this study are elaborately described in this chapter.

Chapter 4 (Results): This chapter illustrates all the findings of the research. These include detailed outcomes of extraction of DNA, design and specificity check of all the biomarkers, sensitivity and validity assessment of the assays under various admixed and processed food matrices as well as PCR products authentication.

Chapter 5 (Discussion): Herein, the research findings and outcomes are elaborately discussed, and they are critically compared with those of previous reports.

Chapter 6 (Conclusions and Recommendation): Finally, the summary of findings of the present study along with prospects and suggestions for future research works are stated here.

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of Animal Materials in Food Chain and Need for their Authentication

Meat is a vital component of healthy, well-balanced diet for its high nutritional values. According to European legislation, meat is defined as the edible portions, obtained from domestic animals like bovine, caprine, ovine and porcine as well as the poultry meat, farmed and wild animals (Pereira & Vicente, 2013). It is a rich source of high-quality proteins, various fats including omega-3 polyunsaturated fatty acids, different vitamins, minerals and micronutrients like iron, zinc, selenium, magnesium, sodium, potassium, vitamins A, and B-complex and folic acid which are very essential for the development, growth, and proper functioning of the body organs. The most common meat source is animal species such as cow, buffalo, pig, sheep, goat and poultry (Herrero et al., 2013). In addition to the nutritional value, they possess economic, cultural and religious significance with the leading consumption rate in most parts of the world. Due to the increase in global populations and rapid growth in income, demand for meat products worldwide has dramatically increased. Meat consumption, in most countries, has been continuously increasing since the 1960s, while a rapid increase was observed from the 1980s decade till today. According to recent studies, meat consumption has been increased as high as 500% (1992 – 2016) (Katare et al., 2020) indicating that nutritional habits had notable changes during the last century. Approximately 2 billion people depend primarily on meat-based food worldwide (Pimentel & Pimentel, 2003). A recent survey reporting the trends in meat consumption habits in Europe shows that nowadays, up to 58% of the total available protein comes from animal-derived products which constitute the major protein source (28 g of protein/person/day) and account for 30% of total calorie consumption (Bonnet et al., 2020). Thus, the ever-expanding markets and huge popularity

of meat and meat products have rendered them especially vulnerable to adulteration and fraudulent labelling.

In addition to affordability, several factors influence decisions in selection, purchasing and consumption of meat. These include availability or convenience to buy or cook, social and cultural values, religious beliefs, geographical locations and age groups. Beef is considered as the preferred meat and has been consumed significantly in almost all parts of the world. Buffalo turnover is remarkable in India, both for domestic consumption and external exports, although there is a restriction on the slaughtering, consumption, and exporting beef in some states of the same country for religious reasons (Girish et al., 2013). The Egyptians and some Europeans prefer buffalo to beef because of the cultural preference and outbreak of the bovine spongiform encephalopathy (BSE) (Sakaridis et al., 2013). In contrast, pork is popular in most of the Western countries despite its complete unacceptability to the Muslims and Jewish people. Poultry meat appears to have little or no detrimental effects on health and thus, its consumption is increasing in all regions – mainly in North America (Basu, 2015). Chicken is widely acceptable to all religions and cultures, cheaper and available in all areas of the world. Sheep and goat meat consumption is normally linked to certain ethnic groups and associated with some religious festivities. Asian, African, and Caribbean people in particular, consume goat meat (Teixeira et al., 2019).

Since meat and meat products are the significant and major component of the regular human diet, their quality and integrity are of increased concern to the consumers, the state regulatory bodies, the food processors and business communities. The higher demand along with the increased cost of meat and meat products make them vulnerable to fraudulent mixing, substitution and false labelling. Because of zoonotic threats, religious sensitivity and social or lifestyle factors, the indiscriminate use of the animal meat creates

concerns among consumers. Some animal species can cause public health threat as they transmit multiple infecting agents to humans, causing a regional emergency. The US department of Agriculture (USDA) alerts that animal products are responsible for approximately 75% of human infections either directly or indirectly (USDA, 2015). Meat consumption is also related to religious as well as social and lifestyle factors. For example, beef is not acceptable to Hindus and pork is completely restricted for the Muslims, Jewish and selective denominations of Christians. Vegetarians and vegans do not allow any kind of animal materials and certain people are allergic to some meat species. In recent years, there have been a number of social outcries over the adulteration/substitution and consumption of some animal meats (Girish et al., 2013). It is of grave concern that meat consumption issue can also provoke social unrest and can lead to the utmost cruelty. For example, there was killing of a man by some excited Hindus in the city of Dadri in India based on false news of keeping beef in his refrigerator; his family members were also beaten seriously (Matthew, 2015). Therefore, animal materials in food, if used indiscriminately, may also destroy social and religious harmony.

Thus, proper labeling of constituents in meat and meat products and their subsequent field monitoring are considered as seriously important issues in modern times to prevent food forgery, safeguard consumers trust, respect religious faith, to enforce acts associated with livestock products, to maintain standards of livestock products, and to maintain sustainable food businesses. Considering the need, most countries have regulatory bodies for tracing and tracking adulterants such as lower grade or lower-priced meats mixed with the higher priced ones. With the use of a reliable traceability system, regulatory authorities or food companies are able to track and trace any foodstuff that does not meet consumer expectations or is not in compliance with the related applicable regulations of the country. The main objective of a traceability system is to disclose a product's story, i.e., specify a definite product batch and the ingredients used in its production and follow

the same throughout the production and distribution chain. Thus, a comprehensive and perfectly managed supply chain system must be ensured to make truly labelled meat products available and for this purpose, an authentic analytical tool is crucially needed. The exact identification and differentiation of meat species help ensure meat product quality and prevent consumers from the deception by fraudulent adulteration.

2.1.1 Food Fraud

Several definitions have been used for "food fraud" but most countries lack established legal definitions. The United Kingdom's Food Standards Agency (FSA) describes food fraud as "the deliberate placement on the market, for financial gain, intending to deceive the consumer, covering two main types of fraud. These include the sale of food which is unfit and potentially harmful as well as the deliberate misdescription of food, such as products substituted with a cheaper alternative" (Johnson, 2014). The United States Pharmacopeial Convention (USP) states: "Food fraud in the context of food ingredients refers to the fraudulent addition of non-authentic substances or removal or replacement of authentic substances without the purchaser's knowledge for the economic gain of the seller". These definitions generally categorize three types of fraud namely: i) total or partial replacement of a food ingredient or costly authentic component with a cheaper counterpart (or alternative cheaper animal species in case of meat and fish) ii) addition of a small quantity of a non-authentic substance to hide an ingredient of inferior quality and iii) removal or deliberate exclusion of an authentic and costly component or ingredient from the food product without the knowledge of purchasers. (Johnson, 2014). Generally, food fraud is committed by persons somehow involved in the food chain.

2.1.1.1 Adulteration of food

Adulteration can be termed as, either mixing or substituting inferior substances with the superior one or removal or the omission of some valuable and important constituents

from a given product (Ruiz-Matute et al., 2007). Adulteration can also be perpetrated (either intentionally or unintentionally) by adding a non-food ingredient to increase the quantity of the food in raw or processed state or to improve the appearance. According to the Food and Drug Administration (FDA), economically motivated adulteration (EMA) may be defined as the "fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production, i.e., for the economic gain" (Johnson, 2014).

According to the Federal Food, Drug, and Cosmetic (FD & C) Act (2002, Sec. 402) of the United States (Rahman, 2015), a food shall be considered to be adulterated:

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

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

d) If 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) If 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) If its container is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health.

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

h) If any valuable constituent has been in whole or in part omitted or abstracted therefrom.

i) If any substance has been substituted wholly or in part, therefore.

j) If damage or inferiority has been concealed in any manner.

k) If 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".

On the other hand, there is not clearly stated "generally acknowledged definition of food fraud" in the EU laws, although there is a vast legislative framework that focuses on food safety. The general guideline of EU regulations requires that food labeling, advertising, presentation, and packaging "shall not mislead consumers" (Johnson, 2014).

2.1.1.2 Mislabeling of food

Mislabeling is a kind of false advertising where the product label contains overemphasized, exaggerated, or sometimes completely wrong statements. Mislabeling of food products is usually done intentionally to achieve financial gain with a view to deceiving the consumers regarding the product within the package. In fact, the authenticity of a food product relies on its compliance with labeling rules and regulations, especially regarding ingredients' composition, manufacturing and processing methods, treatments and practices, packaging time etc. Correct product labeling stating the appropriate description of ingredients helps building consumers' confidence and to assure fair trade.

The Federal Food, Drug, and Cosmetic Act of United States section 403 (MBF, 2002) stated that a food shall be considered as misbranded or mislabeled:

“a) If its labeling is false or misleading in any particular, or its advertising is false or misleading in a material.

b) If it is offered for sale under the name of another food.

c) If it is an imitation of another food, unless its label bears, in type of uniform size and prominence, the word ‘‘imitation’’ and, immediately thereafter, the name of the food imitated.

d) If its container is so made, formed, or filled as to be misleading.

e) If in package form unless it a label containing (1) the name and place of business of the manufacturer, packaging provider, or distributor; and (2) an accurate statement of the quantity of the contents in terms of weight, measure, or numerical count, except that under clause (2) of this paragraph reasonable variations shall be permitted, and exemptions as to small packages shall be established, by regulations prescribed by the secretary.

f) If any word, statement, or other information required by or under the authority of this Act to appear on the label or labeling is not prominently placed thereon with such conspicuousness (as compared with other words, statements, designs, or devices, in the labeling) and in such terms as to render it likely to be read and understood by the ordinary individual under customary conditions of purchase and use.

g) If it purports to be or is represented as a food for which a definition and standard of identity has been prescribed by regulations as provided by section 401, unless (1) it conforms to such definition and standard, and (2) its label bears the name of the food specified in the definition and standard, and, insofar as may be required by

such regulations, the common names of optional ingredients (other than spices, flavoring, and coloring) present in such food".

2.1.1.3 Causes of food fraud

Despite adopting and implementing local and global food labelling regulations, the adulteration or misrepresentation of food products including meat products, to gain unethical financial profit, is rampant in society (Singh & Neelam, 2011). Adulteration of meat has not been practiced from ancient times since marketing and distribution of meat had mostly been performed readily as easily identifiable joints (Nakyinsige & Sazili, 2012). However, given increased demand for commercial meat commodities, the popularity of ready-to-eat packaged food all parts of the world as well as improved technologies and facilities to process meat into value-added products, mislabeling, meat substitution and related fraud have become commonplace in both developing and developed countries (Cawthorn et al, 2013). Meat products that are sold at a high price and undergo several processing steps, are often adulterated with meats of cheaper price or controversial species and thus are falsely labelled (Ballin et al., 2009). The main reasons behind the adulteration of food products may be summarized as follows:

a) For better revenue and profit, practiced as a part of business strategy.

b) To increase the quantity of food item and distribution of nutrition.

c) For imitation of another food item that is usually of high price and high quality.

d) Ignorance and lack of awareness of sufficient food consumption because of illiteracy of the general people.

e) Increased food demand of the growing population as well as its changing lifestyle and food habit trends.

f) Lack of efficient food laws and regulations as well as government initiatives to monitor the market.

2.1.1.4 Regulatory focus

Food fraud practice is not new in society. The existence of rules regarding the adulteration of wines with colors and flavors during the Roman and Greek Empires reflects the prevalence of food fraud during that period (Charlebois & Haratifar, 2015). Various countries adopted several laws and regulations to control such fraudulent activities. France was the first to protect consumers from food forgery by establishing the Conseil de Salubrite in Paris in 1802. England, France, Germany, and other Continental countries had legislations against adulteration of specific items including coffee, tea, wine and beer since the Middle Ages. England passed the first general act in 1860. To establish transparency in food manufacturing as well as food distribution and marketing, many countries have established credible regulatory bodies. For example, Malaysia, Indonesia, Thailand, China, Singapore and Brazil have credible halal certification bodies to verify the halal status of foods (Nakyinsige et al., 2012).

The EU food laws related to food safety aims particularly at protecting consumers' health. The EU requirements of food safety are described as follows:

"i) Food shall not be placed on the market if it is unsafe.

ii) Food shall be deemed to be unsafe if it is considered to be: (a) injurious to health; (b) unfit for human consumption.

iii) In determining whether any food is unsafe, the following criteria should be addressed: (a) if the normal conditions of the food consumed by people and the safety is controlled at each stage of production, processing, and distribution, and

(b) if necessary, information is provided to the consumer, including the general information on the label, or other information concerning the avoidance of specific adverse health effects from a particular food or category of foods.

iv) In defining whether any food is injurious to health, regard shall be had: (a) not only to the probable immediate and/or short-term and/or long- term effects of that food on the health of a person consuming it, but also on subsequent generations; (b) to the probable cumulative toxic effects; (c) to the particular health sensitivities of a specific category of consumers where the food is intended for that category of consumers.

v) In determining whether any food is unfit for human consumption, regard shall be had to whether the food is unacceptable for human consumption according to its intended use, for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay.

vi) Where any food which is unsafe is part of a batch, lot or consignment of food of the same class or description, it shall be presumed that all the food in that batch, lot or consignment is also unsafe, unless following a detailed assessment there is no evidence that the rest of the batch, lot or consignment is unsafe.

vii) Food that complies with specific Community provisions governing food safety shall be deemed to be safe insofar as the aspects covered by the specific Community provisions are concerned".

Malaysia has the Food Act 1983 and Food Regulations 1985 in order to prevent food fraud thereby protecting the consumers from unsafe food. The Food Act 1983 is the parent act. The Food Act 1983 was enacted with a view to protecting the consumers against health hazards and fraud in the preparation, sale, distribution and use of food, and for

matters incidental thereto or connected therewith. The Act consists of thirty-six sections divided into five parts. Part I discusses about preliminary issues like the definitions of basic concepts. Part II deals with the administration and enforcement of the Act. Part III covers the legislation that protects consumers against unsafe food with respect to composition, fake labelling and misleading advertisement. on other hand, importation, warranties and defenses have been discussed in Part IV. According to the contents in Part IV it is notable that the Food Act 1983 oversees imported food also in addition to local food industry. Finally, all miscellaneous issues have been incorporated in Part V (Ismail, 2011).

Malaysian Government has taken the food fraud issue very seriously. Food adulteration is a crime that is defined not only in the Food Act 1983, but also in the Penal Code. Food adulteration includes not only the act of mixing food with harmful or forbidden substances, but also the act of mixing food with permissible substances in greater amounts than the Food Act 1983 or the Food Regulations 1985 allow. Food adulteration also occurs when the original food package is opened, part or whole of the food within is transferred, and the original package is filled with another substance. Regarding labelling and advertisements, the Food Act 1983 states that "preparing, packaging, labelling or selling food in a manner that is false, misleading or deceptive as regards its character, nature, value, substance, quality, composition, merit or safety, strength, purity weight, origin, age or proportion or in contravention of the Food Regulations 1985 is an offence" (Ismail, 2011).

Food Regulations 1985 contains a specific part, Part IV for labelling. According to Food Regulations 1985, it is mandatory that meat and meat products should be labelled as such: "(I) There shall be written in the label on a pack-age containing meat and meat product, in not less than 10 point lettering (a) the common name of the kinds of meat from

which its content has been prepared; and (b) where its content consists of two or more kinds of meat, the common name of the kind of meat present, in descending order of the proportion present" (Chuah et al., 2016). Advertisement of food which is inconsistent with the provisions of the Food Act 1983 and the Food Regulations 1985 is not permitted.

According to Malaysia Food Regulations 1985, it is mandatory that food items containing meat and meat products should be labelled as such: "(I) There shall be written in the label on a pack-age containing meat and meat product, in not less than 10 point lettering (a) the common name of the kinds of meat from which its content has been prepared; and (b) where its content consists of two or more kinds of meat, the common name of the kind of meat present, in descending order of the proportion present" (Chuah et al., 2016).

Malaysia established 'the Department of Standards Malaysia' that aims in protecting consumers' health and safety by ensuring and monitoring the standard in manufacturing and trade of halal food. According to 'the Department of Standards Malaysia,' to be allowed under the Shariah law, food and drink and/or their ingredients must satisfy 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 descendants/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".

Different countries have comprehensive food legislation as well as authorities/bodies to ensure safe food. However, the most important thing we need now is the effective enforcement of the legislation so that consumer safety is guaranteed.

2.1.1.5 Prevalence of meat fraud

Meats obtained from different animals differ in price and taste and the fraud of substituting high-priced meat with a cheaper, lower quality one is commonplace in the food industry (Uddin 2021a). Despite adopting and implementing local and global food labelling regulations, the adulteration or misrepresentation of food products including meat products, to gain unethical financial profit, is rampant in society (Singh & Neelam, 2011). With the increased demand of costly commercial meat commodities, the popularity of ready-to-eat packaged food in the globalized market as well as available technologies and facilities to process meat into value-added products, meat adulteration/substitution and related fraud have been perpetrated for the last few decades (Cawthorn et al., 2013). Horse meat scandal of 2013 in Europe was the most remarkable food fraud outrage covered by global news media (Zhao et al., 2019). Individual surveys on in meat products' fraud conducted in different countries clearly showed that meat fraud has become a burning issue globally. One of the largest surveys on seafood fraud was

conducted by Oceana, a non-profit marine conservation organization over the period 2010 to 2012, where 1,215 samples were collected from 674 outlets in 21 states of the United States (U.S.). They found on an average, a 33% probability of not getting the original product people pay for. The incidence of mislabeling was quite high; among the seafood sold, 74% in sushi restaurants and 18% in grocery stores were found mislabelled as "red snapper" and "tuna" that accounted for 90% and 55% of the relevant, tested products, respectively (Warner et al. 2013). The prevalence of food fraud is also high in Europe. A large investigation on food fraud was conducted by Interpol and Europol in 47 countries; thousands of tons of adulterated food were seized, among them 31 tons of chemically treated seafood from Italy and 35 tons of adulterated butter from Egypt (Oaklander, 2015).

Below are some examples of mislabelling incidences in different countries that have been reported. In Italy, 57% of commercially available processed meat products were found mislabelled while it was up to 35% in the United States (B hme et al., 2019). Individual market survey reports different percentages of meat fraud incidences in various countries; 5% in United Arab Emirates (Premanandh et al., 2013), 22 % in Turkey (Ayaz et al., 2006), 10% in the UK (Shears, 2010), 25 – 30% in India (Singh & Neelam, 2011), 25% in the United States and Canada (Wong & Hanner, 2008), 68% in South Africa (Cawthorn et al., 2013), 15 % in Switzerland, and 8 % in the UK (Ballin et al., 2009). A recent study by Stamatis et al. (2015) revealed that 54, 35 and 34% of pet foods, frozen fish products and processed meat sold in the Greek market were found mislabelled, respectively. A survey in Turkey revealed that 100% beef labeled meatballs contained chicken and turkey instead of beef, and sausages labeled as 5% beef contained no bovine DNA at all (Ulca et al., 2013). Investigation of beef and pasta products in the UK identified horse DNA in 29 out of 2501 samples (Castle, 2013). The Food Safety Authority of Ireland also detected horse DNA in 37% of the tested beef burgers and among them, 85% also contained pig materials. (Walker et al., 2013). In another study,

Cawthorn et al. (2013) demonstrated the presence of undeclared species in 68% (95 out of 139) samples of burger patties, sausages and deli meats. Undeclared pork was detected in 30% of burgers and patties, 32% deli meats, 38% minced meat and 52% sausage products. Undeclared animal species were also detected in 24% of beef burgers and minced meat samples (Al-Nassir et al., 2014). Upon analysis of 105 imported beef products in the Arabian Gulf countries, pig and horse DNA was detected in 26% and 7% of the tested samples (Bourguiba-Hachemi & Fathallah, 2016). In Malaysia, non-halal material was also detected in halal branded food products. Porcine DNA was found in two chocolate and one chicken nugget samples out of total 30 tested food samples (Farouk et al., 2006). Huge amount of fake beef that was fraudulently prepared by chemically treated pork was seized by Chinese police (Tan, 2013). Processed rat meat was also found to be sold as lamb in China (Buckley, 2013). In northern California, human and rat DNA was identified in burger samples (Kowitt, 2016). There are concerns that the documented food fraud incidents might be only the tip of the iceberg representing just negligible portion of actual scenario of global perspectives while the exact extent of worldwide mislabelling or adulteration cases remains undetermined. However, these are sufficient to realize the importance of food product authentication to prevent fraudsters from cheating consumers.

2.1.1.6 Impact of food fraud

Food fraud, whether in the form of adulteration or mislabeling, has been considered as a threat to the integrity of the expanding agri-food system and a major concern for consumers, businessmen, and the government authorities around the world. Consumers are deceived by purchasing lower quality foodstuff against their knowledge and choice. It is not easy to determine how widespread this fraudulent practice is across the entire food supply chain and how widespread its impact on multiple aspects. With the

introduction of food fraud into the supply chain, there may be a number of repercussions ranging from minor to significant, consequently disrupting consumer trust and exerting a deleterious effect on the entire food industry. In general, food adulteration has a long-term impact on producers/farmers, processors, consumers and above all, on the government.

Despite the widespread occurrence of food fraud and its deleterious consequences for consumers and the sectors involved, a systematic analysis of its impact from an economic viewpoint is virtually absent. Most of the previous researchers has focused mainly on product mislabeling and its direct effects on consumers. However, the financial and reputational impact on businesses is very significant. An exact estimation of the monetary cost of food fraud is difficult. A recent report reveals that food fraud causes a financial loss exceeding $40 billion per year to the food industry (B hme et al., 2019).

The food fraud issue becomes the most serious when it affects consumer health. When adulteration makes a food unsafe, then the damage is no longer only economical, rather it becomes health concern resulting in a number of ill effects on public health. Three types of food fraud risks have been identified for public health: direct, indirect, and technical. Consumers are at direct risk when faced with immediate or imminent threat, for example, by mixing acutely toxic or lethal contaminants. Indirect food fraud risk occurs through long-term exposure, such as persistently ingestion of chronically toxic contaminants in low doses. Indirect risk also occurs by omitting the beneficial ingredients, such as vitamins or preservatives from the food. Technical food fraud risk, indeed, is nonmaterial in nature. This may include food documentation fraud occurring when there is deliberate misrepresentation of product content or country-of-origin information. Some incidences of food fraud affecting human health are discussed below. In 1986, methanol contamination with wine caused death of 23 persons in Italy (Tähkäpää et al., 2015). The

Chinese milk scandal in 2008 affected consumers and industries in various countries. The scandal involved the addition of unexpected food adulterant melamine in milk to increase its protein content and pass quality control tests (Mooney, 2008). After ingestion of infant formula and milk contaminated with melamine, 290,000 infants and children were affected of which at least six were died and 52,000 were hospitalized (Reshanov, 2008).

Fraud involving especially meat and meat products exerted financial as well as health impacts in different countries. In 2003, inedible poultry meat of pet food plants entered into the UK food chain (FSA, 2004). In Belgium, a food crisis occurred following contamination of cancer-causing dioxin and polychlorinated biphenyls (PCBs) in 1999. Domestic and export markets of poultry and pork were adversely affected destroying about 2500 poultry and pig farms and the USA imposed import cancellation of certain food products from the entire European Union (Covaci et al., 2008). Ireland also faced the Irish pork dioxin crisis in 2008 resulting in pork market fall; feeding of dioxin-contaminated feed affected 10% pig. Eventually, all pork products that had been prepared during that period were recalled causing huge losses to the manufacturing industries. In addition, there was a report of illegal repackaging and marketing of Poultry and beef of unknown sources for human consumption in Northern Ireland (Tähkäpää et al., 2015).

Apart from economic loss and potential public health threats (zoonotic diseases and allergies), meat adulteration affects consumers from social/religious, lifestyle (e.g., vegetarianism) and moral perspectives (Nešić et al., 2017). For instance, porcine consumption is strictly prohibited in Islam and Judaism while the Hindus forbid any kind of beef and beef products. Meat issues might also destroy social and cultural harmony. A man was brutally killed, and his family members were seriously beaten by a group of radical Hindus in a city in India based on a rumor of consuming beef and storing it in the refrigerator for further use (Matthew, 2015). Vegetarians and vegans do not allow any

kind of animal materials in their diet. In summary, food fraud causes hamper in brand image; economic loss to the food industry; costs to government from public health, and above all, disrupts consumer trust over the food chain system.

2.2 Meat Production and Meat Consumption

Many factors, in addition to affordability, influence decisions to select and consume meat. These include availability or convenience in buying or cooking, its social and cultural values, religious acceptability as well as economics and political economy. There is significant variation in the quantity of meat in human diets among individuals living in a society and across different societies. With the ever-increasing population, global production and consumption of meat are dramatically increasing day by day (Wanapat & Chanthakhoun, 2015). Worldwatch Institute reports that there is three times increase in global meat production during the last four decades with the 20% rise in the last decade (Rousseau, 2016; WWI, 2017). It is speculated that there will be an increase in per capita consumption of global meat by more than 4% over the next 10 years (Reubold, 2015). The projection of the Organization for Economic Cooperation and Development (OECD), regarding the status of annual meat consumption per capita in different countries is given in Figure 2.1 (OECD, 2021). Collecting data from OECD, the total quantity of meat (beef and veal, pork, sheep and poultry) consumed per capita in 2015 was estimated, and a list of the leading meat consuming countries with the total amount (Kg) of meat consumed has been illustrated in Figure 2.2 (OECD, 2017). According to Figure 2.2, the United States tops the leading meat consuming countries with per-person consumption of about 95.4 kg of meat yearly or approximately 260 gm per day. Whereas South Africa is the lowest meat-consuming country where per person consumes 47.8 kg of meat yearly or about 130 gm per day (Reubold, 2015). Increasing trends in the demand for meat may be

driven by many reasons. Meat is considered as a vital source of nutrients for people of low-income group with restricted diets options (Forouzanfar et al., 2015).

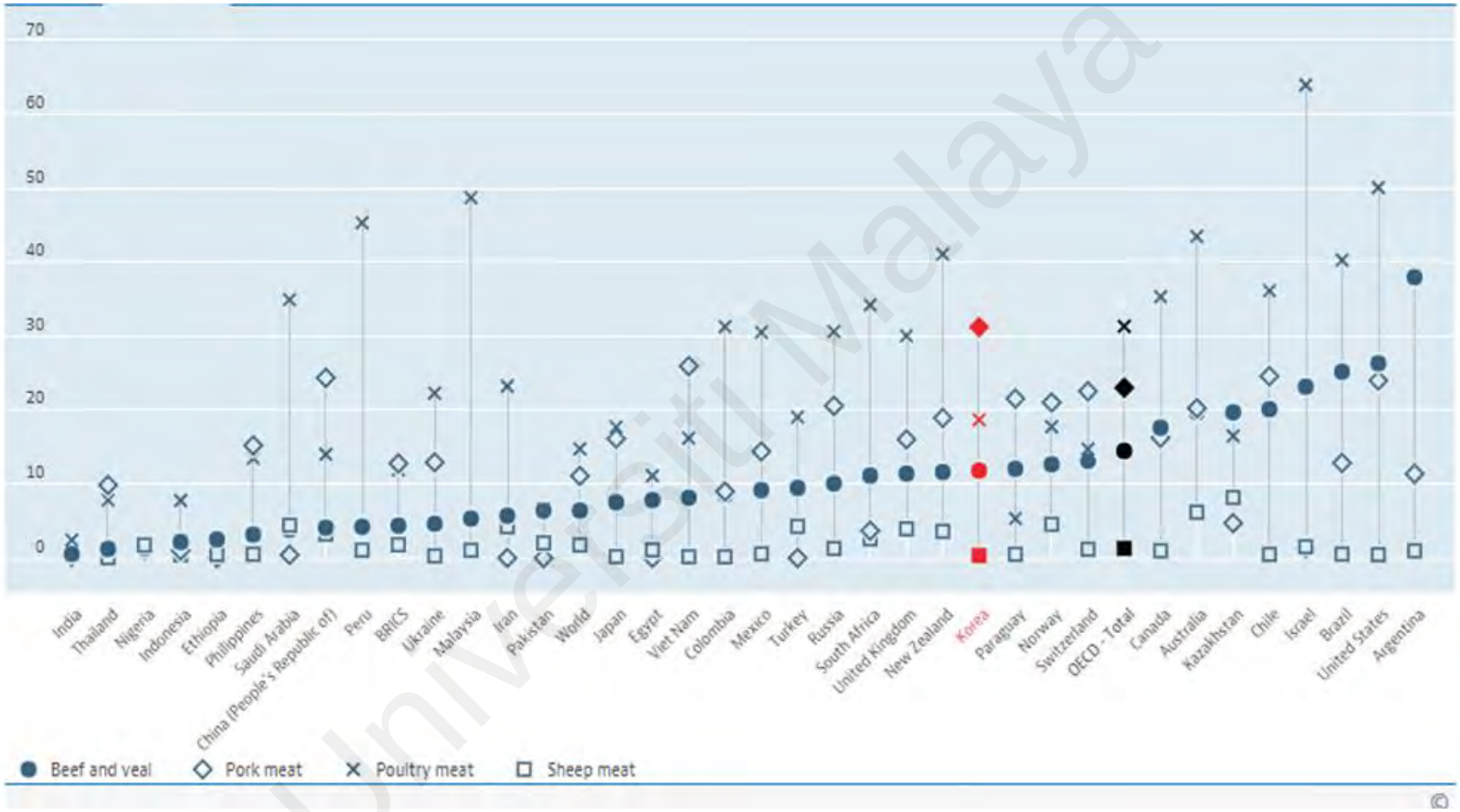


Figure 2.1: Consumption of meat (beef and veal, pork, lamb and poultry) by different countries in Kilograms/capita in 2019 as projected by OECD. Adapted from OECD (2021), Meat consumption (indicator). doi: 10.1787/fa290fd0-en (Accessed on 09 March 2021).

With the increase in average individual income and population growth, both the average meat consumption per capita as well as the total amount of meat consumed have been rising globally (*FAO, FAOSTAT* 2018). Figure 2.3 shows the top 10 countries with the highest annual meat consumption per capita in 2018. In 2018, the U.S., among the OECD countries, was the topmost meat-consuming country followed by Australia. The average annual meat consumption by an American is 219 lbs (99 kg). Chicken constitutes the largest part (110 lbs), followed by beef and veal (58 lbs) and pork (51 lbs). Argentina ranks third with 88 lbs of beef consumption per capita early (McCarthy, 2020). Among the different types of available meats, there are significant increases in chicken and pork consumption (Basu, 2015; Milford et al., 2019). Pork consumption has mainly increased in the Southeast Asian region, while the increase in poultry consumption was observed in all parts – mainly in North America (Basu, 2015). In the recent years, cattle meat consumption has been stable, even with a slightly decreasing trend (Milford et al., 2019). Moreover, a larger proportion of meat that we eat nowadays is processed before purchasing.

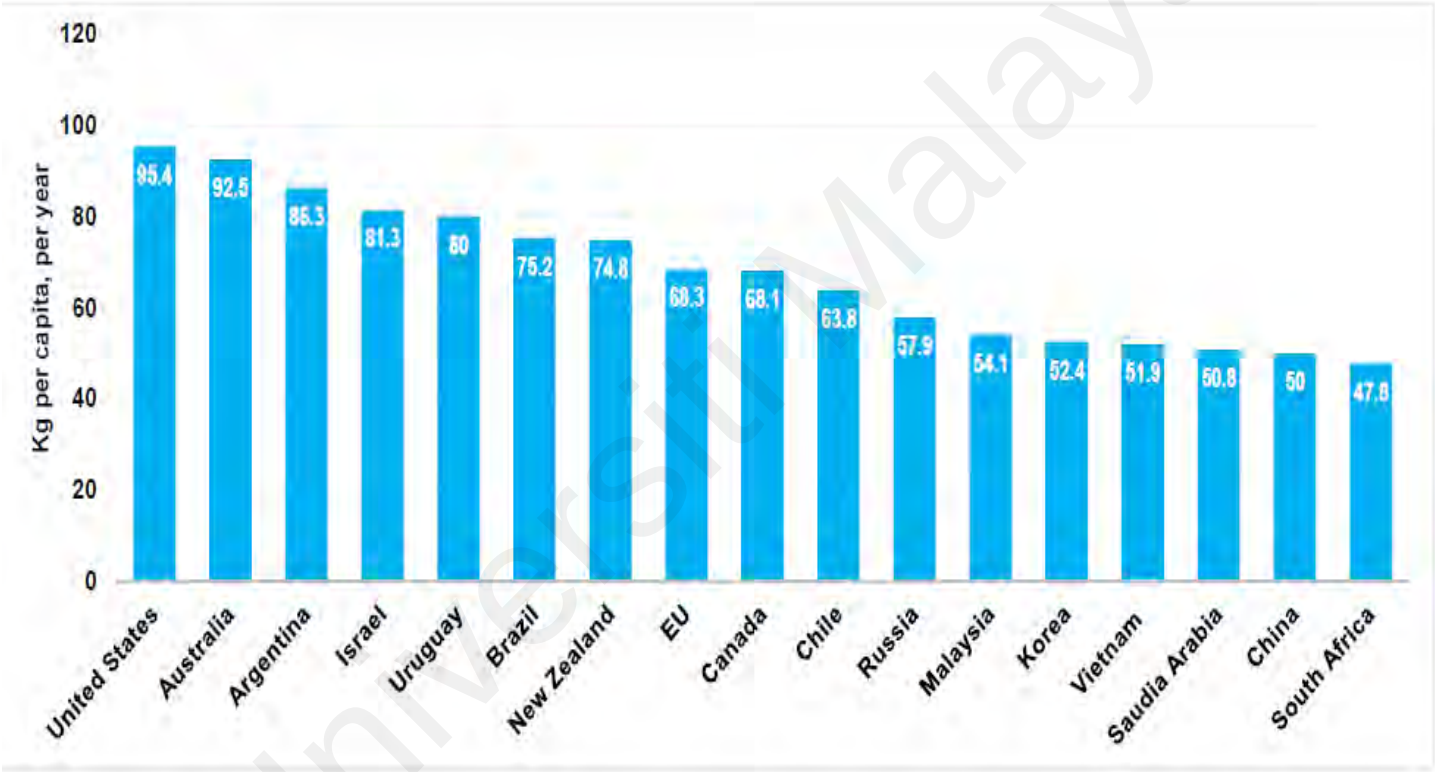


Figure 2.2: List of top meat (beef and veal, pork, sheep and poultry) consuming countries in 2015 (kg per capita, per year) (Data source: OECD, 2017)

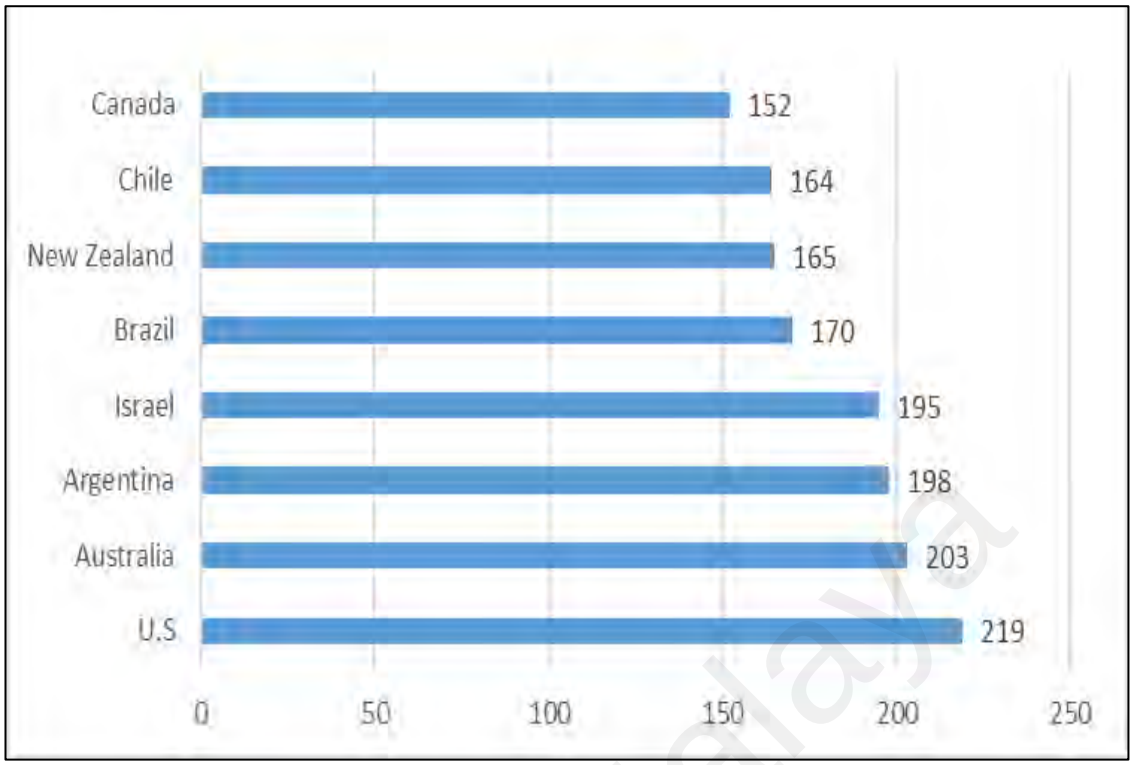


Figure 2.3: Countries with the highest annual meat consumption per capita in 2018 (in lbs) (Source.: OECD).

In meat production, Brazil, China, the European Union, the Russian Federation, and the United States continue to dominate. Due to the impact of African Swine Fever (ASF) in China, global meat production has decreased to 325 Mt in 2019. China increased overall meat import in all categories by 62% (around 2 Mt) in 2019. There is a projection of growth increase in meat consumption by 12% over the next decade (by 2029) as compared to the base period (2017 to 2019 average) (OECD/FAO., 2020). Global consumption per capita is expected to increase by more than 1% as compared to the base period. In fact, this increase in per capita consumption is mainly due to higher consumption of poultry meat. However, this growth is supposed to increase in developing countries (approximately five times that of developed countries) because of increased population and growth rates. In the higher-income countries, the consumption level is, in certain cases, close to saturation (OECD/FAO., 2020).

The recent outbreak of COVID-19 around the world has significantly affected the global meat market. Since the starting of 2020, the labour-intensive meat processing industries have faced the lack of workers (abattoirs). Moreover, the transportation bottlenecks have resulted in a shortage of meat leading to increase in their prices (FAO, 2020).

2.2.1 Domesticated Animals as a Source of Meat

Cow, buffalo, pig, goat, sheep and poultry are the major domestic animals that are commonly consumed worldwide, and their production rates are increasing gradually day by day. The global consumption of some commonly consumed animal species in 2020 is presented in Figure 2.4, indicating that poultry and pig meat were consumed mostly. Moreover, Figure 2.5 presents their consumption in different years (1990-2020). There is a gradual decrease in beef consumption whereas poultry consumption shows an increasing trend as reflected in the following four pie charts in Figure 2.5.

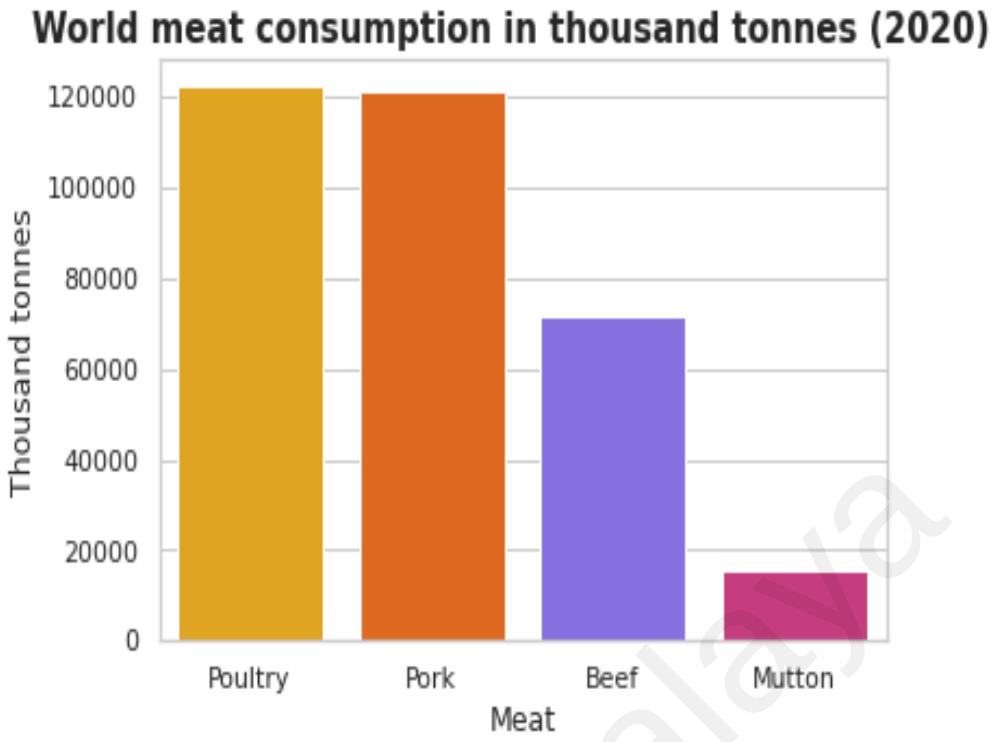


Figure 2.4: Global consumption of Poultry, Pork, Beef and Mutton in 2020 (Data source: Worldwide Meat Consumption, https://www.kaggle.com/elisthefox/meat-consumption-of-today-and-tomorrow) (Accessed on March 10, 2021)

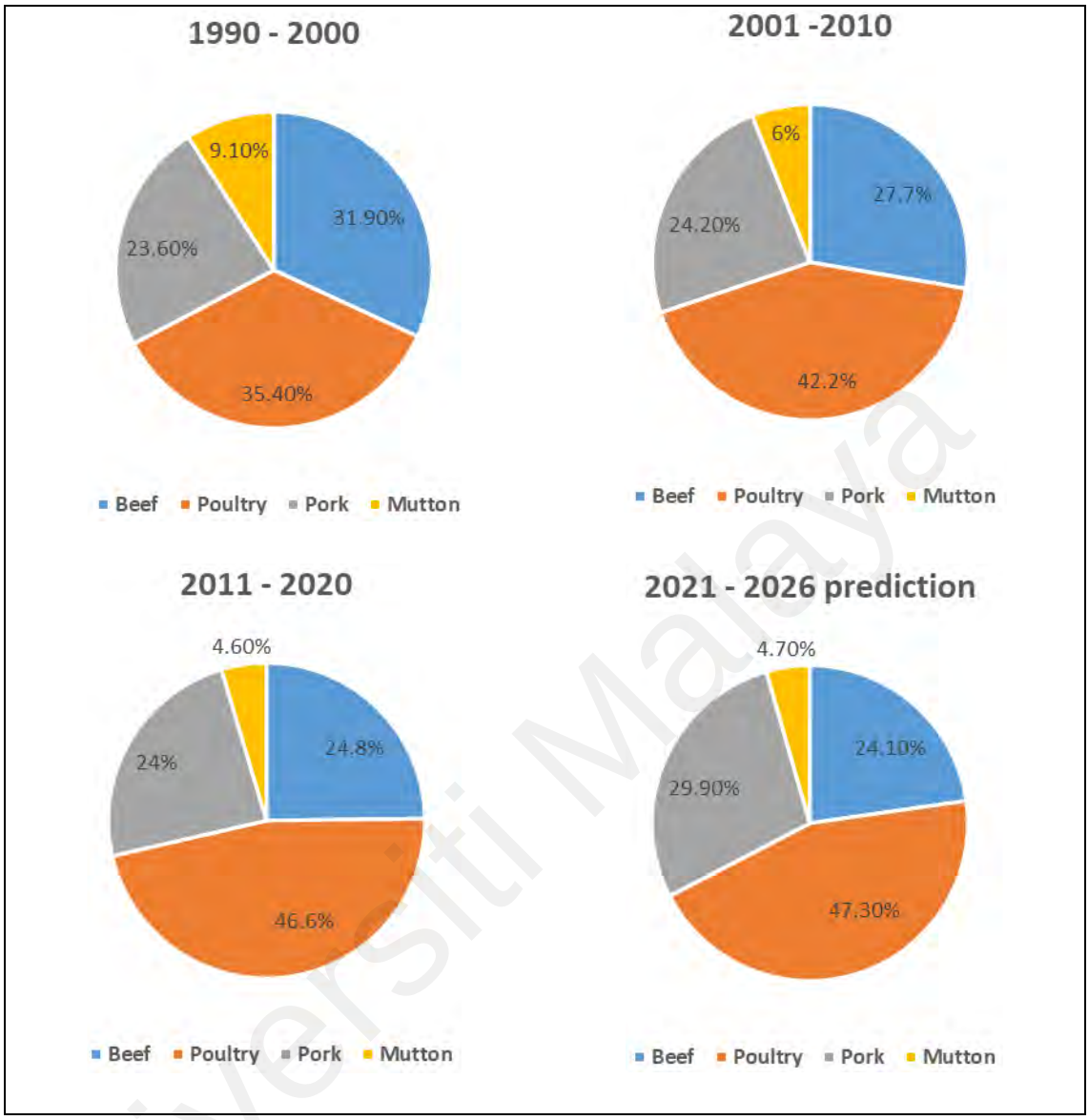


Figure 2.5: World meat consumption from 1990 to 2020 and prediction of consumption for 2021-2026 (Data source: Worldwide Meat Consumption, https://www.kaggle.com/elisthefox/meat-consumption-of-today-and-tomorrow (Accessed on March 10, 2021).

2.2.1.1 Cow

Cow is the largest and most common among domesticated animals. Cows belonging to the family Bovidae and genus Bos were domesticated about 10,500 years ago (Bollongino et al., 2012). They are basically classified as three different species, based on the region: i) Bos taurus, which are known as "taurine" cattle or European cattle, ii) Bos indicus, which are known as zebu and iii) Bos primigenius which are called as the

aurochs and now become extinct. Again, the above three groups have been classified as one species: Bos taurus (Wilson & Reeder, 2005).

Domesticated cows are considered as a major part of human food since they provide meat and milk. In recent years the growth of cattle meat consumption has been static, even with slightly decreasing trend (Milford et al., 2019). According to a report by Statista, in 2019, there was the highest consumption of beef and veal in the United States amounting to about12.41 million metric tons. China and Brazil ranked second and third in beef consumption (Figure 2.6) (Shahbandeh, 2020a). Leading beef and veal producing countries in the world in 2020 are indicated in Figure 2.7. According to forecasts by USDA and FAO for 2020, the United States is expected to produce the highest amount of beef and veal (12.5 million tons) this year followed by Brazil (10.3 million tons) (Figure 2.7). India unexpectedly ranks 5 on the list (Buchholz, 2020). Most of the Indian population are Hindus and the cows, being considered as holy, are restricted to consume there. The slaughtering of cows is restricted in many states of India and some violence has occurred involving cow smuggling and consumption issues. In Malaysia, the cattle production in 2019 reached to approximately 683.5 thousand heads (Figure 2.8). This indicates a slight increase from the previous year, although during 10-year time duration, cattle production per head has been on the decline (Hirschmann, R, 2021).

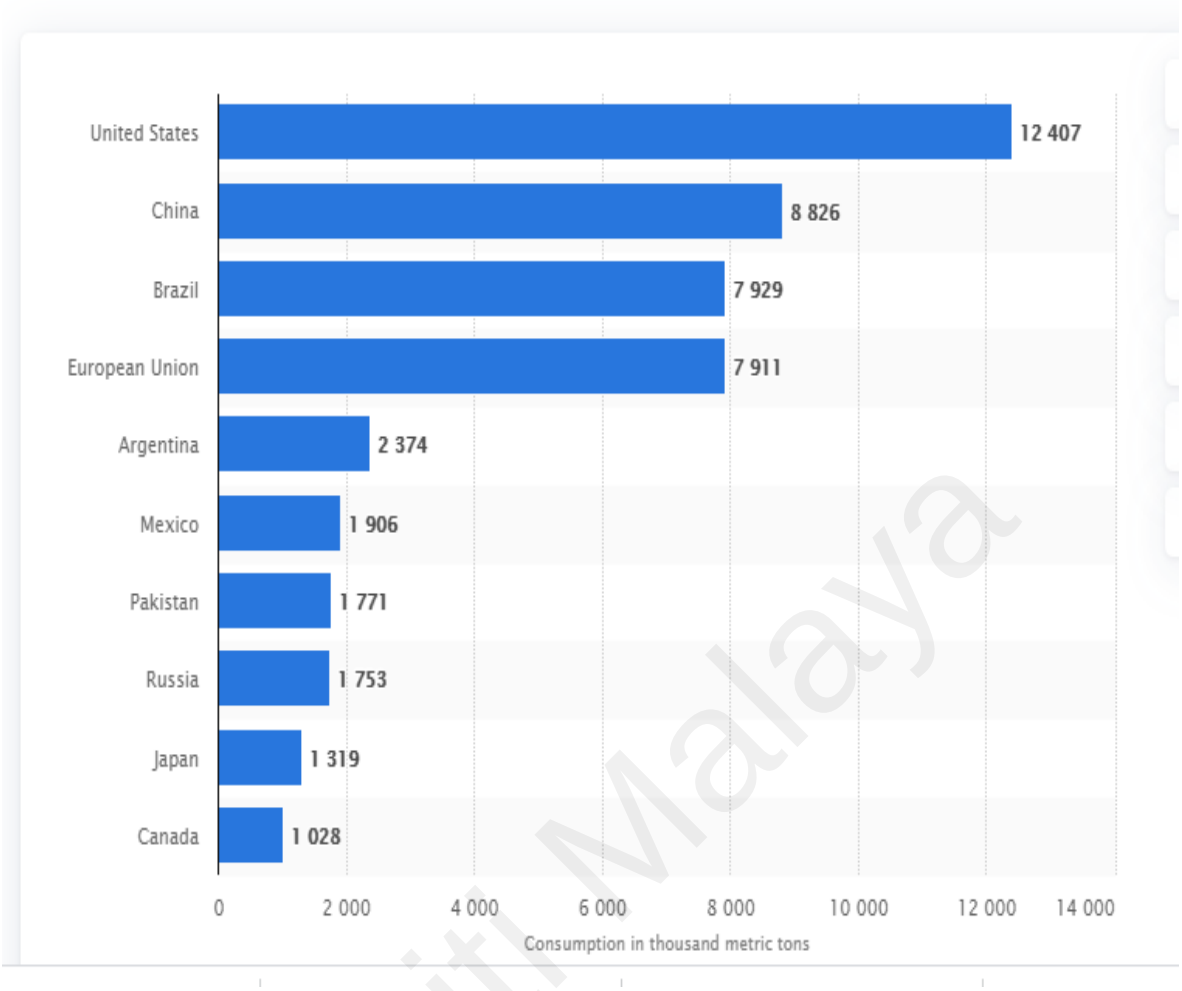


Figure 2.6: Domestic consumption of beef and veal in selected countries in 2019 ((in 1,000 metric tons). Includes other bovines (water buffaloes). (Source: Statista) (Shahbandeh, 2020a).

According to the FAO projection report, beef consumption is expected to increase to 76 Mt over the next 10 years accounting for 16% of the total increase in meat consumption as compared to the base period (2017 to 2019 average). In developing countries, beef consumption per capita will continue to remain lower at a volume of about one-third compared to developed countries. On the other hand, only Asia is expected to show an increase in per capita beef consumption over the projection period. Some countries having high per capita beef consumption will show the declining trend of beef consumption due to the cheaper price of poultry meat and pig meat (OECD/FAO, 2020).

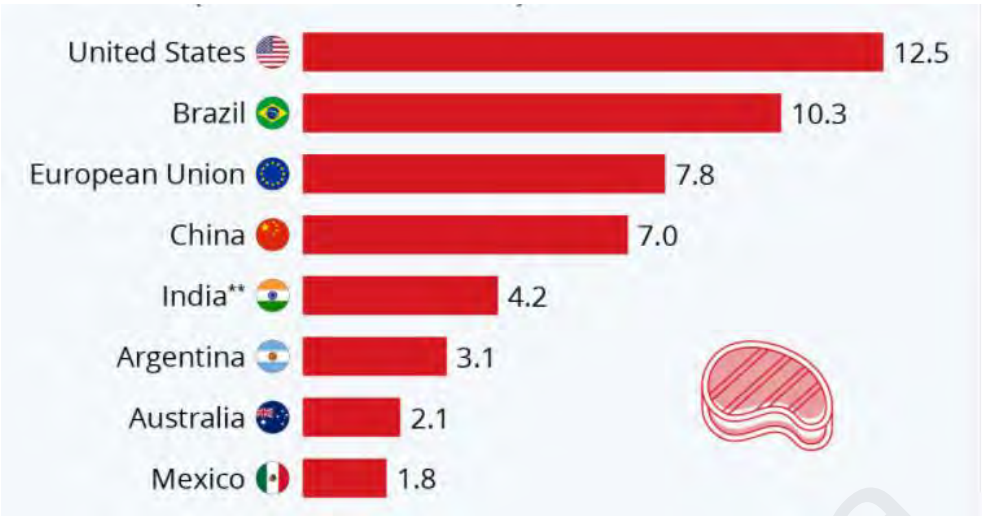


Figure 2.7: Leading beef and veal producing countries in the world in 2020 (in million metric tons). Includes other bovines (water buffaloes). (Sources: FAO, US department of agriculture) (Buchholz, 2020).

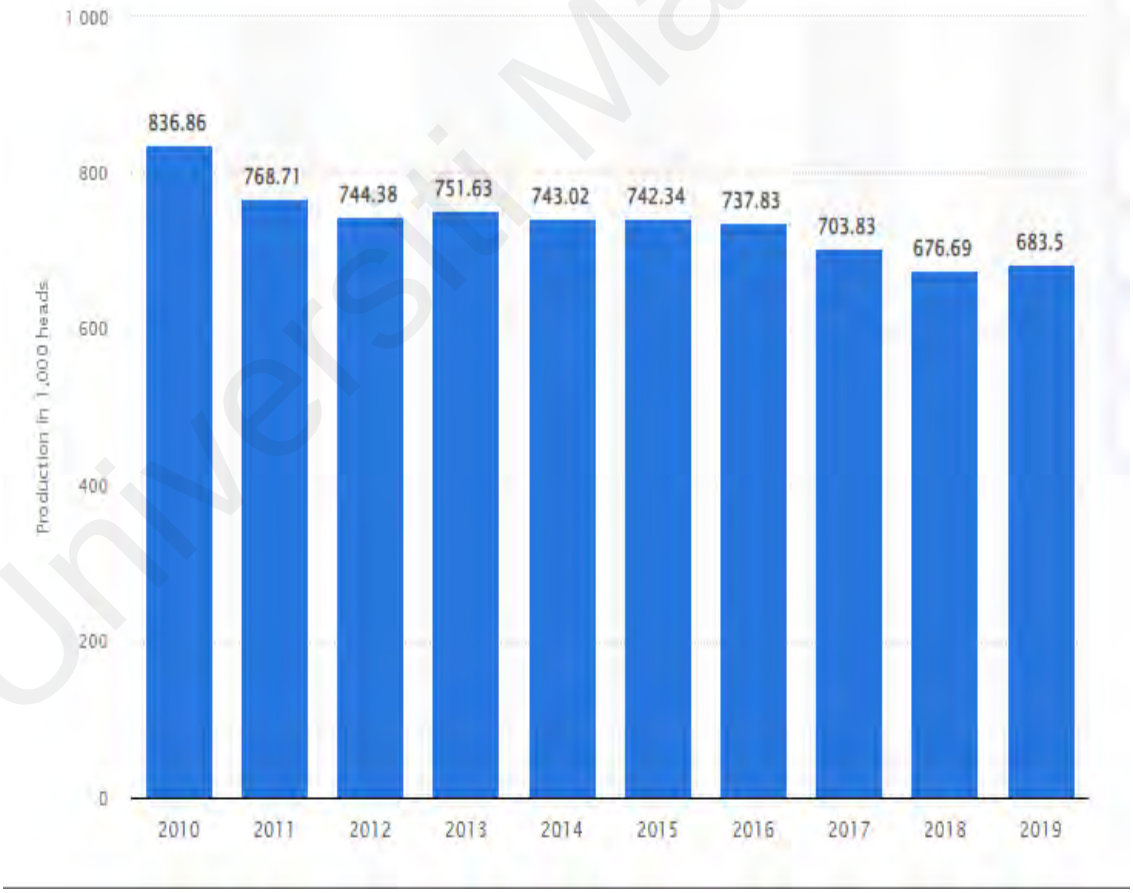


Figure 2.8: Cattle production in Malaysia from 2010 to 2019 (in 1,000 heads). (Source: Statista, 2021) (Hirschmann, R, 2021).

2.2.1.2 Water Buffalo

Water buffaloes, based on behavioral and morphological characteristics are classified into two types: i) River buffalo is available in Indian subcontinent and has been domesticated for about 5,000 years and ii) Swamp buffalo is available in China, and it is domesticated about 4,000 years ago (Yang et al., 2008).

Water buffalo belongs to the family Bovidae, genus Bubalus and species bubalis. The domestic water buffalo has been found to be the same as the ancestor of wild water buffalo (*Bubalus arnee*) (Lau et al., 1998). The scientific name of domestic buffalo is *Bubalus bubalis* whereas the wild species is known as *Bubalus arnee* (Gentry et al., 2003). Buffalo meat has been considered as a very rich source to meet the increasing demand of global meat. Buffalo meat, because of its reduced fat (cholesterol) and other important nutritional attributes, is especially demandable in some Asian countries (southeastern and middle eastern part) and Africa. India is the most important country in connection to buffalo production and population in the world. Buffalo meat has no religious taboo against its consumption, is considered as a rich source of red meat, and is gaining increased popularity in many regions of the globe. The buffalo meat sector has huge potential to flourish since this meat possesses similarity with beef (cattle meat) and is increasingly acceptable to people of different cultures. Buffalo export is forecast to reach 11.5 million tonnes in 2020, a 4% increase in 2019. The growth in global buffalo consumption is forecast to be 1.2% per annum in 2022 as supported by population and economic growth (Harrington., 2021). However, the African Swine Fever (ASF) causes a huge amount of pigs' death and consumers around the world might find buffalo as the alternative protein source. The ongoing drought situation in Australia affects buffalo supply in the short term. Moreover, because of the rapid expansion of the poultry industry, global buffalo meat consumption might decline slightly (Harrington, 2021). India with about 304 million of cattle and water buffalo is considered to have the largest buffalo

population. The next leading markets are Australia, Brazil, New Zealand and the United States of America.

In terms of global buffalo exports, India possesses 15% share, although in global production, it has only 6% share (Figure 2.9) (Harrington., 2021).

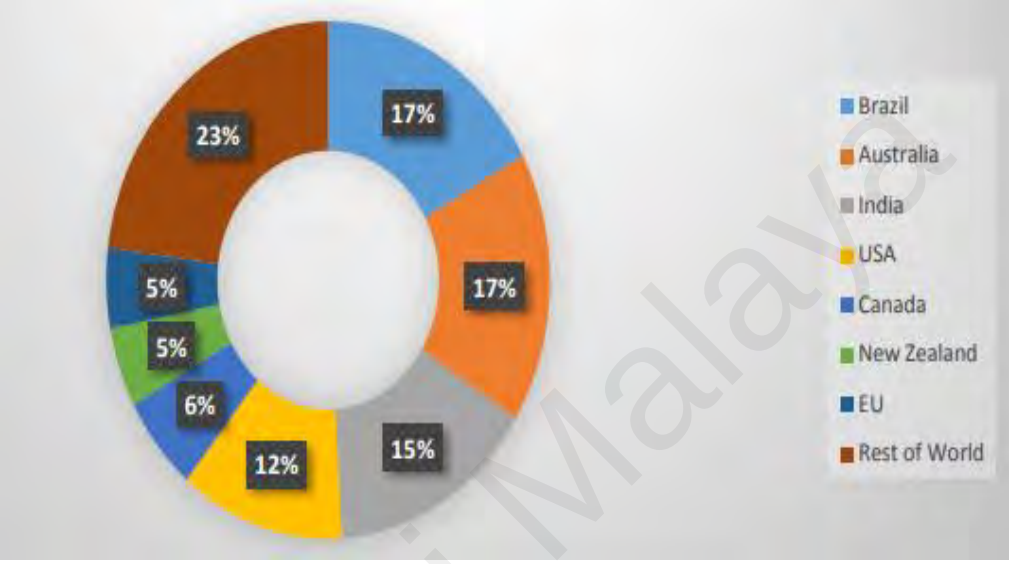


Figure 2.9: Share in global buffalo exports (source: Meat and Livestock Australia). (Harrington, 2021).

2.2.1.3 Pig

Pigs (*Sus scrofa*) are thought to have been domesticated from wild boar some 9,000 years ago. They originated in Europe and portions of Asia but have since spread to many regions of the globe (Compassion, 2017). *Sus scrofa* is the scientific name for the domestic pig, while some scientists refer to it as *Sus scrofa domesticus*. Pork meat has been a vital part of human nutrition for thousands of years. Pig breeding is a highly successful livestock industry in all continents. Despite the fact that many people do not consume pork because of religious restrictions (e.g., Muslims and Jews), the pig livestock sector is the World's largest.

China was the world's leading pork producer in 2020 producing 34 million metric tonnes of pork. The European Union came in second and the United States came in third, respectively. Global pork production in that year was around 94.33 million metric tonnes (Figure 2.10) (Shahbandeh, 2020d). Figure 2.11 depicts global pork production in 2020 by country (Shahbandeh, 2020c). The demand for pork in China has risen in tandem with the country's rapid economic growth.

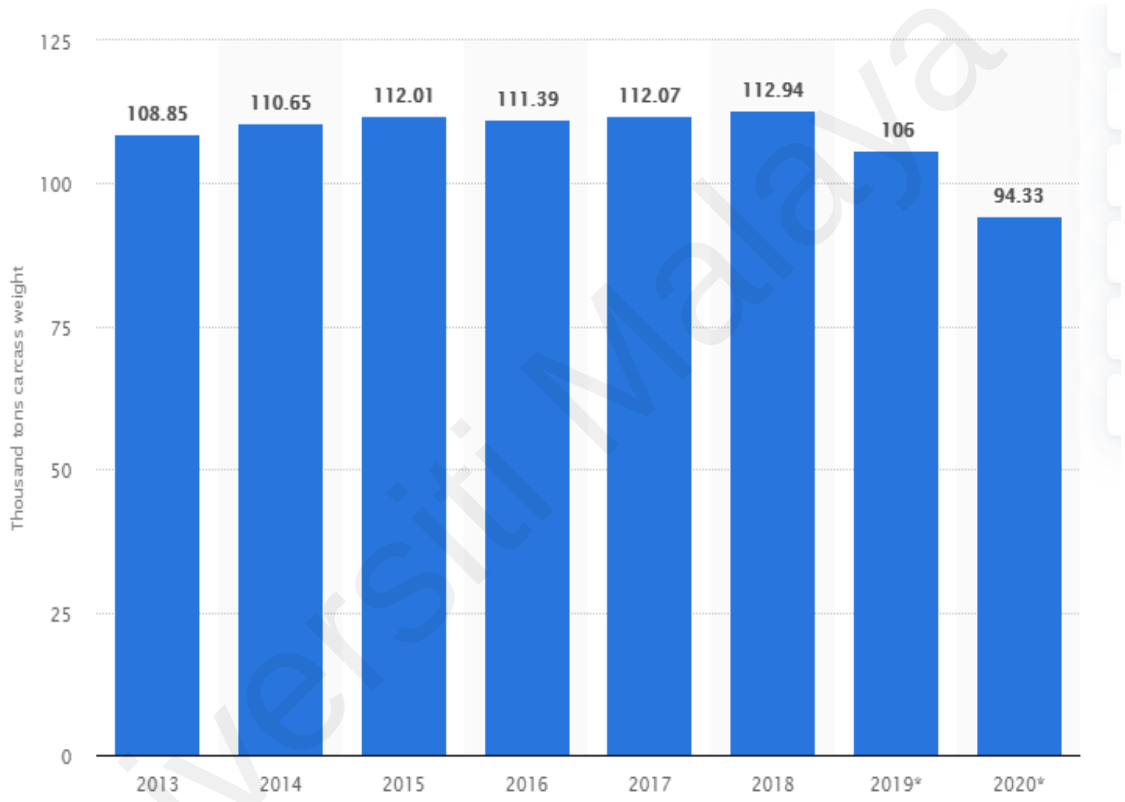


Figure 2.10: Production of pork worldwide from 2013 to 2020 (in million metric tons)** Forecasted as of April 2020. **carcass weight equivalent (CWE). (Source: Statista 2021) (Shahbandeh, 2020d).

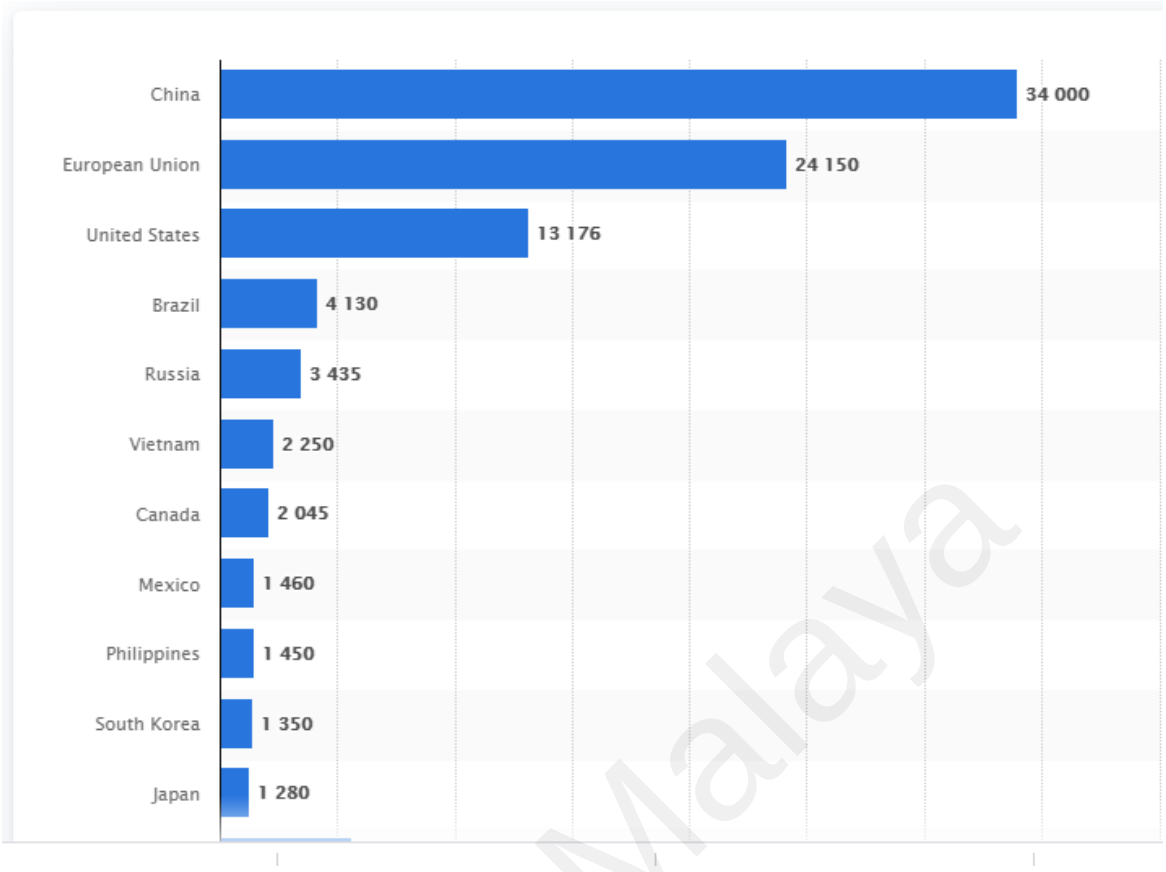


Figure 2.11: Global pork production in 2020, by country (in 1,000 metric tons) * Forecasted as of April 2020. *carcass weight equivalent (CWE). (Source: Statista 2021) (Shahbandeh, 2020c).

Global pig meat consumption is expected to rise to 127 Mt over the next ten years, accounting for 28% of the entire growth in meat consumption, according to the OECD-FAO Agricultural Outlook 2020-2029 report. Once the impact of ASF has faded, several countries in Asia that traditionally consume pork are expected to boost consumption per capita (OECD/FAO, 2020).

2.2.1.4 Poultry

Domestic fowls, such as chicken, turkey, goose, and duck, are kept for meat or eggs and are classified as poultry. Poultry meat is one of the richest sources of protein for human consumption all around the world. The most prevalent type of fowl in the world is domesticated chicken (*Gallus gallus domesticus*) that originated in Asia. Except for the

Muscovy duck, it is commonly accepted that the mallard (Anas platyrhynchos) is the ancestor of all domestic duck breeds.

In recent years, the poultry business has been the most important contributor to the animal industry's growth. Poultry production has increased dramatically during the previous century, contributing significantly to the reduction of global hunger. Commercial broiler chickens (especially for meat) and layer hens (especially for egg) varieties have increased the supply of affordable, nutritious animal protein in both established and developing nations. In 2020, The United States, the world's leading producer of broiler meat, was estimated to produce around 20.51 million metric tonnes of broiler meat. In that year, China was estimated to have produced 15.5 million metric tonnes of broiler meat. Even though dozens of countries import broiler meat in some capacity, Japan led the list of broiler meat importers in 2019. With 3.78 million metric tonnes and 3.25 million metric tonnes, respectively, Brazil and the United States are the world's biggest exporters of broiler meat (Shahbandeh, 2020b).

Duck meat and eggs are still produced at a lower rate than chickens. In 2016, the world's duck population (Anas spp.) reached 1.24 billion, with 1.1 billion in Asia. With a share of 82.2 percent, Asia is the largest region in duck meat production, followed by Europe with 12.4 percent (Sumarmono, 2019). According to the OECD-FAO report, worldwide Poultry meat consumption is estimated to rise to 145 Mt throughout the forecast period (2020-2029), with poultry accounting for half of the increased meat consumed (OECD/FAO, 2020).

2.2.1.5 Goat

Goat meat, the meat of the domestic goat (*Capra aegagrus hircus*), is a vital nutrient source, particularly for those living in developing countries, primarily in the tropics.

People from Asia, Africa, and the Caribbean, in particular, eat goat meat. When compared to other proteins, goatmeat has the benefit of having no religious taboos, and it plays a distinctive role in religious and traditional family festivities in several societies.

Per capita goat meat consumption varies widely between nations and is mostly influenced by local production and tradition. The Asia-Pacific goat meat industry grew fast in 2019, reaching $30.1 billion, up 9.9% from the previous year. Between 2007 and 2019, goat meat consumption in China climbed at an annual pace of + 1.9 percent on average. China ($22.7 billion) leads the market in terms of value. India ($2.4 billion) was placed at second followed by Pakistan (Global Trade, 2020). In 2019, goat meat production in Asia-Pacific increased by 2% to 3.9 million tonnes, up from 2018. The market is likely to maintain its upward consumption trend over the next decade, owing to rising demand for goat meat in Asia-Pacific. (Global Trade, 2020).

Figure 2.12: Global goatmeat exports (Data source FAO, 2016) (Global snapshot goatmeat, 2019) https://www.goatindustrycouncil.com.au/wp-content/uploads/2020/10/2020-mla-ms_global-goatmeat_v2.pdf. (Accessed on December 25, 2021).

2.2.1.6 Sheep

Sheep are one of the first domesticated animals. Sheep meat is a good source of high-quality protein with a low-fat content and is preferred by a wide range of consumers in Europe, Asia, and Africa. The meat of young domestic sheep is known as lamb (*Ovis aries)*. Lamb refers to the flesh of young sheep within their first year, whereas mutton refers to the meat of adult sheep. Its rank in global consumer diets varies widely, depending on a variety of cultural, social, economic and geographic considerations. Many countries, particularly those with largely Muslim populations and a history of sheep meat production and eating, consider it the preferred meat, but it occupies a niche in many developed markets. However, consumption in the Muslim world surges during the Islamic festivals of Eid Al-Fitr and Eid Al-Adha. Through cultural or religious practices, developed markets, such as China and the Middle East, have a high demand for sheep meat, although price remains a key obstacle for many customers.

Lamb is a niche commodity in many developed countries, including the United States, Japan, and Korea, where it is not widely available or consumed (except in certain demographic parts) (Figure 2.13). Sheep meat production has been increasing over the world, though at a slower rate than poultry and pork. However, Over the next decade, sheep meat around the world will remain a minor protein, accounting for less than 5% of global meat production (MLA, 2020).

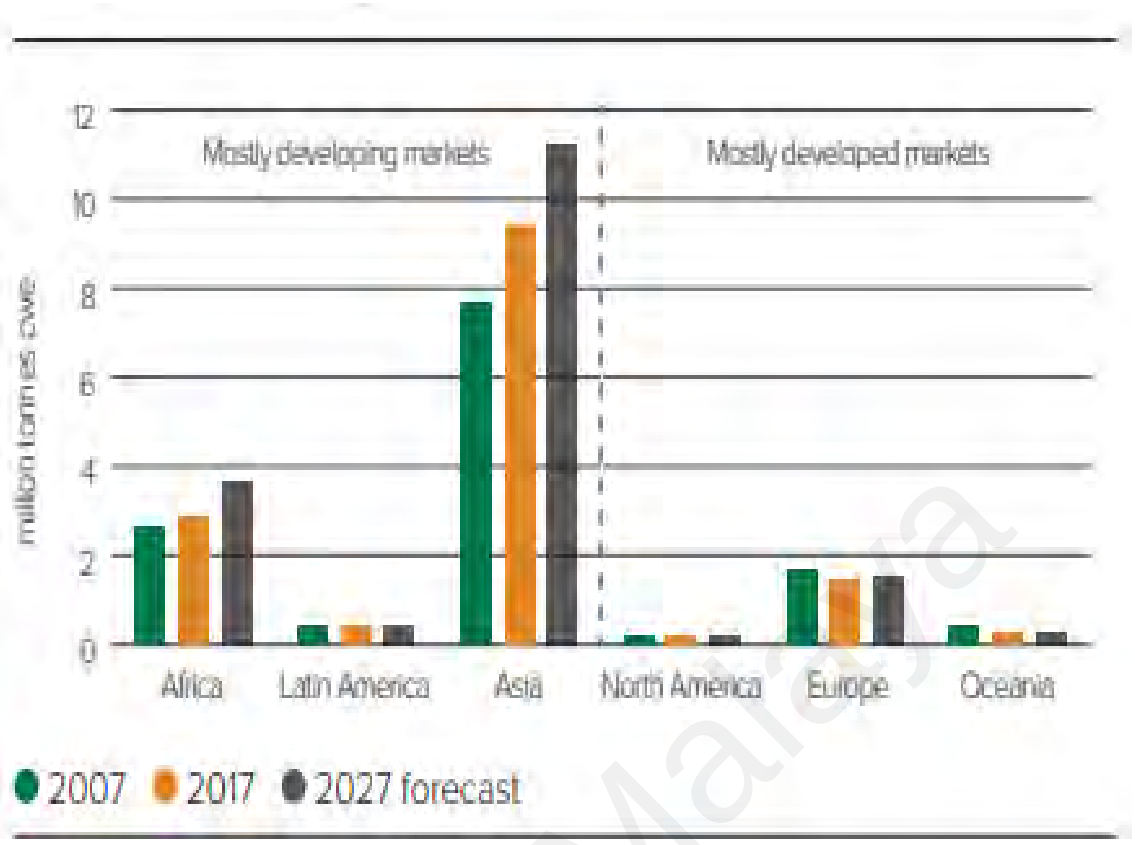


Figure 2.13: Sheep meat consumption. Source: OECD-FAO 2018 Agricultural Outlook. Middle East and North Africa (MENA) split across Africa and Asia (MLA, 2020).

Australia exported the most sheep and lamb meat in terms of cash value in 2019. Australia, New Zealand, the United Kingdom, Ireland, the Netherlands, Spain, Belgium, India, Uruguay, and Germany are the top 10 nations that exported the most sheep and lamb meat in dollar terms in 2019 (Workman, 2020). According to the OECD-FAO report, global sheep meat consumption is expected to increase by 2 Mt over the outlook period (2020-2029), accounting for 6% of the additional meat consumed. Sheep meat consumption on a per capita basis is predicted to increase little over the forecast period as costs may remain high (OECD/FAO, 2020).

2.2.2 Religious Issue in Meat Consumption

Religious belief is one of the vital factors in selecting food for consumption. Most religions contain respective dietary laws pertaining to acceptance and avoidance of food

items particularly meat originated foods; certain meats are permitted to consume, and others are restricted. For instance, beef is strongly forbidden for Hindus since the cow is treated as sacred animal, although milk and milk-derived products are allowed. The recent assassination of a man and beating of his family members by a mob of radical Hindus in the Indian city of Dadri, based on a false information that he kept beef in his refrigerator, revealed that beef is a sensitive subject in Hinduism that might cause social unrest and even claim human lives (Matthew, 2015). The Muslims take halal branded meat and meat products while the Jews prefer Kosher ones. Kashrut (food law of Jews) permits the ruminant animal species provided that the animal is a ruminant (one that chews its cud) and have split hooves. For example, pigs having cloven hooves are non-Kosher as they are not ruminants. Thus, cow, goat, deer, sheep and bison are allowed, but pig, camel and rock Hyrax are prohibited for the Jews. They are forbidden to eat both meat and milk together. Birds of prey, non-domesticated birds, and insects are not permitted to consume. Animals which died in a natural manner or were slaughtered in a way other than the specified one are also thought to be non-Kosher. Over the past decade, the demand for kosher food (food acceptable to the Jews) has been increased significantly and at present, it has been the new trend for food products (Solanki, 2016). Kosher market is growing mostly in the United States and Europe (Jayalal, 2015). In a current study, it was projected that among the total Kosher food consumers (12.5 million) in the USA, only 20% are Jewish while the remaining 80% are non-Jewish people (Yang, 2017). It is estimated that around 40% of the shelfs' products in the superstores of the USA are Kosher and 125,000 Kosher foods are available in US super stores and about 3,000 more are being included every year and the number of Kosher consumers is over 45 million around the world (KLBD, 2017). It has been reported that more than 10,000 American companies produce Kosher foodstuffs, Europe being the second highest contributor in the global Kosher food business (Solanki, 2016). According to the yearly food trades report, Kosher products

consist of nearly 200 billion US dollars (40%) among the total food sales of 500 billion US dollars (Buckenh€uskes, 2015). Mintel performed a consumer survey among adults who usually buy Kosher food stuff. They found that there are three main causes for which people purchase Kosher food. First, food quality (62%), the second is 'general healthfulness' (51%) and the third is food safety (34%) (Mintel, 2009).

The food allowed to eat for the Muslim is classified as halal food. Thus, halal animals include cows, goats, buffaloes, chicken, sheep, camel, goose, duck, turkey, ostrich, etc. if they are slaughtered in accordance with Sharia law (Uddin et al., 2021b). The list of various haram or forbidden foods includes pork and its by-products, meat of animals that are shot or died of disease and those which were not slaughtered or slaughtered in a name other than that of Allah, foodstuffs prepared with alcohol, blood and other intoxicating components. Other non-halal foods include pig, dog, horse, donkey, frog, cat, rat, carnivorous animals etc. If the animal is slaughtered and prepared following halal rules, all organs or parts of the carcass may be eaten without any restrictions. In Islamic rule, the animals should be slaughtered according to the shariah manner, and the name of Allah must be declared upon the slaughter of each animal individually. Muslims are permitted to eat all the entire animal from a halal-slaughtered and -dressed animal except blood. Only the Muslim people do not consider halal foods as their sole religious requirement; the non-Muslim consumers now also began choosing this category of food as they realize that halal foods are much fresher, congenial to health and safe (Zulfakar et al., 2014). The Worldwide market for authorized halal food products including meat and meat products, is growing fast and it has a massive potential for expansion as the halal products are accepting global recognition as a scale for food safety and quality assurance (Majid et al. 2015). The projected growth of the halal product market is projected to increase to more than 58.3 billion U.S. dollars by 2022 from 45.3 billion in 2016 (Shahbandeh, 2019).

Thus, halal foods are a new and better alternative for individuals requiring safer, fresher, and healthier foods.

The Malaysian government established the halal Development Corporation (HDC) in 2008 in order to monitor and support halal products and Malaysia has been considered around the world as the international halal hub (GVR, 2017).

Although vegetarianism is not rigidly recommended for Muslims, Christians, or Jews from a religious standpoint, ancient Indian religions like Hinduism, Buddhism, and Jainism prefer the vegetarian lifestyle because of their religious rituals as well as their caring and empathetic attitude toward all other animal life (Davidson, 2003).

2.2.3 Zoonotic Threats to Meat Industry

Domestic animals have an important role in the spread of many diseases to humans. Germs that transfer between animals and people cause zoonotic diseases (also known as zoonoses). Any disease or infection that is naturally transmitted from vertebrate animals to human beings or from humans to animals is designated as a zoonosis by the World Health Organization (WHO) (Rahman et al., 2020). Bacteria, viruses, parasites and fungi are among the pathogens that cause zoonotic infections. The majority of infectious diseases that affect human beings are caused by animals. Approximately 61 percent of human pathogens are zoonotic (Taylor et al., 2001). Zoonoses are a major public health concern as well as a direct human health risk that can result in mortality. The 13 most prevalent zoonoses, out of 200 known zoonoses, have had the greatest impact on poor livestock workers in low- and middle-income countries, making about 2.4 billion people sick and 2.7 million deaths each year, in addition to their harmful influence on human health (Rahman et al., 2020). The majority of these diseases have a negative impact on animal health and reduce livestock productivity. Domestic animals such as cattle, goats,

sheep, pigs, dogs, cats, horses etc. serve as reservoirs for pathogens that cause domestic zoonoses and can spread diseases to people (Samad, 2011). Pathogen transmission can occur through direct contact or foods derived from animals. Below are examples of some common zoonotic diseases that might be transmitted to human beings from domesticated animals: anthrax, rabies, tuberculosis, brucellosis, leptospirosis, campylobacteriosis, toxoplasmosis, ancylostomiasis, balantidiasis, toxocariasis, listeriosis, bovine pustular stomatitis, rotavirus infection, and Q fever (Ghasemzadeh & Namazi, 2015).

Anthrax, caused by the bacteria Bacillus anthracis, is one of the most dangerous zoonotic diseases spread by domestic animals. Humans can be infected through coming into intimate contact with sick animals (such as cattle and goats) or their products (meat, hides, skin, or bones). Anthrax cases impact roughly 2,000–20,000 people worldwide every year (Goel, 2015). People from Bangladesh, India, Pakistan, Zimbabwe, United States, Iran, South Africa, Iraq, Turkey is sometimes affected. Anthrax continues to pose a threat to developing countries whose economies are largely based on agriculture.

"Mad cow disease," also known as bovine spongiform encephalopathy (BSE), is a deadly neurological disease that has its origins in scrapie (spongiform encephalopathy) of sheep and goats, which was first recognized in Europe in the 18th century (Brown et al., 2001). When BSE outbreaks occurred in the United Kingdom, most European countries prohibited the import of British beef. The cost of putting in place rigorous control measures, such as culling all sick cattle and slaughtering at-risk animals, was too expensive. The BSE outbreak in Toronto, Canada, led to a 0.5 percent drop in the city's GDP. Many countries have banned international trade with Canada when the illness was discovered in millions of animals (Mitura, 2004). By 2004, the Canadian cattle industry had lost around 5.3 billion dollars (Charlebois & Haratifar, 2015). Following the discovery of BSE in the United States in 2003, many nations banned the import of

American beef, resulting in significant economic losses. Tuberculosis is another most important zoonotic disease among bovine zoonoses with serious public health implications. The disease has been resulting in huge economic losses in animal production. Despite the fact that bovine tuberculosis has been almost eradicated in affluent countries, other parts of the world are still dealing with major zoonotic consequences. Direct contact between sick animals and humans, such as farm employees, abattoir workers, veterinarians, or village residents can represent a serious risk.

Buffalopox, a disease of domestic buffaloes that causes reduced productivity and increased morbidity (Singh et al., 2006) and infects both cattle and humans, is also a serious zoonotic disease. In 2003, a buffalopox outbreak broke out in Aurangabad, India, infecting domestic buffaloes, cows, and humans at the same time (Gurav et al., 2011). The brucellosis pathogen is also carried by buffalo. Brucellosis is one of the most frequent bacterial zoonotic illnesses, affecting over 500,000 humans worldwide each year (Hull & Schumaker, 2018). This dangerous disease has been causing infertility in humans and animals alike. More than 3,000 buffaloes had been killed in the Greater Yellowstone Area in 1984 to limit the spread of brucellosis (Kats-korner, 2017).

Swine influenza, Q fever, brucellosis, leptospirosis, Rabies, Cryptosporidiosis, Ringworm, Anthrax, and Campylobacteriosis are among the zoonoses that pigs can transmit to humans (KingCounty, 2016). Huang et al. (2002) detected the swine Hepatitis E virus (HEV) in American pigs and revealed that it is genetically related to human HEV. Consequently, hepatitis E could potentially be classified as a zoonosis. HEV is causing an outbreak in various nations, notably the United States, and has become a major public health concern.

The remarkable zoonotic diseases are listed in Table 2.1, along with their etiological agents, animal hosts and major symptoms. The highly pathogenic avian influenza (HPAI)

H5N1 virus, which spreads zoonotically from affected poultry to human beings and can be lethal, continues to spread and represent a serious threat to animal and human health. H5N1 viruses are seriously damaging the poultry sector in many developing nations, affecting both economic and social well-being directly or indirectly. The impact of this virus (and the human response to its spread) on wildlife and ecology is enormous. The HPAI H5N1 virus was initially discovered in ill geese in China's Guangdong Province (Peiris et al., 2007). With 18 human cases and six deaths, the H5N1 bird flu outbreak in Hong Kong in 1997 was the first recorded instance of a completely avian virus producing serious human sickness and death (Claas et al., 1998). This outbreak was terminated by slaughtering all (1.5 million) chickens in Hong Kong's farms and marketplaces. From December 2003 to the present, outbreaks of the H5N1 virus have been documented in South Korea, Japan, Vietnam, Indonesia, China, Thailand, Laos, Cambodia and Malaysia (Peiris et al., 2007).

Table 2.1 Major Zoonotic Diseases, their causative agents, animal hosts, and the major symptoms in humans (Adapted from Rahman et al. (2020)).

Disease	**Etiology**	**Animal Host**	**Major Symptoms, System or Organs Involved**
Anthrax	*Bacillus anthracis*	Cattle, horses, sheep, pigs, dogs, bison, elks, white-tailed deer, goats, and mink	Skin, respiratory organs, or GI tract
Tuberculosis	*Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microti*	Cattle, sheep, swine, deer, wild boars, camels, and bison	Respiratory organs bone marrow
Brucellosis	*Brucella abortus Brucella melitensis, Brucella suis, Brucella canis,*	Cattle, goats, sheep, pigs, and dogs	Fever, back pain, joint pain, poor appetite, and weight loss
Mad Cow Disease, also known as BSE (Bovine spongiform encephalopathy)	Prion protein	Cattle, sheep, goats, mink, deer, and elks	Ataxia, jerky movements, seizures, dementia, memory loss, and personality changes
Arcobacter infections	*Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii*	Cattle, sheep, pigs, and chickens	Abdominal pain, fever, and vomiting
Actinomycosis	*Actinomyces bovis*	Cattle, sheep, horses, pigs, dogs, and other mammals	Swelling of lymph nodes, soft tissues, skin, and abscess

Table 2.1, continued

Disease	**Etiology**	**Animal Host**	**Major Symptoms, System or Organs Involved**
Buffalopox	Buffalopox virus (BPXV)	Buffalo and cattle	Febrile illness with rash, malaise, pain at site of lesion
Avian influenza	Influenza A virus Genus—Alphainfluenzavirus Family—Orthomyxoviridae	Ducks, chickens, turkeys, dogs, cats, pigs, whales, horses, seals, and wild birds	Flu like symptoms, diarrhoea, and pneumonia
Campylobacter enteritis	*Campylobacter jejuni,* *Campylobacter coli*	Cattle, sheep, chickens, turkeys, dogs, cats, mink, ferrets, and pigs	Enteric disorder
Campylobacter fetus infection	*Campylobacter fetus* subsp. fetus, *Campylobacter fetus* subsp. testudinum	Cattle, sheep, and goats	Enteric disorder
Q-Fever	*Coxiella burnetti*	Cattle, sheep, goats, dogs, cats, chickens, and wild animals	Fever, and skin rash
Toxoplasmosis	*Toxoplasma gondii*	Pigs, sheep, goats, poultry, and rabbits	Lymphadenopathy, fever, malaise, night sweats, myalgia, sore throat, and maculopapular rash

Table 2.1, continued

Disease	**Etiology**	**Animal Host**	**Major Symptoms, System or Organs Involved**
Enterohemorrhagic Escherichia coli infections	*E coli* O157:H7	Cattle, sheep, pigs, deer, dogs, and poultry	Enteritis and Hemolytic–uremic syndrome (HUS)
Helicobacter infection	*Helicobacter pullorum, Helicobacter suis*	Poultry and pigs	Peptic ulcer
Rift Valley fever	Rift Valley fever virus Genus—Phlebovirus Family—Bunyaviridae	Buffaloes, camels, cattle, goats, and sheep	Influenza- like fever, muscle pain, joint pain, and headache
Pasteurellosis	*Pasteurella multocida*	Poultry, pigs, cattle, buffaloes, sheep, goats, deer, cats and dogs	Fever, vomiting, diarrhoea, and gangrene
Hydatidosis	*Echinococcus granulosus*	Buffaloes, sheep, goats and adult stray or shepherd dogs	Hydatid cysts in liver, lungs, bones, kidneys, spleen, abdominal pain, and respiratory problem
Cryptosporidiosis	*Cryptosporidium parvum*	Cattle, sheep, pigs, goats, horses, and deer	Diarrhoea lasting 3–14 days. Abdominal pain, nausea and malaise are frequent.
Fascioliasis	*Fasciola hepatica, Fasciola gigantica*	Cattle, sheep, goats, and other ruminants	Intense internal bleeding, fever, nausea, swollen liver, skin rashes, and extreme abdominal pain

2.3 Analytical Methods to Detect Species in Meat and Meat Products

Researchers have focused more on developing authentic and precise techniques for detecting different animal species because of the ever-increasing meat fraud incidences around the world. To detect species origin in food products, a large range of analytical approaches have been developed so far. Food identification was initially focused on morphological characteristics like colour, flavour, taste, form and look. Honey, for example, was physically checked for purity by duly designated honey inspectors known in England as "Aletasters" and in Germany as "Bierkiesers" (Winterhalter, 2006). Due to vast and ongoing improvements in processing and packaging technologies, physical attribute identification of food components is currently unattainable. Furthermore, the microscopic approach is inadequate for identifying meat products because it fails to determine the precise animal species in food items. Various analytical methodologies to identify fraudulent mixing in foods have been documented to date. Protein detection has always been the most reliable method for identifying animal species (Zhao et al., 2019). Protein-based approaches are sometimes unsuitable because they are time-consuming, target-biomarkers are frequently modified by heat or chemical treatments, and hence cannot accurately discriminate species in highly processed foods (Lago et al., 2011). On the other hand, DNA-based approaches are more advantageous, they have been increasingly popular in recent years for meat authentication purposes in real-world samples.

2.3.1 Protein-based Methods

The major protein-based methods include immunological techniques. These techniques have been extensively applied to identify the species origin of animal materials in meat and meat products. For applications in regulatory purposes, enzyme-linked immunosorbent assays (ELISAs), have been successfully used since it is simple, specific,

sensitive, and provides high throughput screening requiring little cost and short time (Carrera et al., 2014). It is a suitable technique for analysing higher number of samples at a time (Singh et al., 2014). According to ELISA principle, either antibody (Ab) or antigen (Ag) is fixed to a surface followed by measurement of antigen-antibody interactions with the help of the labeled enzyme (E) which converts specific substrate into a colored product. The measurement of the produced color is an indicator for the identification and quantification of the sample (Pokhrel, 2015). The most commonly applied ELISA methods in authenticating meat and meat products include indirect and sandwich ELISA (Asensio et al., 2008).

2.3.1.1 ELISA assays

ELISA assays are applicable for both qualitative and quantitative analysis. Qualitative analysis provides either positive or negative results for the sample, but the quantitative assays generate a standard curve by interpolating the fluorescence or optical density of the serially diluted antigen concentration (Asensio et al., 2008). Herein, antibodies are developed against the target antigens. The antibodies used in these techniques to authenticate food ingredients are of two types namely monoclonal (Chen et al., 2004) and polyclonal (Berger et al., 1988) antibodies. Polyclonal antibodies (PAbs) are more appropriate for the testing of denatured protein samples as they are competent to recognize the antigens from a mixture of different epitopes with little changes in the property of antigen, such as denaturation or polymerization (Asensio et al., 2008). However, PAbs have some limitations including limited yield, variable affinity and extensive purification steps needed to overcome cross-reactivity for the detection of specific species. On the contrary, monoclonal antibodies (MAbs) are produced homogenously by using hybridoma techniques with high yield, specific biological activity and high specificity (Asensio et al., 2008).

To date, various ELISA assays have been reported for food authentication using both MAbs and PAbs based on structural and soluble proteins of the muscle cell (see Table 2). Some factors like high temperature may denature the protein and thus, they cannot be detected in immunoassays and therefore, the species identification in processed food (by heat treatments etc.) becomes troublesome. To overcome this limitation, Berger et al. (1988) raised PAbs against the antigen of pork and chicken muscle tissues, which are heat resistant. Similarly, Rencova et al. (2000) developed an ELISA method to detect heat-treated samples. Poultry, kangaroo, rat and horse species were successfully identified with a sensitivity of 1-5% by developing PAbs against muscular tissue under heat treatments at 1000 or 1200 ^{0}C for 30 min. To detect the adulterated pork in beef admixture, sandwich ELISA technique was introduced by raising PAbs against muscle soluble protein with the detection limit of 1% adulteration level (Martín et al., 1988). Later, Liu et al. (2006) developed the sandwich ELISA using MAbs raising from soluble myofibril proteins extracted from heat-treated ovine muscle. The ELISA system was highly reactive to heat-treated (1000 C for 30 min) sheep muscle proteins. The limits of detection were 0.25%, 0.5% and 0.5% (w/w) for cooked ovine muscle adulterated with chicken, beef, and pork, respectively. ELISA technique was also used to authenticate certain food products like frankfurter, cooked salami and fermented sausage (Ayaz et al., 2006) and hamburger (Macedo-Silva et al., 2000).

Researchers also introduced ELISA methods for the quantitative determination of adulteration in meat samples. For example, the ELISA method quantitatively measured raw pork in the admixture of raw beef with the quantification limit down to 1% (Martín et al., 1988). Chen and Hsieh (2000) reported the ELISA assay to quantify pork in various processed meat products such as salami spread franks, sausage bologna ham and luncheon meat using MAbs which was raised against heat-stable muscle protein of pig. The detection limit was found 0.5% (w/w) pork in various meat mixtures and the precision of

the developed method was confirmed by a comparative study with a commercial PAbs test kit. Similarly, Liu et al. (2006) developed MAbs based quantitative Sandwich ELISA assay for the determination of pork in raw and heat-treated (1320ºC for 2 h) meat samples with the lower detection limit of 0.05% (w/w) of pork in adulterated chicken mixture and 0.1% pork in beef admixture. At Present, different ELISA test kits of specific meat species are commercially available for the analysis of raw, processed, cooked meat, meat products and feedstuffs (Asensio et al., 2008).

Perestam et al. (2017) performed a comparative study between ELISA and DNA-based methods (real-time PCR) based on specificity, sensitivity, analysis time and cost, and purpose of application. They found that both the methods are suitable for the detection of species origin in raw meat and meat products, but ELISA is unsuitable in cases of highly processed food, particularly when a lower detection limit is a required. Other researchers also commented on the lower sensitivity of the ELISA assay and suggested that it is not suitable for the differentiation of species in mixed matrices, particularly in closely related species (Martin et al., 1988). Moreover, immunoassays were often interrupted due to cross-reactions occurrence between closely related species since these techniques are based on the raised antibodies against a specific protein (Fajardo et al., 2010).

Although the ELISA method offers enormous advantages, the technique also suffers from some limitations. For example, heat or other processing treatments might denature the target Ag resulting in distortion of epitopes' original forms that interferes with the binding of Ab to the specific antigen. Ag and Ab binding may also be interrupted especially in severely processed foods since the food ingredients such as carbohydrates, lipids, nucleic acids, salts, and other components might exert inhibitory effects. In addition, the quantity of Ag in the food products is also important. The amount below the LOD level may make the method unable to detect it (Nhari et al., 2019).

2.3.2 DNA-based Methods

Researchers, nowadays, are paying increased attention to the DNA-based methods to detect, quantify and monitor any species adulterated in meat and meat products due to their specificity, sensitivity, accuracy, reliability and rapidity and consequently, these methods are now being widely used for food authentication purposes. The DNA-based methods are considered as highly useful tools in practical fields due to the exceptional properties of DNA molecules including codon degeneracy, thermal stability, presence in huge copies in most living cells along with intra-species conserved and inter-species polymorphic regions. (Mafra et al., 2008). The stability of biomarkers is important for the detection of species, especially in processed food products that undergo thermal treatments during preparation. In contrast to protein biomarkers that are denatured by heat treatments, DNA biomarkers retain their stability under extreme processing treatments (Mane et al., 2012a). Moreover, DNA-based techniques need a small quantity of samples for species detection since huge copies of DNA can be found in one cell. Given the above-mentioned advantages, DNA-based techniques have become increasingly popular for species authentication even in the complex background of severely processed foods. Among the DNA based assays, polymerase chain reaction (PCR) has evolved as the most robust and reliable approach because of specificity, sensitivity, reproducibility and accuracy where DNA is used as a detection target to be amplified from a single copy into multiple copies (Aida et al., 2005).

2.3.2.1 Polymerase chain reaction (PCR) based assays

PCR is an in vitro process where a selected target of DNA fragment is amplified from a single DNA copy or small number of DNA to a huge number of DNA through a simple enzyme-catalyzed reaction under defined conditions (Garibyan & Avashia, 2013).

The PCR reaction uses some major components, including specific primers, template DNA, DNA polymerase enzyme and nucleotides (Garibyan & Avashia, 2013). PCR assay involves simply three-step cycling reactions such as

(i) Double stranded DNA denaturation

(ii) Primer annealing

(iii) Primer extension

When RNA is to amplify, firstly, a complementary DNA (cDNA) of that RNA should be synthesized through reverse transcription prior to running PCR. During the PCR reaction, the building block nucleotide molecules (adenine, guanine, cytosine and thymine) become associated together with the catalyzation of DNA polymerase to give final PCR products. The primers are short length sequences of single-stranded DNA that are complementary to the desired DNA sequence of target species either from 5'-end or 3'-end. Primers become Annealed with the dissociated DNA stands, thereby facilitating the DNA polymerase to start new stands to extend. Thus, after successfully completing each cycle, the copy number of DNA becomes double, finally resulting in millions of DNA after 30 to 40 cycles. After adding all the PCR components proportionately in the PCR tubes, they are placed in a 96-well plate in the Thermal Cycler to run amplification reaction steps (Figure 2.14) (Garibyan & Avashia, 2013). Amplified PCR products are then separated and visualized under an electrophoresis system with the use of an appropriate DNA size marker.

A crucial stage in the development of PCR assays is designing specific biomarkers for the target species. Both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been used in several studies to design biomarkers in order to meet the study requirements (Morin et al., 2007). Because of the extra advantages of mtDNA over nDNA in species authentication of meat products, researchers have paid special attention to it. Mt-genes

are found in large quantities in every cell, are maternally inherited, are protected by membranes, and are extensively conserved in nature, with no sequence discrepancies (Murugaiah et al., 2009). They ensure that the appropriate PCR results can be obtained even when the DNA has been severely damaged by excessive processing methods (Mane et al., 2012a). They also allow for the distinction of closely related animal species in admixture, as opposed to the nuclear sequence (Gupta et al., 2011).

The specific gene is selected to design the species-specific primer set for a successful PCR assay. The selection of genes depends on the inter species conserved region and intra species hyper variable region which facilitates to increase the specificity of the PCR assay. Generally, the mitochondrial Cytb gene is targeted to design the PCR biomarkers. This gene contains both conserved and variable regions which facilitate to clarify deeper evolutionary relationships and resolve divergence at the population level, respectively. Besides this, researchers also use other mitochondrial-encoded genes, namely, 12S rRNA, 16S rRNA, D-loop, NADH dehydrogenase subunit 5 (ND5), and ATPase6/ATPase8 depending on the target species as well as assay design (Mohamad et al., 2013).

Several PCR techniques have been developed and validated for the discriminatory detection of different species in raw as well as normal and extremely processed food matrices. Two main types of PCR methods are being used for food authentication, namely, end point (conventional) and real-time PCR. Both methods can adapt singleplex (simplex) and multiplex systems. Simplex system refers to single species detection technique whereas multiplex system offers multiple species detection in a single assay platform. In End point (EP) PCR, usually, a target region of a mitochondrial gene is amplified using a set of species-specific primer pairs along with a buffer, magnesium chloride and specific enzymes and then the amplified product is visualized on an agarose gel using ethidium bromide or other non-carcinogenic dyes. EP-PCR gives results only

at the end of the reaction in the form of bands in the gel. In contrast to conventional or EP-PCR assays, qPCR techniques, in addition to detection, also offer quantification opportunities of the analyte targets in real-time. This system allows quantification of the PCR products at an initial stage of the reaction. The various steps in the development of EP-PCR as well as real-time PCR are presented in Figure 2.15. Several simplex and multiplex EP-PCR methods have been reported for species authentication (see Table 2.2). A brief description of the different PCR-based assays is illustrated below under different subheadings:

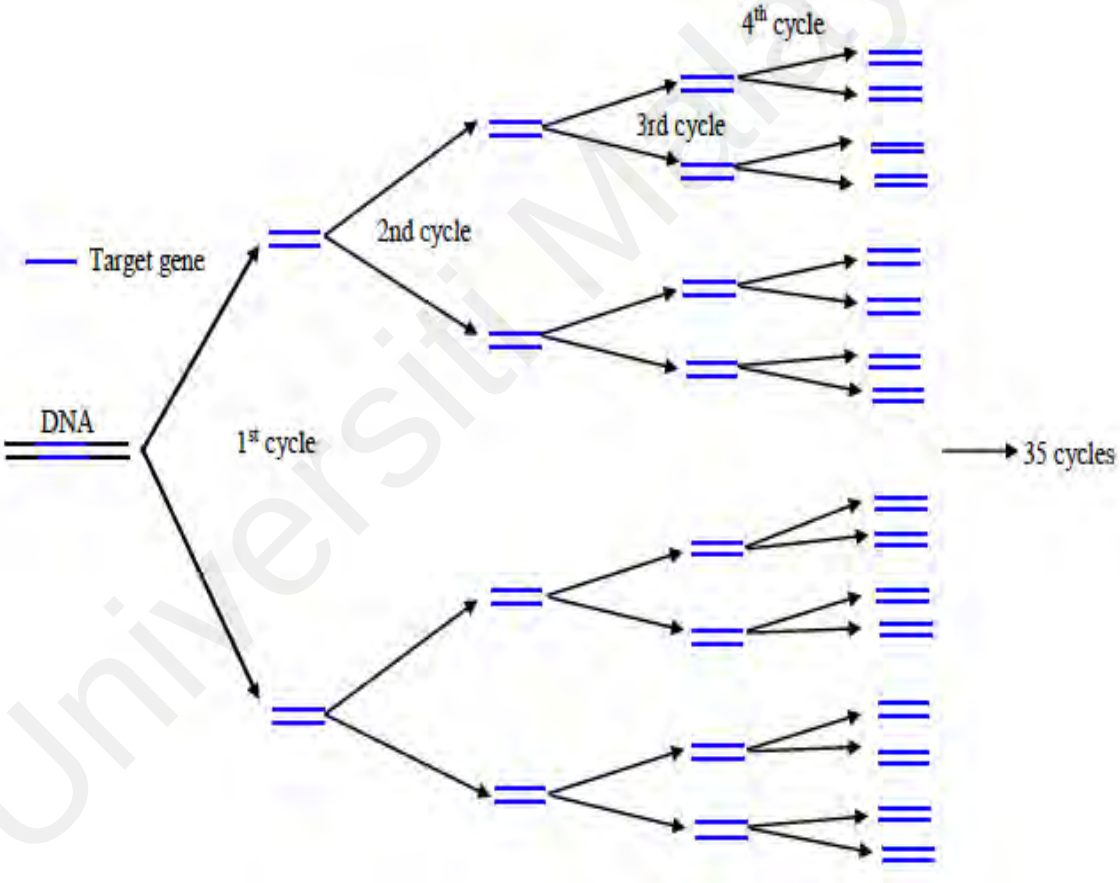


Figure 2.14: Amplification of target gene by PCR reaction (Hossain, 2017).

(a) ***Simplex end point PCR***

This method is usually applied to detect single species in a reaction. Due to their sensitivity, precision, and robustness, a large number of simplex PCR assays for the detection of numerous species with varying target (amplicon) sizes have been documented up to now. Mane et al. (2012a) developed a beef-specific PCR assay based on 513-bp amplicon from the mitochondrial D-loop gene to detect raw and processed beef and beef products. Arslan et al. (2006) also documented a simplex PCR assay to detect beef under different heat treatments including boiling, roasting, pressure cooking and pan frying through amplification of 271 bp mitochondrial DNA fragment. Girish et al. (2013) amplified 482 bp fragment from mitochondrial D-loop gene in detecting buffalo species. A buffalo specific simplex PCR was also reported by amplifying 537 bp amplicon from the same gene (Mane et al., 2012b). Beef and buffalo specific simplex assays were developed to amplify 126 bp and 226 bp products for these species, respectively with a detection limit of 0.47 ng (beef) and 0.23 ng (buffalo) DNA (Vaithiyanathan & Kulkarni, 2016).

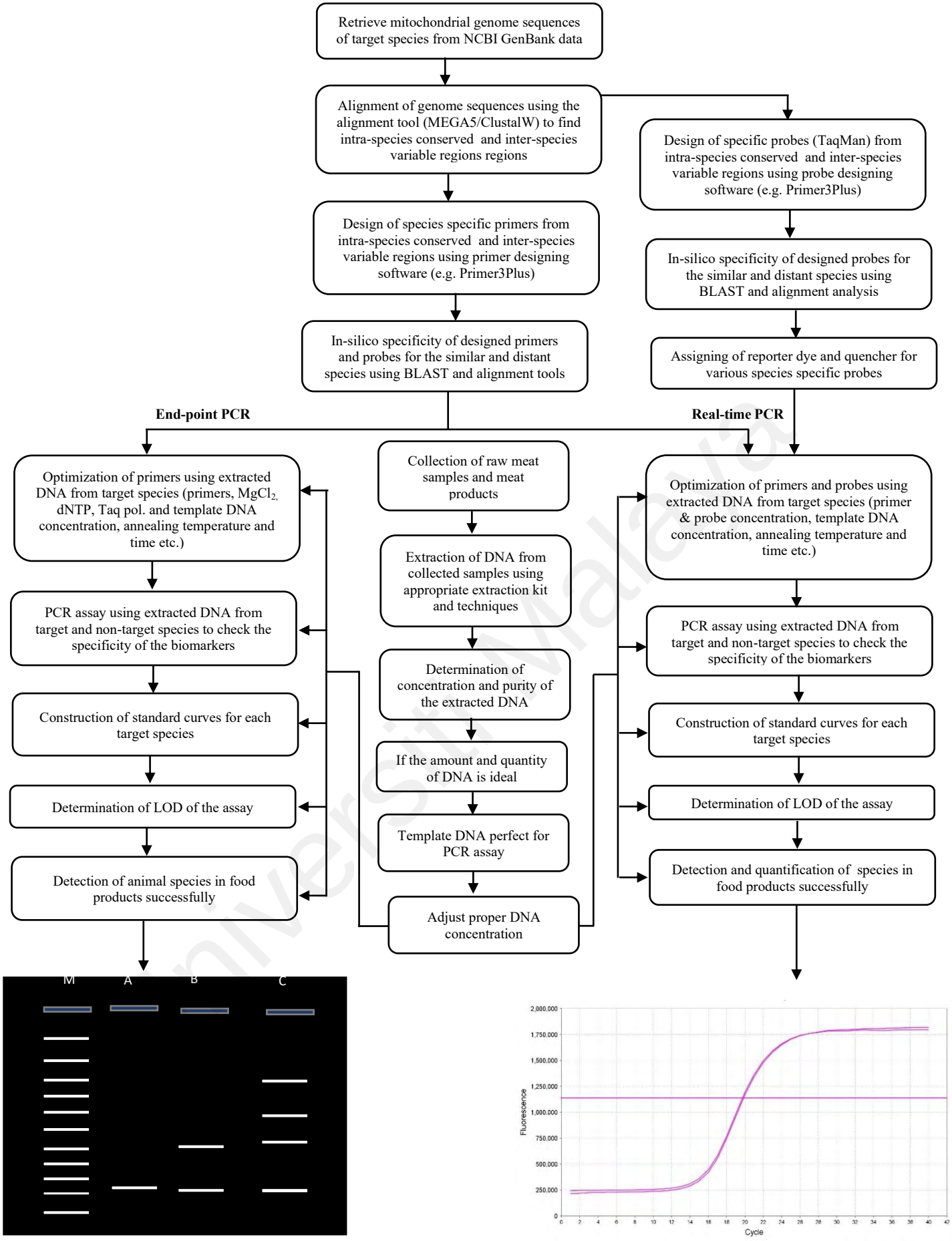


Figure 2.15: Various steps involved in the development of end-point and real-time PCR (qPCR) systems to authenticate meat and meat products (Hossain et al., 2020).

Another simplex PCR assay was reported by Hopwood et al. (1999) to detect chicken in raw or mixed cooked meat containing other species of beef, pork, lamb, duck, horse and pheasant. This method was also applied by Kitpipit et al. (2013) to discriminate among mutton, pork and chicken meat. Chikuni et al. (1994) differentiated between goat and sheep meats under heat treatment using 374 bp fragments. Again, a highly specific simplex PCR was documented by Kumar et al. (2011) for detecting goat using mitochondrial D-Loop region targeting 294 bp PCR product. A simplex PCR of high sensitivity was developed to detect sheep in raw and heat-treated meat mixtures using mitochondrial 12s rRNA gene (Rodríguez et al., 2004). Karabasanavar et al. (2011) also detected raw and heat-treated sheep meat using mitochondrial D-Loop gene targeting 329-404 bp amplicons. Several simplex PCR assays were also documented for the authentication of porcine material in food staff. For example, Barakat et al. (2014) introduced porcine specific simplex PCR method targeting two different mitochondrial genes; cytb (117 bp) and D-loop (185 bp), for raw and cooked sausage samples. The developed method was highly sensitive; hence up to 0.01% pork adulteration in beef could be detected by using species-specific genes under raw and heat-treated samples. Other pork specific PCR systems were developed using different sized amplicons targeting mitochondrial genes of cytb (Aida et al., 2005), 12S rRNA (Man et al., 2007) and D-loop (Karabasanavar et al., 2014).

(b) ***Multiplex end point PCR***

In (mPCR) system, multiple targets of DNA fragments are simultaneously amplified. Multiplex PCR techniques, using species-specific primers, are greatly promising for simultaneous identification and differentiation of several species at a reduced cost and time, and have been widely used for food products authentication. Multiplex PCR technique was first introduced by Matsunaga et al. (1999) to detect five different meat

species such as cattle, pig, goat, sheep and horse using a common forward primer from the mitochondrial cytb gene and a species-specific reversed primer. A duplex PCR system was developed by Rea et al. (2001) to detect bovine and water buffalo milk and mozzarella cheese targeting 113 bp and 152 bp fragments, respectively from cytb gene. The assay sensitivity was 1 pg under raw and 1% in the adulterated state. Another duplex assay was developed to detect beef and buffalo fat using mitochondrial D-loop gene to amplify 126 bp and 226 bp products from cow and buffalo, respectively with the detection limit of 0.12 ng (buffalo) and 0.47 ng (cow) (Vaithiyanathan & Kulkarni, 2016). A common primer mPCR was developed for the simultaneous detection of four species, namely pig, cattle, chicken, and horse which amplified 412, 292, 239 and 451 bp fragments from pig, cattle, chicken and horse, respectively. The sensitivity of the system was found to be 0.1 ng DNA. The use of a common forward primer for all target species offers more advantages over conventional mPCR systems by reducing the competition among primers in the reaction mixture resulting in increased specificity and sensitivity (Bai et al., 2009). Zhang (2013) introduced another type of mPCR system following two strategies; applying semi-nested mPCR and shortening the number of primers to enhance the sensitivity of mPCR. They used a pair of common primers to perform the first PCR assay, followed by the use of the amplified product as the template of the second mPCR. This method was very effective to detect meat species in processed foods increasing the sensitivity of the mPCR assay by 3-fold compared to conventional system. The LOD of semi-nested mPCR was found to be 1 pg for simultaneous detection of pork, chicken, beef and mutton species. Another rapid and cost-effective mPCR method was successfully developed for the first time without requiring the extraction of DNA. The method was able to identify six common species like pork, beef, chicken, lamb/mutton, ostrich meat, and horse meat in highly degraded and processed food samples with the detection limit of 7 fg (Kitpipit et al., 2014). To identify pig, cattle, poultry (chicken and

turkey), and equine (horse and donkey) species simultaneously, mPCR was applied by İlhak and Güran (2015). The market survey of 50 sausage samples (beef and poultry) revealed that the developed assay successfully identified 23.3% poultry adulteration in beef sausage samples and equines were positive for 2% samples, but porcine material was absent in all tested samples. A hexaplex PCR was developed for detecting simultaneously five meat species, namely pig, cow, poultry, sheep and horse as well as one plant species such as soybean. The assay sensitivity was found to be 0.01% in complex matrices (Safdar & Junejo, 2016).

Recently, researchers are more interested in using fully automated multi-capillary electrophoresis devices instead of conventional agarose gel electrophoresis to separate and visualize the amplified PCR products. Conventional gel electrophoresis cannot clearly distinguish DNA fragments of less than 50 bp length difference whereas, the capillary system effectively enhances sensitivity and resolution (~5 bp length difference) saving analysis time and also minimizing the manual handling errors. In addition to gel images, it also clearly indicates the PCR product size through electropherograms (Hossain et al., 2017a).

Thus, the multiplex PCR is a highly promising and effective approach for the discriminatory identification of several species. As a result, both labour and time can be saved. Simplex PCR tests, on the other hand, require many independent assays because they use individual set of species-specific biomarkers separately. However, most reported assays used longer DNA targets that are often unsuitable for analyzing severely degraded samples because of the target amplicon breakdown (Rashid et al., 2015).

Although PCR based methods became a popular technique for the authentication of meat products due to their stability, specificity and sensitivity, however, PCR amplification could be affected by food ingredients such as fats and proteins (Nhari et al.,

2016). This limitation can be overcome by modifying DNA extraction method making it more suitable to extract highly purified DNA from the food products.

Table 2.2: Application of DNA-based methods for the detection of species in meat and meat products (Adapted from Hossain et al., 2020)

Methods	Target species	Target gene	State	Target size (bp)	Limit of detection	References
Simplex PCR	Pork	12S rRNA	Normal	387	Not given	Man et al. (2007)
Simplex PCR	Cat, dog and rat or mouse	12S rRNA	133°C for 20 min at 300 kPa	108, 101, & 96	0.1%	Martin et al. (2007)
Simplex PCR	Pork	Cytb & D-loop	Cooked	117 & 185	0.01%	Barakat et al. (2014)
Simplex PCR	Dog	Cytb	Autoclaved for 2.5 h	100	0.2%	Rahman et al. (2014)
PCR-RFLP	Pork	Cytb	Normal	359	0.1%	Erwanto et al. (2014)
PCR-RFLP	Pig, cattle, goat, buffalo, and sheep	Cytb	Normal	609	Not given	Kumar et al. (2014)
PCR-RFLP	pork, goat, beef, buffalo, chicken, rabbit and quail	Cytb	Normal	359	1-5%	Murugaiah et al. (2009)
PCR-RFLP	Macaque	D-loop	Normal	120	0.1%	Rashid et al. (2015)
PCR-RFLP	Dog, cat, horse and donkey	Cytb	Normal	672, 808, 221 and 359	-	Abdel-Rahman et al. (2009)
PCR-RFLP	Beef, buffalo and pork	Cytb and ND5	Autoclaved for 2.5 h	73, 90, 106, 120, 138 and 146	0.1%	Hossain et al. (2016)
Multiplex PCR	Pig, cattle, chicken and horse	Cytb	Normal	412, 292, 239 & 451	0.1 ng	Bai et al., 2009
Multiplex PCR	Chicken, goat, cow, camel and donkey	Cytb, 12S rRNA, ND 2	Normal	183, 157, 274, 200 & 145	0.1 ng	Nejad et al. (2014)
Multiplex PCR	Chicken, beef, mutton and pork	Cytb	Cooked & further-processed foods	216, 263, 322 & 387	1 pg	Zhang (2013)
Multiplex PCR	Pork, lamb/mutton, chicken, ostrich, horsemeat and beef	cyt b, cytochrome oxidase I (COI), & 12s rRNA	Highly processed food	100, 119, 133, 155, 253, & 311	7 fg	Kitpipit et al. (2014)

Table 2.2, continued

Methods	Target species	Target gene	State	Target size (bp)	Limit of detection	References
Multiplex PCR	Pig, cattle, poultry (chicken and turkey), equine (horse and donkey)	Cytb, 12S rRNA	Processed food	212, 256, 183 & 439	Not given	Ilhak and Guran (2015)
Multiplex PCR	Cat, dog, pig, monkey and rat	ND5, ATPase 6, & cytb	121^0 C for 2.5 h	172, 163, 141, 129 & 108	0.01–0.02 ng (pure) & 1% (admixed)	Ali et al. (2015b)
Multiplex PCR	Horse, soybean, sheep, poultry, pork and cow	cyt b, lectin, 12S rRNA, 12S rRNA, ATPase 6 & ATPase 8	Normal	85, 100 119 183 212 & 271	0.01%	Safdar and Junejo (2016)
Multiplex PCR	Crocodile	Cytb and ATP6	Boiling, autoclaving and microwave cooking	77 and 127	0.01−0.001 ng and 1%	Ahmad Nizar (2018)
Simplex qPCR (TaqMan)	Pork	12S rRNA	Normal	411	0.5%	Rodriguez et al. (2005)
Simplex qPCR (TaqMan)	Pork, beef, mutton, chicken and horse	Cytb	Normal	Not given	100 fg	Tanabe et al (2007)

Table 2.2, continued

Methods	Target species	Target gene	State	Target size (bp)	Limit of detection	References
Simplex qPCR (Molecular Beacon)	Pork	Cytb	Normal	119	0.0001 ng (pure) & 0.1% (admixture)	Yusop et al. (2012)
Multiplex qPCR (TaqMan)	Chicken, pork, turkey, horse, beef, sheep (mutton) and goat	Cytb, Beta-actin-gen & Prolactin receptor	Normal	76, 80, 83, 85, 96, 101 & 140	2%	Köppel et al. (2009)
Multiplex qPCR (TaqMan)	Chicken, pig and duck	Transforming growth factor, beta actin & T cell growth factor	Normal	76, 111 & 212	0.15 ng (pure) & 1% (admixed)	Cheng et al. (2014)
Multiplex qPCR (TaqMan)	Pork and beef	Cyclic-GMP-phosphodiesterase & Beta-actin	Normal	Not given	20 genome equivalents	Iwobi et al. (2015)
Multiplex qPCR (TaqMan)	Beef, buffalo and pork	ND5 & cytb	Normal	106, 90 & 146	0.003 ng (pure) & 0.1% (admixed)	Hossain et al. (2017a)
DNA barcoding	Beef, chicken, lamb, turkey, pork and horse.	COI	Normal	658	-	Kane and Hellberg (2016)
DNA barcoding	Poultry	COI	Heavily processed products	658 and 127	-	Hellberg et al. (2017)
DNA barcoding	Beef, chicken, lamb, goat, buffalo, pork, duck, prawn and fish.	COI	Chefs and cooking	~650	-	Ahmed et al. (2018)

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

PCR restriction fragment length polymorphism (PCR-RFLP) is one of the most promising molecular techniques because, in addition to amplification of specific targets, it offers the opportunity of authentication of a product by its restrictive digestion using one or more restriction enzymes (REs) followed by fragment separation (Rashid et al., 2015). They are especially applicable in meat speciation because they utilize the sequence variations existing within a selected region of target DNA and allow very closely related species to be easily differentiated with the use of selected restriction enzymes (Ali et al., 2015a). RFLP has advantages over other existing DNA based methods given its simplicity, rapidity, reproducibility and low cost and thus, it is easily applicable in the routine analysis of meat and meat products (Sivaraman et al., 2018).

Species-specific PCR assay, although often provides a conclusive result, may be proved unsuitable to be considered a definitive analytical tool due to certain features (Yang et al., 2005). For instance, it sometimes may produce artifacts because of contamination by alien DNA at a very small level and may cause non-specific target amplification (Doosti et al., 2014). However, to eliminate such ambiguities, amplified product verification is effective that certainly increases assay reliability. Verification of PCR products could be performed through at least one of the techniques, namely, probe hybridization, DNA sequencing, and PCR-RFLP (Maede, 2006). Among them, the PCR-RFLP assay has attracted researchers' attention worldwide because of its low cost, simplicity and reliability (Hashim & Al-Shuhaib, 2019). It authenticates the PCR product amplified from a selected gene fragment by generating species-specific fragments through digestion with restriction enzymes (Pereira et al., 2008) followed by separation and visualization of the DNA fragments by gel electrophoresis. Thus, it enables the

distinction of the artificial PCR product from the original with the help of the restriction fingerprints.

PCR-RFLP systems have been especially used for differentiating very closely related species like goat-sheep and cattle-buffalo (Girish et al., 2005), cattle-yak (Chen et al., 2010), swine-wild boar (Fajardo et al., 2008), beef, buffalo and pork (Hossain et al., 2016), as well as rabbit, rat and squirrel (Ali et al., 2018). A variety of PCR-RFLP assays have been documented for animal authentication in food products using several mitochondrial genes, including cytb (Ali et al., 2015), 16S rRNA (Sharma et al., 2012), 12S rRNA (Chen et al., 2010) and ND5 (Hossain et al., 2016). A powerful, simple and sensitive PCR-RFLP method was reported for the detection of porcine material in the meatball. In this assay, cytb gene-targeted 359 bp amplified product was digested with BseDI restriction enzymes which generated 131 and 228 bp fragments. A survey on Indonesian beef meatball products found porcine positive in nine samples out of twenty (Erwanto et al., 2014). Kumar et al. (2014) used a pair of forward and reverse primers from the conserved region of mitochondrial cytb gene amplifying 609 bp products from five most commonly consumed animals, namely pig, cattle, goat, buffalo, and sheep. The amplified PCR products were digested with AluI and TaqI REs which resulted in distinctive digestion maps able to discriminate each species. The developed method is suitable to distinguish the meats of closely related species of domestic livestock. Similarly, PCR-RFLP technique was applied for differentiating seven species such as pork, goat, beef, buffalo, chicken, rabbit and quail targeting 359 bp mitochondrial cytb gene. To differentiate the meat species AluI, BsaJI, RsaI, MseI, and BstUI enzymes were selected which generated individual restriction pattern for each species (Murugaiah et al., 2009). PCR RFLP assay was also reported to detect cat, dog, donkey and horse and the authenticity of the amplified fragments were verified by RFLP analysis (Abdel-Rahman et al., 2009).

*(d) **DNA barcoding***

Among the available techniques in species detection, DNA barcoding is one of the most promising candidates with high accuracy and efficiency for differentiating meat of various animal species. DNA barcoding is a sequencing-based method involving COI gene-targeted biomarker that amplifies approximately 650 bp fragments through PCR reaction. The mitochondrial COI gene has been appropriate for species discrimination because it shows relatively high level of sequence divergence between species and low level of divergence within species. Compared to several other available techniques, DNA barcoding seems to be more time-consuming, but it has an extra advantage of allowing for a comprehensive approach for species detection favored by a high level of genetic information. Moreover, this technique can easily support high-throughput automation (Hellberg et al., 2017). However, animal meats in food products are usually processed through boiling, canning and stir-frying which can cause degradation of DNA, resulting in difficulties for the amplification of target fragments of full-length DNA barcodes. To overcome this limitation using a full-length barcode, researchers have paid more attention to the mini-barcoding technique where the sequence of target length is reduced by targeting a shorter fragment within the standard barcode region, improving the amplification possibility and capability. A number of recent studies have documented successful amplification and sequencing of a variety of mini barcodes from different processed products. Thus, both full-length DNA barcoding (Haye et al., 2012; Hellberg et al., 2017; Kane & Hellberg, 2016) and mini barcoding (Hajibabaei et al., 2006) techniques have been widely applied for meat and meat product authentication (see Table 2.2).

(e) Quantitative real-time PCR (qPCR)

Due to the inability of the EP-PCR to provide quantitative information of the target species originally present in the specimens, researchers have been paying increased attention to automated real-time PCR (qPCR). In contrast to conventional PCR assays, qPCR techniques are especially promising because of full automation, rapidity and high sensitivity. In addition to detection, they also offer quantification opportunities of the analyte targets at real-time, eliminating the time-consuming post PCR analysis step like electrophoresis (Cheng et al., 2014). Particularly, qPCR involves direct monitoring of the generation of PCR products during each amplification cycle and can measure the exponential phase of the reaction where there is no need to complete the reaction. Unlike EP-PCR assay, this system allows quantification of the PCR products at an initial stage of the reaction with more precision and accuracy. As fluorescent molecules are used to collect real-time data, there is a high correlation between the intensity of the fluorescent dye and the quantity of PCR products (Fajardo et al., 2010). Two general types of fluorescent chemistries are commonly used in qPCR systems, namely double-stranded (ds) DNA-intercalating dyes such as SYBR Green (Asing et al., 2016) or Eva Green (Safdar and Abasıyanık, 2013) and probe-based chemistry such as TaqMan (Ahamad et al., 2019) or Molecular Beacon (Hadjinicolaou et al., 2009) probes. The main drawback of DNA-intercalating dye chemistry is that it non-specifically binds any dsDNA including primer-dimers that are available in the reaction tube making the detection false positive and unreliable (Arya et al., 2005). Moreover, some dyes are known to inhibit the PCR reaction (Gudnason et al., 2007). In contrast, TaqMan probe-based technique is widely acceptable since both the primers and probes find their complementary sites in the template DNA and thus offer the chance of double-checking which increases the specificity and reliability of the technique (Hossain et al., 2017a). After hybridizing the specific probe, fluorescent signal is generated as the DNA polymerase moves by and

cleaves off the probe's reporter and quencher molecules. In addition, TaqMan probe techniques are advantageous in developing multiplex qPCR (mqPCR) systems, because labelling of specific probes can be done with different reporter dyes that allow the identification of amplified targets formed by single or multiple primer sets in one assay tube (Arya et al., 2005). Hence, unlike singleplex qPCR, mqPCR could be advantageously applied for detection and quantification of multiple target oligos in one platform, which can save both analytical time and cost (Iwobi et al., 2015). The various stages in the development of qPCR are given in Figure 2.13.

The qPCR methods have been used to identify and quantify animal species in various meat and meat products (see Table 2.2). A rapid qPCR assay was introduced to detect trace amounts of beef, pork, chicken, mutton and horsemeat in processed foods. Herein, cytb gene was targeted to design the primers and TaqMan probes. The limit of quantification of this method was found to be 0.0001% (10 fg/μL) of each species in pure state (Tanabe et al., 2007). The qPCR assay was improved targeting shorter DNA fragments (109 bp) from cytb gene for the detection and quantification of pork in meatball and burger using TaqMan probe. The developed method could detect down to 0.01% pork adulteration in beef meatballs and burgers under cooking and grilling conditions (Ali et al., 2012a).

Furthermore, to increase the assay specificity, Yusop et al. (2012) introduced qPCR assay in detecting pork using molecular beacon (MB) probe from cytb gene targeting 119 bp amplicon. The LOD was found to be 0.0001 ng DNA in pure state and 0.1% (w/w) pork in pork–beef admixture. It is known that MB enhances the assay sensitivity and specificity since it can identify single nucleotide polymorphism (SNP). MBs are hairpin-shaped oligonucleotide probes which contains a stem and loop with fluorescent reporter dye at 5′end and quencher dye at 3′end. These two dyes are in close contact with each

other at the end of the stem of the hairpin. The loop consists of 15-20 nucleotides which are complementary to the target sequence. This expedites the MB-based assay to amplify only target species by eliminating the possibility of non-specific binding. The Tm values of the primers as well as stem and loop region of the probe should be within the range which is suitable for perfect amplification (Mohamad et al., 2018).

A triplex TaqMan probe-based qPCR assay was documented to identify chicken, pig and duck in blood curds. Total DNA from blood curds samples was extracted using three different kits, namely TIANamp®Blood DNA Kit, phenol/chloroform extraction method and TIANamp®GenomicDNA Kit to compare DNA yields and purity by measuring the concentration of DNA and the ratio of the absorbance at 260 and 280 nm. Better efficiency was found for the TIANamp®GenomicDNA Kit and modified phenol/chloroform extraction method compared to TIANamp®Blood DNA Kit. The sensitivity of the assay was 0.15 ng under pure state (1:103 dilution) and 1% under ternary admixture for each target species (Cheng et al., 2014). Another triplex qPCR assay was developed to quantify and differentiate between beef and pork in minced meat products. In this technique, cow and pig specific primers and probes were used against mammals and poultry species-specific myostatin universal system. The sensitivity of the assay was 20 genome equivalents with the measurement of uncertainty at 1.83% and the assay showed good reproducibility and reliability upon screening of several commercially available minced meat products (Iwobi et al., 2015). A tetraplex qPCR assay with TaqMan Probes was reported for the quantitative detection of cattle, pig and buffalo. To avoid ambiguity in molecular diagnostics because of breakdown of target DNA, the amplicon lengths were kept very short (90-146 bp). To avoid the false negative detection, 141 bp eukaryotic 18S rRNA endogenous control was used as internal amplification control (IAC). The LOD of the system was 0.003 ng under pure state and 0.1% under ternary admixture showing 84−115% target recovery for the three target species (Hossain

et al., 2017a). Recently, Khairil Mokhtar et al. (2020) developed a pork specific qPCR assay to establish a rapid, cost-effective and simple DNA extraction technique applicable in both raw and processed meat products. They tested three different formulations of lysis buffer namely LB1 (5% Chelex-100 in water suspension), LB2 (0.2M Tris-HCl, 0.01M EDTA, 0.5M NaCl and 1% SDS), and LB3 (5% Chelex-100 in LB2 suspension) to extract the DNA from meat samples. The LB3 buffer was proved to be the most effective for high quality DNA extraction.

Although several mqPCR systems have been reported to date, to the best of our knowledge, no mqPCR system is available for quantitative detection of cow, buffalo, goat, sheep, chicken, duck and pig in food products.

2.3.2.2 Validation of PCR methods

(a) Definition:

Method validation is a process to establish the performance characteristics and limitations of an analytical method. According to Green (1996), "Method validation is the process of proving that an analytical method is acceptable for its intended purpose". According to the criteria of the definition, the PCR method must be adequately optimized, standardized and developed so that it can be adjusted to achieve performance characteristics commensurate with the assay's objective. After the initial method optimization and development, method validation is accomplished by performing experiments to determine performance characteristics and quantify performance of the method.

(b) Practical evaluation of parameters and acceptance criteria:

The Codex Alimentarius Commission places an emphasis on the acceptance of methods of analysis which have been validated through a collaborative trial conforming to an internationally accepted protocol according to ISO 5725:1994 or the AOAC/IUPAC Harmonized Protocol. These guidelines provide information on criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences of interest that may be present in foods (Codex Alimentarius Commission, 2010).

To ensure that the method's performance is fit for purpose, certain parameters of the PCR assay must be checked. If a method meets the predetermined criteria, it can be used for routine analysis. The following parameters have to be evaluated during development and in-house validation of singleplex and multiplex PCR assays (Broeders et al., 2014).

i) Applicability

Under the applicability statement, the developer should explicitly explain the scope of the method that describes the name of the target species, the type of samples, indication of the matrix (e.g., processed food, raw materials, etc.), the amount of DNA to be analyzed and the range to which the method can be applied. Relevant limitations of the method should also be addressed (e.g. interference by other analytes or inapplicability to certain situations). Limitations may also include possible restrictions due to the costs, equipment or specific and non-specific risks implied for either the operator and/or the environment (Codex Alimentarius Commission, 2010). The method needs to be evaluated under different matrices including raw and processed materials, food and feed, genomic DNA and plasmid DNA etc. Additionally, varying amounts of DNA need to be analyzed to find possible PCR inhibitors. Reproducible results are to achieve under as many matrices as possible (Broeders et al., 2014).

ii) Practicability

Practicability of the assay can be evaluated by testing blind samples in the routine laboratory. In this case, the novel method can be applied in conjunction with current methods that had already been tested in the lab under the same conditions. The developed method can be transferred to a second laboratory to ensure the reproducible results (Broeders et al., 2014).

iii) Optimization and standardization of reagents and determination of critical Control parameters

Sample collection and preparation as well as DNA extraction procedures are considered critical parameters in assay performance, therefore, these have to be optimized to get satisfactory results. Depending on the sample type, several DNA extraction procedures are used. DNA extraction from raw meat samples, for example, is simpler, whereas the extraction from complex matrices is more complicated.

To eliminate the presence of PCR inhibitors, internal amplification controls should be incorporated in the assay design. Internal controls are amplified using primer and probe sets that are different from those used for amplification of the targets.

It needs to assess the capability of the assay to remain unaffected by minor modifications in the major parameters. It is also important to evaluate the critical parameters of the method such as concentration of primers, $MgCl_2$, buffer, dNTP and DNA Taq polymerase as well as annealing time and temperature (Belak & Thorén, 2004).

iv) Repeatability

Replicate analyses can be used to assess the analytical precision. Each replicate should be treated as an independent sample. It is noteworthy that, using single DNA extract for

the analysis of a replicate e.g., triplicate amplifications, is unacceptable. In case of qPCR assay, inter-run coefficient of variation could be calculated from the Ct-values generated from the replicated samples (Belak & Thorén, 2004).

v) Analytical specificity and sensitivity

Specificity of the PCR assay refers to the ability of the system to distinguish between the target species and other non-target species. Specificity checking of a new assay can be performed in several phases as discussed below:

Theoretical test for specificity - Theoretical specificity between the closely related and distant species can be checked by conducting a computer-aided ("in-silico") test by searching suitable databases (e.g., the online Basic Local Alignment Search Tool (BLAST) in the NCBI database) between the oligonucleotide sequences (primer, probe) and the amplicon sequence for similarities to other sequences.

Experimental test for specificity - The method should be tested by analyzing DNA/RNA extract from target species and genetically related species. Acceptable cross-reactivity depends mainly on the desired purpose of the assay, and it needs to be determined for individual case.

Limit of detection (LOD) or sensitivity of the assay is the lowest quantity of DNA that could be detected. Sensitivity is determined by using serially diluted DNA extract starting from higher to lower concentrations till the assay is unable to detect the target analyte in more than 5% of the replicates (Belak & Thorén, 2004). Following determination of the LOD of the assay, further experiments should be run to determine the LOD under different food matrices.

In a real-time PCR method, there should be a Ct (Threshold cycle) cutoff value and any result above the cutoff value is considered negative. The laboratory should establish what cutoff value should be fixed depending on assay optimization, validation data etc.

vi) Reproducibility of the assay

Determination of reproducibility is important in evaluating the assay precision. The assay reproducibility is determined by applying an identical method (protocol, reagents and controls) in different laboratories. Test results of at least three different laboratories using the same specimens' set (at least 20 samples) of similar aliquots are usually considered in validating the reproducibility as well as ruggedness of the assay (Belak & Thorén, 2004).

(c) Validation of quantitative PCR methods

For the analysis of nucleic acid, especially in processed foods, very minute quantities of target-specific DNA/RNA are required. In a quantitative PCR assay, the results are often expressed in percent as the amount of target nucleic acid in relation to an endogenous control. Consequently, two PCR-based determinations are involved here: one for the target-specific DNA/RNA sequence and another for the comparator. As a result, it's critical that both measurements are thoroughly validated. Quantitative PCR analysis requires the fulfilment of the following basic performance characteristics:

(i) Specificity (Selectivity)
(ii) Sensitivity (Limit of Detection-LOD)
(iii) Sensitivity (Limit of Quantification-LOQ)
(iv) PCR Efficiency
(v) Precision-Repeatability and Reproducibility Standard Deviations

Standard Curves - Optimization of quantitative PCR assays require the generation of standard curves. Standard curves should be constructed using pure samples at the early

stages of method development. However, in food analysis assays, standard curves prepared using relevant food matrix or matrices should also be included. These standard curves are used to determine the PCR efficiency and limit of quantification, which are discussed below.

PCR Efficiency - PCR efficiency refers to the closeness of the observed reaction to a true statistical doubling of amplified PCR product during PCR cycles and this is based on Ct values. To evaluate the efficiency, a standard curve is generated using diluted DNA template and the Ct value is determined for each dilution. The standard curve has the average slope between -3.1 and -3.6, which correspond to PCR efficiency ranging from 90%-110%. Most current real-time PCR analysis software packages calculate the slope and PCR efficiency based on the CT values of the standard curve. Deviations in efficiency significantly from the recommended value range may be due to the presence of PCR inhibitors and this suggests the need for further optimization of the assay.

Limit of Quantification (LOQ) - The limit of quantification refers to the minimum amount of analyte in a sample that could be successfully quantified. The LOQ can be determined in a variety of ways: by analyzing spiked samples containing a known amount of analyte or by assaying a large number of samples that contain known amount of analyte. The target species is first spiked with a relevant food matrix and then sample preparation and DNA/RNA extraction are performed.

Repeatability and Reproducibility Standard Deviations ($RSD_{repeatability}$ and $RSD_{reproducibility}$) - $RSD_{repeatability}$ refers to the relative standard deviation of results obtained by testing the same samples repeatedly and independently using the same method, in the same laboratory, by the same analyst, using the same equipment over a short period of time. The $RSD_{repeatability}$ should be less than 25% over the whole dynamic

range throughout the assay. The relative standard deviation of results between laboratories ($RSD_{reproducibility}$) should not exceed 35%.

(d) Validation of qualitative and quantitative multiplex PCR assays

When multiplex assays are performed, all the method validation steps must be carried out in multiplex format and performance metrics discussed above must be verified for each of the individual targets as it performed under multiplex conditions. In case of probe-based techniques, the fluorescence signals from different targets must not interfere with each other. Multiplex intercalating dye-based techniques cannot be considered quantitative because of the inability of intercalating dyes to distinguish different targets in a multiplex format.

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".

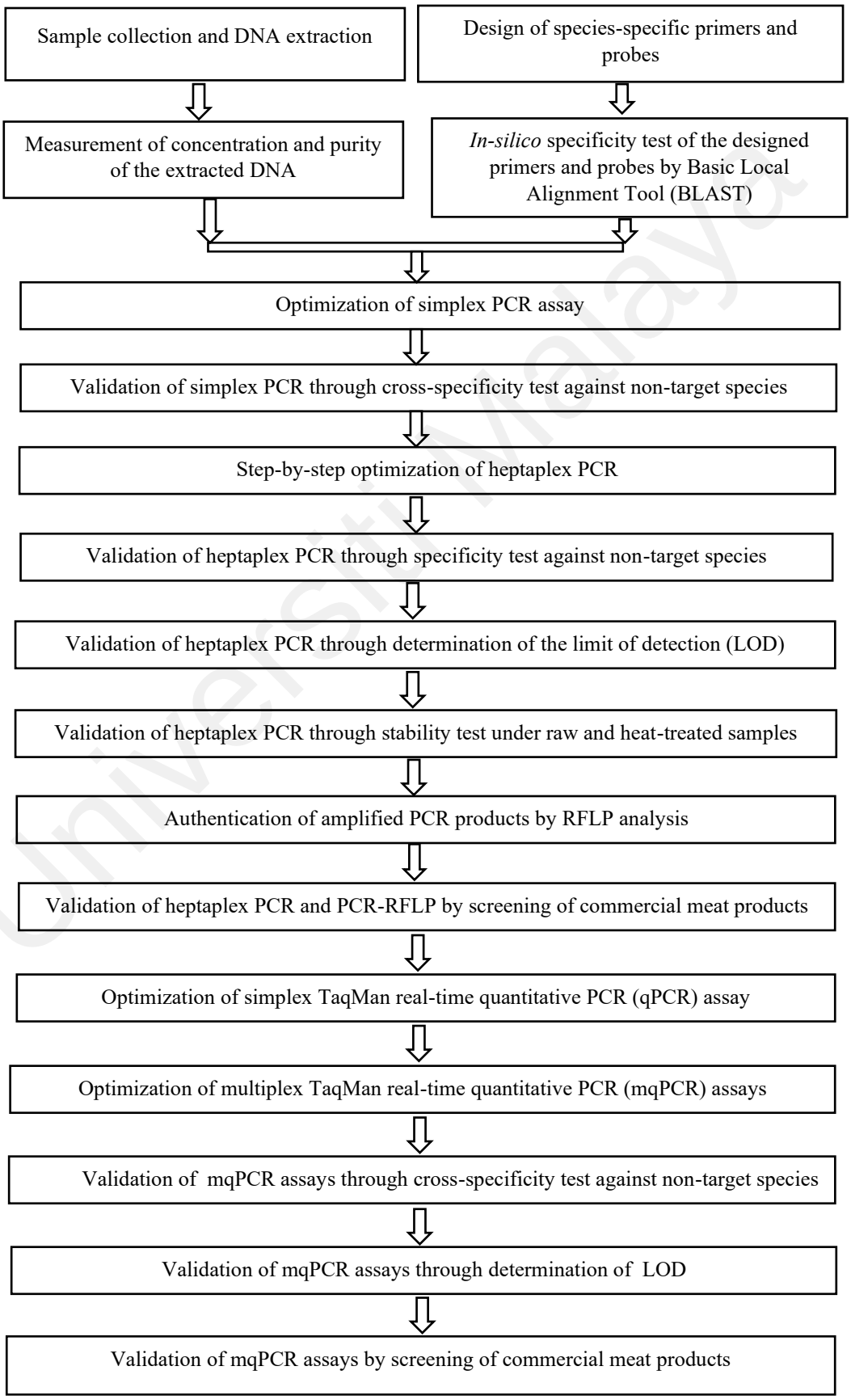
Amplicon length:

"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".

CHAPTER 3: MATERIALS AND METHODS

3.1 Overview of the Methodology

An overview of the methodology adopted in the present research is presented below:



3.2 Sample Collection

Raw meat samples of six target species like cow (*Bos taurus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), sheep (*Ovis aries*), chicken (*Gallus gallus*) and duck (*Anas platyrhychos*) were purchased directly from different butchers in Kuala Lumpur, Malaysia to guarantee their authenticity. Samples of different species, including pigeon (*Columba livia*), quail (*Coturnix coturnix*), tuna (*Thunnus orientalis*), tilapia (*Oreochromis niloticus*), sardine (*Sardinella longiceps*), rabbit (*Oryctolagus cuniculus*), frog (*Rana kunyuensis*), ostrich (*Struthio camelus*), squirrel (*Callosciurus notatus*) and turtle (*Cuora amboinensis*) were collected from several wet and supermarkets at Kuala Lumpur in Malaysia. Pig (*Sus scrofa*) meat was purchased from a Chinese market in Selangor, Malaysia. Meat from euthanized dog (*Canis familiariz*), cat (*Felis catus*), and rat (*Rattus rattus*) were donated by Dewan Bandaraya Kuala Lumpur (DBKL). Crocodile (*Crocodylus porosus*) meat was taken from Pearl Point Shopping Center, KL, Malaysia. Different plant samples like onion *(Allium cepa)*, ginger (*Zingiber officinale*), garlic (*Allium sativum*), pepper (*Capsicum annuum*) and wheat flour (*Triticum aestivum*) were bought from grocery shops. Commercial chicken, beef and pork meatballs, frankfurters, burgers, sausages etc., of various popular brands were purchased from five different super shops in Malaysia. Moreover, curry and cooked whole muscle meat samples were purchased from ten different restaurants. All the samples were collected in triplicates on different days. All the samples were maintained under 4°C during transportation and then preserved at -20°C until DNA extraction.

3.3 Extraction of DNA from Samples

Total DNA was extracted from raw meat and fish samples using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) adhering to the manufacturer's instructions (Rashid et al., 2015). Briefly, about 20 mg of each sample

was homogenized in a 1.5 mL tube followed by addition of lysis buffer (to break the cell wall/cell membrane and release DNA) and proteinase K. The tube was then incubated in a water bath maintaining 60°C for 30 min with inversion for a few times. Absolute ethanol was added (for removal of protein part) followed by centrifugation. Spin columns were used that allowed the binding of the sample DNA to the glass fiber matrix during centrifugation. Ethanol-added wash buffer helped to remove potential contaminants. Finally, elution buffer was used followed by centrifugation to elute the purified DNA. Total DNA was extracted from food products (model and the commercial meat products) by using DNeasy mericon Food Kit (QIAGEN GmgH, Hilden, Germany) taking about 200 mg of samples (Sultana, Hossain, Zaidul, & Ali, 2018). On the other hand, plant DNA was extracted by the DNeasy Plant Mini Kit (QIAGEN GmgH, Hilden, Germany) (Hossain et al., 2017b). The yield and purity of all the extracted DNA were determined using a UV−Vis spectrophotometer (NanoPhotometer Pearl, Implen GmbH, Germany) based on absorbance at 260 nm and absorbance ratio at 260/280 nm (Napolitano et al., 2014).

3.4 Development of Biomarkers for Multiplex PCR

The design of suitable biomarkers is crucially important for successful PCR amplification. Primer design, especially for mPCR assays, is extremely critical since they demand more stringent specificity and melting temperature (Tm) needs to be identical for all primers to anneal with their respective targets under the same set of PCR conditions (Ali et al., 2014).

The following criteria and instructions were followed when designing specific primers for this study:

i) **Primer length**: The length of primers should be kept reasonable. Too long or too short primers may cause secondary structure formation or reduce specificity resulting in non-specific amplification (Abd-Elsalam, 2003). An ideal primer should be of 18-28 nucleotides length (Dieffenbach et al., 1993). All the primers in this study were kept within the recommended nucleotide range.

ii) **Guanine-cytosine (GC) content**: A reasonable GC content is important to have a good PCR product since Tm and annealing temperature (Ta) are fully dependent on GC (%) (Rychlik et al., 1990). In designing primers, the GC content criteria were maintained in this study.

iii) **Melting temperature and annealing temperature:** Melting temperature (T_m) has an important role in primer annealing. Both forward and reverse primers should be of similar Tm. Melting temperature (Tm) needs to be closely spaced since all primers in mPCR assay have to anneal with their respective binding regions under the same set of PCR conditions (Ali et al., 2014). Primers with Tm extremely lower or higher than the annealing temperature (T_a) of PCR, seriously affect amplification. In this study, the Tm values were cautiously evaluated for every primer sets during primers design.

iv) **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 of PCR, DNA polymerase starts attaching nucleotides from the 3′-end of a primer. Therefore, primers, at their 3′-ends, should possess mismatching with non-target species since it inhibits the amplification (Ali et al., 2014). This study critically calculated primer mismatching at their 3′-ends.

3.4.1 Design of Species-specific Primers

Seven sets of species-specific primers were designed targeting cytb and ND5 genes of mitochondrial origin, given their higher degree of divergence. They showed adequate conserved regions within the same species but sufficient polymorphism among the closely related species. Cytb gene was targeted to design the chicken primer set while the primer sets for the other six species of cow, buffalo, duck, goat, sheep and pig were designed targeting ND5 gene. The gene sequences of cow, buffalo, duck, chicken, goat, sheep and pig as well as other non-target species have been retrieved from NCBI database. The MEGA7 alignment software (http://www.megasoftware.net/) was utilized for sequence alignment to detect conserved and hyper-variable regions among the target and non-target species. The online software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was applied to design the primer sets. The developed primers were checked for theoretical specificity between the closely related and distant species using the online Basic Local Alignment Search Tool (BLAST) in the NCBI database (http://blast. ncbi.nlm.nih.gov/Blast.cgi). The degree of mismatches among target and non-target species was evaluated *in silico* by using CLUSTALW multiple sequence alignment tool (http://www.genome.jp/ tools/clustalw/) and MEGA7 software. This study evaluated both intra-species (within species) and inter-species base mismatch in the primer annealing regions (Tables 4.2 - 4.8). The primers were aligned against three individuals of the same species and 7–11 species of the same genus (depending on the availability of corresponding gene sequences from the NCBI database) as well as 30 different nontarget species including 19 land animal species such as, cow (*B. taurus*), goat (*C. hircus*), buffalo (*B. bubalis*), sheep (*O. aries*), duck (*A. platyrhynchos*), pig (*S. scrofa*), chicken (*G. gallus*), rabbit (*O. cuniculus*), dog (*C. familiaris*), cat (*F. catus*), rat (*R. norvegicus*), quail (*C. coturnix*), deer (*Cervus nippon*), monkey (*Macaca fascicularis*), horse (*Equus caballus*), donkey (*Equus asinus*), turkey (*Meleagris gallopavo)*, ostrich (*S.*

camelus) and pigeon (*C. livia*); 9 aquatic species including salmon (*Salmo salar*), cod (*Gadus morhua*), tilapia (*O. niloticus*), pangas (*pangasius pangasius*), rohu (*Labeo rohita*), tuna (*T. orientalis*), turtle (*C. amboinensis*), crocodile (*C. porosus*) and frog (*R. kunyuensis*) and, 3 plant species: pepper (*C. annuum*), onion (*A. cepa*) and wheat (*T. aestivum*). Thus, sequence matching was critically evaluated within species (intraspecies) as well as with other non-related species (Tables 4.2 - 4.8). The pair-wise distances among the target and non-target species were determined and phylogenetic trees were constructed from the alignment by MEGA7. Finally, absolute specificity was confirmed through a PCR assay against templates of 7 target and 19 non-target species. The sequences of our designed primers and the primers taken from another published article are given in Table 3.1. The required primers were synthesized by Integrated DNA Technologies (IDT), Singapore.

Table 3.1: Names and sequences of primers used in simplex and multiplex conventional PCR assay in this study

Species/Primer	Target gene	Sequence (5′-3′)	Amplicon size (bp)	Tm values	GC content (%)	Reference
Cow	ND5	Forward: GGTTTCATTTTAGCAATAGCATGG Reverse: GTCCAATCAAGGGTATGTTTGAG	106	61.0 59.8	37.5 43.5	Hossain et al. (2017b)
Buffalo	ND5	Forward: TCGCCTAGCTTCTTACACAAAC Reverse: TGGTTTGTGACTGTGATGGAT	138	58.7 58.8	45.5 42.9	Hossain et al. (2017b)
Chicken	Cytb	Forward: CTTTGCAATCGCAGGTATTACTAT Reverse: GGAATGGGGTGAGTATGAGAGT	161	58.8 59.3	37.5 50.0	Hossain et al. (2019b)
Duck	ND5	Forward: CACCGTAAAAGCTGCATTTCTAA Reverse: ACATGGCGAATTGTAGAATGG	203	60.6 59.8	39.1 42.9	This study
Goat	ND5	Forward: TGTTTTCTTCTCTTGCACTAACCAC Reverse: CTTGAAGCTGAGCGATAATTTAAGG	236	61.0 61.7	40.0 40.0	This study
Pig	ND5	Forward: GATTCCTAACCCACTCAAACG Reverse: GGTATGTTTGGGCATTCATTG	73	58.6 60.1	47.6 42.9	Hossain et al. (2017b)
Sheep	ND5	Forward: TTCCTCCCTCACACTAGTCACC Reverse: CTGGAACGAATATTATTGAGAAGAAGTC	263	60.5 61.0	54.5 35.7	This study
Universal eukaryotic primer	18S rRNA	Forward: AGGATCCATTGGAGGGCAAGT Reverse: TCCAACTACGAGCTTTTTAACTGCA	99	-	-	(Safdar & Junejo, 2015)

3.4.2 Construction of Pairwise Distance and Phylogenetic Tree

The pair-wise distances among the target and non-target species were determined and phylogenetic trees were constructed by aligning each of the amplicon sequences with the respective gene sequences of the target and other 30 non-target species using the neighbour-joining method of MEGA7 software (Tamura et al., 2011). For example, the sequence of cow ND5 amplicon was aligned along with the ND5 gene of cow and other 30 non-target species including pig, cow, buffalo, chicken, duck, goat, sheep, cat, dog, rat, rabbit, crocodile, horse, donkey, turkey, quail, pigeon, ostrich, cod, salmon, pangas, rohu, tuna, sardine, tilapia, frog, turtle, onion, pepper and wheat. For other six amplicons, the same procedure was followed.

3.5 Development of Simplex PCR Assay

3.5.1 Optimization of Simplex PCR Assay

Simplex PCR assay was optimized for every target species with an individual set of primers. PCR reaction was carried out in a final 25 µL volume which contains 5 µL of 5× GoTaq Flexi Buffer, 2.5 mM of $MgCl_2$, 0.2 mM each of dNTP, 0.625 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI), 0.2 µM of corresponding primer, and 1 µL (20 ng/µL) of extracted DNA (Table 3.2). The optimum annealing temperature for perfect amplification was obtained by checking all the primer sets within a defined temperature range from 56º to 61°C in the gradient system. A negative control containing deionized water in place of DNA was included with each PCR run to check any DNA contamination in the reaction mixture. Then universal eukaryotic primer set (0.2 µM) targeting 99 bp site of the 18S rRNA gene (Muhammad Safdar & Junejo, 2015) was included in all the simplex PCR assays. All the PCR assays were carried out in a 96-well thermal cycler (ABI Veriti, Applied Biosystems, Foster City, CA). PCR was conducted as follows: the initial denaturation was performed at 95 °C for 3 min. In the subsequent steps of 35 cycles

there were denaturation (95°C for 30 s), annealing (60°C for 40 s), extension (72°C for 40 s), and lastly, final extension (72°C for 5 min) (Table 3.3). PCR products were preserved at -20°C for future analysis.

Table 3.2: Concentration of simplex PCR components

Primer	$MgCl_2$ (mM)	dNTP (mM)	Primer (µM)	Taq pol (unit)
Cow ND5	2.50	0.20	0.20	0.625
Buffalo ND5	2.50	0.20	0.20	0.625
Chicken cytb	2.50	0.20	0.20	0.625
Duck ND5	2.50	0.20	0.20	0.625
Goat ND5	2.50	0.20	0.20	0.625
Sheep ND5	2.50	0.20	0.20	0.625
Pig ND5	2.50	0.20	0.20	0.625

Note: 5 µL of 5× GoTaq Flexi Buffer, 0.2 µM universal eukaryotic primers and 1 µL (20 ng) of template DNA from each target species was used in all PCR experiments.

Table 3.3: Cycling parameters of simplex PCR reactions

Type of PCR assay	Initial denaturation	PCR reactions (35 cycles)			Final extension
		Denaturation	Annealing	Extension	
Cow ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Buffalo ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Chicken cytb	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Duck ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Goat ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Sheep ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Pig ND5	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min

3.5.2 Gel Electrophoresis

For separation and visualization of species-specific PCR amplified products, initially, conventional gel electrophoresis (agarose gel electrophoresis) was performed and finally, an automated QIAxcel Advanced Capillary Electrophoresis System (QIAxcel, QIAGEN GmbH, Hilden, Germany) was used for higher resolution.

3.5.2.1 Conventional gel (agarose gel) electrophoresis

Conventional agarose gel electrophoresis was performed using 2% (w/v) agarose gel following the procedure as follows (Hossain, 2017):

Initially, 3 g of agarose was added in a 250 ml beaker containing 150 ml of 1× Tris-borate-EDTA (TBE) buffer. After mixing well, it was dissolved completely by heating in a microwave oven. After the gel temperature decreases to 50-60^{o}C, 5-6 µL of a DNA stain (fluorosafe, 1st Base Laboratories, Malaysia) was added and mixed well. The gel mixture was then poured into a horizontal electrophoresis tray containing the comb wells and allowed for 20-30 minutes to solidify. Upon placing the gel tray inside the 1× TBE buffer containing tank, 6 µL PCR products were loaded into the wells followed by 50 bp DNA ladder (Promega, USA). The gel electrophoresis (SUB13, Hoefer, Inc., California, USA) was then performed for about 70 min at 120 volts to separate the PCR products based on their molecular size. Finally, the separated PCR products gave banding profile which was then observed under a gel documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA).

3.5.2.2 Advanced capillary electrophoresis system

The QIAxcel Advanced Capillary Electrophoresis System is fully automated, highly sensitive, rapid and gives high resolution (capable of separating the products having 3-5 bp length difference). This electrophoresis instrument uses disposable, multiple-use cartridges in a cost-effective manner and enables analysis of up to 96 samples within 25 minutes. The QIAxcel Advanced is set up with a gel cartridge, running buffer and wash buffer, and calibrated using intensity markers. The samples for analysis were placed on the sample plate holder. Required data collection settings were selected and the samples were passed through the capillaries of the QIAxcel gel cartridge.

Electrical current was applied to a gel-filled capillary cartridge via respective electrode of every capillary and the system offered both gel images and electropherograms of the PCR product in one assay platform. Data was analysed using the QIAxcel ScreenGel Software.

3.5.3 Specificity Test of Simplex PCR Assay

The simplex PCR Assay was checked for species specificity by cross-testing with the extracted DNA (20 ng) from one target and 25 nontargets of terrestrial and aquatic animal species as well as plant species that we usually use in food preparation (cow, buffalo, goat, sheep, chicken, duck, pig, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic and wheat). The simplex PCR assay was checked for specificity through the use of universal eukaryotic primer (0.2 μM) that targeted the 99 bp site of 18S rRNA gene.

3.5.4 Sequencing of PCR Products

All the PCR products were purified using a PCR purification kit, the Promega Kit (Promega, Madison, WI) and then sent to IDT for bidirectional sequencing using forward and reverse primers. The PCR product sequencing was performed for determining the actual order of the nucleotides in the products. The derived sequences were then compared with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov) using nucleotide basic local alignment search tool (BLAST) to determine any species match. Sequence alignment was also done with specific sequences using the MEGA7 software to observe similarities with specific species.

3.6 Development of Heptaplex (multiplex) PCR Assay

3.6.1 Optimization of Heptaplex (multiplex) PCR Assay

After confirmation of simplex PCR, multiplex PCR assays were sequentially optimized starting from duplex to finally heptaplex (Tables 3.4 and 3.5). A duplex PCR assay for cow and sheep was optimized and developed using individual primer sets. This was followed by triplex of cow, sheep and chicken; tetraplex of cow, sheep, chicken, and pig; pentaplex of cow, sheep, chicken, pig and buffalo; hexaplex of cow, sheep, chicken, pig, buffalo and duck and, finally, heptaplex PCR of cow, sheep, chicken, pig, buffalo, duck and goat. All the PCR reactions were carried out in a final 25 µL volume which contains 5 µL of 5× GoTaq Flexi Buffer and other PCR components, using Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The optimum concentrations of PCR components and cycling parameters are summarized in Tables 3.4 and 3.5, respectively. A negative control containing deionized water in place of DNA was included with each PCR run to check any DNA contamination in the reaction mixture. Agarose gel, giving poor resolution was not applied to separate and visualize PCR products; instead, an automated Advanced Capillary Electrophoresis (ACE) system (QIAGEN, Hilden, Germany). The system gave both gel images and electropherograms.

Table 3.4: Concentrations of PCR Components of different PCR assays

PCR	$MgCl_2$ (mM)	dNTP (mM)	Primer (µM)	Taq pol (unit)
Duplex and triplex	3.0	0.20	0.28-0.56	0.625
Tetraplex	3.5	0.20	0.20-0.40	0.94
Pentaplex	3.5	0.20	0.20-0.80	0.94
Hexaplex	4.0	0.25	0.20-0.80	1.25
Heptaplex	4.0	0.25	0.24-0.80	1.25

Note: 5 µL of 5× GoTaq Flexi Buffer and 1 µL (20 ng) of template DNA from each target species was used in all PCR experiments

Table 3.5: Cycling parameters of different PCR reactions

Type of PCR assay	Initial denaturation	PCR reactions (35 cycles)			Final extension
		Denaturation	Annealing	Extension	
Duplex, triplex and tetraplex	95°C for 3 min	95°C for 30s	60°C for 40s	72°C for 40s	72°C for 5 min
Pentaplex, hexaplex and heptaplex	95°C for 3 min	95°C for 40s	60°C for 60s	72°C for 50s	72°C for 5 min

3.6.2 Specificity Test of Heptaplex PCR Assay

The heptaplex PCR assay was checked for species specificity by cross-testing with the extracted DNA (20 ng) of seven targets (pig, cow, buffalo, chicken, duck, goat and sheep) and 14 non-targets of terrestrial and aquatic animal species (cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle) as well as five plant species (onion, pepper, ginger, garlic and wheat) that we usually use in food preparation.

3.6.3 Limit of Detection (LOD) of Heptaplex PCR Assay under Raw State

To determine the LOD of the heptaplex assay, a mixture of extracted DNA from the seven target species was first prepared comprising of 10 ng/µL DNA from each target. Lower concentrations (1.0, 0.1, 0.01 and 0.005 ng/µL) were then made through dilution with the required amount of deionized water. All diluted DNA mixtures were amplified using the developed multiplex PCR assay.

3.6.4 Target DNA Stability Test under Heptaplex PCR Assay

The developed heptaplex PCR assay was checked for stability. For simulation of traditional food preparation processes, the raw meat samples were subjected to three thermal treatments separately: boiling, microwaving and autoclaving (Table 3.6). The beef, buffalo, goat, sheep, chicken, duck and pork samples were boiled in water for a duration of 60 min and 90 min on a hot plate. The usual canning and steam cooking

processes were imitated by autoclaving the meat samples at 121 °C under 15 psi pressure for 20 min (Ali et al., 2015). The meat samples were heat-treated through microwave cooking (as practiced in modern life) at both 600 W and 700 W for 30 min (Hossain et al., 2017b). The thermally treated samples were stored at - 20 °C until DNA extraction.

Table 3.6: Different thermal treatments applied to target Meat samples

Heat Treatment	Condition	Time (min)	Pressure (psi)	References
Boiling	100°C	60	-	Ali et al. (2016)
Boiling	100°C	90	-	Ali et al. (2016)
Autoclave	121°C	20	15	Ali et al. (2015).
Microwave	600 W, 700 W	30	-	Hossain et al. (2017b).

3.6.5 Sensitivity Test of Heptaplex PCR Assay under Commercial Products (Meatballs and Frankfurters)

After the heptaplex PCR assay was optimized and performed under pure states, it was subsequently validated under raw and heat-treated commercial products. In order to screen commercial products applying the developed heptaplex system, three categories of ready-to-eat model meatballs and frankfurters of chicken, beef and pork were made in the laboratory according to Ahamad et al. (2017) and Hossain et al. (2016) with modification (Table 3.7). The prepared beef, chicken and pork products were then deliberately adulterated by mixing a balanced amount of other species meat. Beef products were adulterated by spiking with 5%, 1%, and 0.5 % (w/w) of buffalo, goat, sheep, chicken, duck, and pork. Adulteration of chicken meatballs and frankfurters was performed by spiking with 5%, 1%, and 0.5 % (w/w) of beef, buffalo, goat, sheep, duck, and pork. Similarly, pork products were contaminated by mixing with the same proportions of six other target species in the same percentages. Then, the prepared 0.5 % adulterated beef, chicken and pork products were heat-treated by boiling at 100 ºC for 90 min and autoclaving at 121ºC under 15 psi pressure for 20 min. The samples were preserved at − 20◦C for DNA extraction.

Table 3.7: Formulation of model meatball and frankfurter

Ingredients	Meat ball (≥50 g/piece)			Frankfurter (≥80 g/piece)		
	Beef	Chicken	Pork	Beef	Chicken	Pork
Minced meat	31.0[a]	31.0[a]	31.0[a]	53.0 [a]	53.0[a]	53.0[a]
Soy protein	4.5	4.5	4.5	9.0	9.0	9.0
Breadcrumb	7.5	7.5	7.5	7.5	7.5	7.5
Chopped ginger	0.15	0.15	0.15	0.20	0.20	0.20
Chopped onion	1.3	1.3	1.3	3.0	3.0	3.0
Garlic power	0.70	0.70	0.70	0.50	0.50	0.50
Cumin powder	1.0	1.0	1.0	1.0	1.0	1.00
Tomato paste	1.9	1.9	1.9	2.5	2.5	2.5
Black pepper	0.17	0.17	0.17	0.30	0.30	0.30
Butter	1.8	1.8	1.8	2.5	2.5	2.5
Salt	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]
Others[b]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]

Note: [a] A 5%, 1%, and 0.5% portion of beef, buffalo, chicken, duck, goat, sheep and pork, meat was mixed with a balanced amount of respective minced meat to prepare ≥ 50 g meatball and ≥80 g frankfurter specimens, [b] Flavouring agents and enhancers; [c]SA, suitable amounts.

3.7 Enzymatic Digestion and RFLP Analysis

The sequences of amplicons of all the targets were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) and online available NEBcutter version 2.0 software (http://tools. neb.com/NEBcutter) was used to select the specific and appropriate restriction endonucleases of all PCR amplicons to develop a PCR-RFLP, so that a highly distinguishable RFLP pattern between target species could be produced. The restriction pattern of the PCR amplicons of beef, buffalo, goat, sheep, chicken, duck and pork mitochondrial cytb and ND5 genes along with selected restriction fragments is given in Table 3.8.

Table 3.8: Digestion of PCR products with selected restriction enzymes

Target	Restriction enzymes	Amplicon size (bp)	Restriction Fragment size (bp)
Cow	*Fat*I	106	87, 19
Buffalo	*HPY188*I	138	70, 68
Chicken	*Bfa*I	161	93, 68
Duck	*Bfa*I	203	141, 62
Goat	*Bfa*I	236	130, 106
Sheep	*Fat*I	263	153, 110
Pork	*Fat*I	73	52, 21

3.7.1 RFLP Analysis of Beef, Buffalo, Goat, Sheep, Chicken, Duck, and Pork PCR Products

The PCR products of cow, sheep and pig were digested with *Fat*I restriction endonuclease (New England Biolab, Ipswich, MA, United States). On the other hand, Chicken, duck and goat products were digested with *Bfa*1 endonuclease. *HPY*188I endonuclease was used to digest Buffalo products. The total reaction volume of each digestion was 25 µL consisting of 1 µg of PCR product (unpurified), 1× digestion buffer, 1U of each enzyme, and an adjusted amount of sterilized deionized water. After gentle mixing, the reaction mixtures were spun down and incubated at 55°C with *Fat*I and at

37°C with *Bfa*I and *HPY*188I in a shaking water bath for 60 min. Finally, DNA digestion was stopped by placing the mixtures in another water bath at 80°C for 20 min for *Fat*I and *Bfa*I and at 65°C for 20 min for *HPY*188I. The digested products were then separated and visualized by running in an automated Advanced Capillary Electrophoresis System that uses QIAxel DNA High-resolution Kit (QIAGEN GmbH, Hilden, Germany).

Table 3.9: Reaction conditions of restriction enzymes for the digestion of target PCR products

Target	**Restriction enzyme**	**Amount of PCR products (µg)**	**Incubation temp. and time**	**Deactivation temp. and time**
Cow ND5	*Fat*I	1.0	55°C for 60 min	80°C for 20 min
Buffalo ND5	*HPY188*I	1.0	37°C for 60 min	65°C for 20 min
Chicken cytb	*Bfa*I	1.0	37°C for 60 min	80°C for 20 min
Duck ND5	*Bfa*I	1.0	37°C for 60 min	80°C for 20 min
Goat ND5	*Bfa*I	1.0	37°C for 60 min	80°C for 20 min
Sheep ND5	*Fat*I	1.0	55°C for 60 min	80°C for 20 min
Pork	*Fat*I	1.0	55°C for 60 min	80°C for 20 min

3.7.2 Authentication of PCR Products of Frankfurters by RFLP Analysis

After the heptaplex PCR-RFLP assay was optimized and performed under pure states, it was subsequently validated under raw and heat-treated commercial products. In order to authenticate the PCR products of cow, buffalo, chicken, duck, goat, sheep and pig by RFLP analysis, deliberately adulterated (5, 1 and 0.5%) model beef, chicken, and pork frankfurters were prepared and subjected to boiling (100°C for 90 min) and autoclaving (121°C under 15 psi pressure for 20 min). The PCR products from raw, heat-treated samples were digested and their restriction digestion patterns were analyzed.

3.8 TaqMan Probe-based Real-time PCR Assay

3.8.1 Design of Primers and TaqMan Probes

The oligonucleotide primers and probes were designed targeting mitochondrial cytb genes of cow (*Bos taurus*), buffalo (*Bubalus bubalis*), chicken (*Gallus gallus*) and pig (*Sus scrofa*), and ND5 genes of duck (*Anas platyrhychos*), goat (*Capra hircus*) and sheep (*Ovis aries*) (Table 3.10). Previous studies revealed that both genomic and mitochondrial genes could be considered as suitable targets for species detection in real-time PCR assays (qPCR). The mitochondrial genes are abundantly present compared to nuclear DNA. Moreover, they are highly conserved, protected by the mitochondrial double membrane, contain a short sequence with polymorphic characteristics between intra- and inter-species, thus facilitating in designing the specific primer and probe sets (Alikord, Momtaz, Kadivar, & Rad, 2018). Primers and probes were designed using Primer3Plus software following a standardized protocol as described in section 3.4.1. The Tm values of probes were kept minimum 8ºC higher than that of the primers. The Tm values differences between all primers were kept very close to each other (less than 2ºC). Similarly, the Tm values differences between all probes were kept less 2ºC. In addition, the GC content of all primers and probes were with the recommended range. The designed primers and probes were ensured regarding specificity through different testing steps. First, the basic local alignment search tool (BLAST) was used against nonredundant nucleotide sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the target species and the dissimilarity index value with other species. Second, the primers/probes were aligned against target species as well as 30 other nontarget animal and plant species using a ClustalW sequence alignment program (http://www.genome.jp/tools/clustalw/) and the MEGA7 software to determine in-silico specificity. Thus, sequence matching was critically evaluated among targets and other non-related species.

As a requirement of the TaqMan probe-based qPCR assay, all the probes were labeled with fluorescent reporter dyes at the 5' end and quencher at the 3' end. For instances, beef probe was labeled with TAMRA at the 5' end and ZEN/IOWA BLACK FQ at the 3' end; buffalo and chicken probes were labeled with HEX at the 5' end and ZEN/IOWA BLACK FQ at the 3' end, duck and goat probes were labeled with CY5 at the 5' end and ZEN/IOWA BLACK FQ at the 3' end, sheep and pork probes were labelled with FAM at the 5' end and ZEN/IOWA BLACK FQ at the 3' end. For endogenous control (IAC), eukaryotic 18S rRNA specific primers and TaqMan probe were used (M. E. Ali, U. Hashim, et al., 2012). The IAC probe was labeled with the fluorescent reporter dye TEXAS RED at the 5′ end and ZEN/IOWA BLACK FQ at the 3′ end (Table 3.10). All the designed primers and probes were supplied by Integrated DNA Technologies (IDT), Singapore.

Table 3.10: Names and sequences of primers and probes used in real-time PCR assays

Species/Primer	Target gene	Sequence (5′-3′)	Amplicon size (bp)	Final concentration (nM)	Reference
Cow	Cytb	Forward: CGGCACAAATTTAGTCGAAT Reverse: TGGACTATGGCAATTGCTATG Probe: TAMRA-TTCTTCGCTTTCCATTTTATCCTTCCA-ZEN/IOWA BLACK FQ	120	400 400 250	This study and Hossain, et al. (2017b)
Buffalo	Cytb	Forward: GGGTTCTAGCCCTAGTTCTCTCT Reverse: ATGGCCGGAACATCATACTT Probe: HEX-AATCCTCATTCTCATGCCCCTGCTACA-ZEN/IOWA BLACK FQ	90	600 600 400	This study and Hossain, et al. (2017b)
Chicken	Cytb	Forward: CTTTGCAATCGCAGGTATTACTAT Reverse: GGAATGGGGTGAGTATGAGAGT Probe: HEX-CCCCTAGGCATCTCATCCGACTCT-ZEN/IOWA BLACK FQ	161	400 400 250	This study and Hossain et al. (2019b)
Duck	ND5	Forward: CACCGTAAAAGCTGCATTTCTAA Reverse: ACATGGCGAATTGTAGAATGG Probe: CY5-CCGGACTAGAATCCATTACCTGCCAC-ZEN/IOWA BLACK FQ	203	600 600 400	This study
Goat	ND5	Forward: TGTTTTCTTCTCTTGCACTAACCAC Reverse: CTTGAAGCTGAGCGATAATTTAAGG Probe: CY5-CCGCACCCATCATAATAACCAACCTC-ZEN/IOWA BLACK FQ	236	500 500 300	This study
Pig	Cytb	Forward: TATCCCTTATATCGGAACAGACCTC Reverse: GCAGGAATAGGAGATGTACGG Probe: FAM-CCTGCCATTCATCATTACCGCCC-ZEN/IOWA BLACK FQ	146	500 500 300	This study and Hossain, et al. (2017b)
Sheep	ND5	Forward: TTCCTCCCTCACACTAGTCACC Reverse: CTGGAACGAATATTATTGAGAAGAAGTC Probe: FAM-CTAACCATACCCATCGCAGCAATCAA-ZEN/IOWA BLACK FQ	263	800 800 500	This study
Universal eukaryotic primer	18S rRNA	Forward: GGTAGT GACGAAAAATAACAATACAGGAC Reverse: ATACGCTATTGGAGCTGGAATTACC Probe: TEXAS RED-AAGTGGACTCATTCCAATTACAGGGCCT-ZEN/IOWA BLACK FQ	141	300 300 150	This study and Ali et al. (2012b)

3.8.2 Multiplex Real-time PCR Conditions

Multiplex real-time PCR assays of the seven target species were performed in two tubes consisting of i) beef, buffalo, goat and sheep specific primers and probes in one tube (indicated as mqPCR-1) and ii) chicken, duck and pork specific primers and probes in another tube (indicated as mqPCR-2). In addition, internal amplification control (IAC) specific primers and probes were used in both the tubes. A Quant Studio 12K flex real-time PCR system (Applied Biosystems, Foster City, CA) was used for the assay. The final reaction volume of 20 µL comprised 1× GoTaq Probe qPCR Master Mix (Promega, Madison), 60 ng of template DNA for each target species, and required amount of nuclease-free water. The concentration of primers and probes are given in Table 3.10. The amplification process involves with the initial denaturation at 95 °C for 10 min. The subsequent steps were completed with 40 cycles of denaturation at 95°C for 20 s followed by annealing and extension at 60°C for 60 s.

3.8.3 Specificity Test of mqPCR Assay

Specificity of both the mqPCR systems (mqPCR-1 and 2) were analyzed by testing mqPCR template DNA from all targets and 23 non-targets including animal (dog, rabbit, turtle, rat, pigeon, quail, monkey, donkey, ostrich, crocodile, frog, tortoise, cat, salmon, tuna, sardine, rohu, cod, tilapia) and plant species (wheat, onion, garlic and pepper, which are commonly used in food preparation).

3.8.4 Limit of Detection (LOD)

To determine the limit of detection, mqPCR assays (mqPCR-1 and 2) were performed with serially diluted DNA extracts from a mixture consisting of equal amounts of DNA from each target. Initially, two separate mixtures were prepared with equal amount of extracted DNA from cow, buffalo, goat and sheep (mqPCR-1) and chicken, duck and pig

(mqPCR-2) to make concentration of 30 ng/μL of corresponding species in each tube. Next, the DNA mixtures were tenfold serially diluted with nuclease free water (Cheng et al., 2014) and the concentrations of the diluted DNA samples were 3, 0.3, 0.03, 0.003 ng/μL in each dilution. In this study, 2 μL of each diluted DNA solution was added to 20 μL of both multiplex reaction mixture. As a result, each reaction mixture contained 60, 6, 0.6, 0.06, 0.006, ng of mixed DNA respectively. The mqPCR for each diluted template was carried out in 6 replicates.

3.8.5 Generation of Standard Curves and Target DNA Quantification and Calculation of PCR Efficiency

The standard curves in qPCR assays are considered as the indicator of performance evaluation in terms of analytical sensitivity and efficiency. Standard curves were generated to determine qPCR efficiency and to quantify targets. For this purpose, two different admixtures with equal ratio of target meats were prepared. The 50 gm of cow, buffalo, goat and sheep meats were mixed for mqPCR-1 while chicken, duck and pig meats were mixed for mqPCR-2. The total DNA was extracted from both admixtures using DNA extraction Kit (Yeastern Genomic DNA Mini Kit, Yeastern Biotech Co., Ltd., Taipei, Taiwan). The concentration of the extracted DNA was made 30 ng/μL (100%) DNA of each species in each admixture. The mixed DNA was then tenfold serially diluted to make the DNA concentrations of 3, 0.3, 0.03, 0.003 ng/μL. This gives the mixtures containing 100–0.001% of DNA for each species. Then, 2 μL of each diluted DNA was added to 20 μL of multiplex reaction mixture so that the final quantity of each mixture contained 60, 6, 0.6, 0.06, 0.006, ng of mixed DNA respectively. The Ct values (three closely spaced values) of each target species were then plotted against the logarithmic concentration of DNA of each species (Cheng et al., 2014; Iwobi et al., 2015). Finally, the efficiency of the assay was determined from the slope of the generated standard curve

according to the equation (Druml, Mayer, Cichna-Markl, & Hochegger, 2015) stated below:

$$E\ (\%) = [10(-1/\text{slope}) - 1] \times 100 \quad (3.1)$$

The acceptance criteria regarding the standard curves of qPCR assays can be stated as follows: The range of qPCR efficiency should be from 90 to 110% that corresponds to a slope of regression between − 3.1 and −3.6 and an R^2 value of ≥0.98 (Iwobi et al., 2015). To quantify beef, buffalo, chicken, duck, goat, sheep or pork in an unknown sample, the C_t value of the unknown sample was extrapolated in the standard curve. Thus, a semilogarithmic correlation was observed among the variables, C_t value, and concentration (Maria Rojas et al., 2010):

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

where m is the slope and c is the intercept.

The target species DNA concentration of each unknown sample was calculated by interpolating its Ct value in the standard curve in the following procedure:

$$\text{Content of target species DNA} = 10^{[(Ct-c)/m]} \quad (3.3)$$

Here, parameter m is the slope, and parameter c is the intercept of the standard curve. Then target meat quantity was determined as follows:

Target meat (%) = (target species DNA content derived from the target species-specific system/total DNA content (40 ng/reaction) × 100. (3.4)

3.8.6 Multiplex Real-time PCR Sensitivity and Applicability Test

To assess the sensitivity and applicability of the mqPCR assay in food products analysis, two different model meat products (meatballs and frankfurters) were made in the laboratory. Each model meat products (Meatballs and frankfurters) were prepared in two different sets, one set for mqPCR-1 (beef and goat meatballs and frankfurters) and another set for mqPCR-1 (chicken and pork meatballs and frankfurters). Beef products (meatballs and frankfurters) were deliberately adulterated with 10, 5, and 1% (w/w) of buffalo, goat, and sheep meat; goat products were adulterated with 10, 5 and 1% (w/w) of beef, buffalo and sheep meat. Similarly, chicken (meatballs and frankfurters) products were adulterated with duck meat and pork while pork products were adulterated with chicken and duck meat in the same ratio (10, 5, and 1% w/w) (Table 3.11). Total DNA was extracted from the prepared meat products, and nuclease free water was used to adjust the concentration to 40 ng/μL.

Table 3.11: Formulation of model meatball and frankfurter

Ingredients	**Meat ball (≥50 g/piece)**				**Frankfurter (≥80 g/piece)**			
	Beef	**Goat**	**Chicken**	**Pork**	**Beef**	**Goat**	**Chicken**	**Pork**
Minced meat	31[a]	31[a]	31[a]	31[a]	53.0 [a]	53.0 [a]	53.0[a]	53.0[a]
Soy protein	4.5	4.5	4.5	4.5	9.0	9.0	9.0	9.0
Breadcrumb	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Chopped ginger	0.15	0.15	0.15	0.15	0.20	0.20	0.20	0.20
Chopped onion	1.3	1.3	1.3	1.3	3.0	3.0	3.0	3.0
Garlic power	0.70	0.70	0.7	0.7	0.50	0.50	0.50	0.50
Cumin powder	1.0	1.0	1.0	1.0	1.00	1.00	1.00	1.00
Tomato paste	1.9	1.9	1.9	1.9	2.50	2.50	2.50	2.50
Black pepper	0.17	0.17	0.17	0.17	0.30	0.30	0.30	0.30
Butter	1.8	1.8	1.8	1.8	2.5	2.5	2.5	2.5
Salt	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]
Others[b]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]	SA[c]

Note: [a] A 5%, 1%, and 0.5% portion of beef, buffalo, chicken, duck, goat, sheep and pork, meat was mixed with a balanced amount of respective minced meat to prepare ≥ 50 g meatball and ≥80 g frankfurter specimens, [b] Flavouring agents and enhancers; [c]SA, suitable amounts.

CHAPTER 4: RESULTS

4.1 Quantity and Quality Assurance of Extracted DNA

Total genomic DNA was extracted from authentic raw meat and fish tissues, admixtures, and various meat products (meatball, burger, frankfurter, sausage, cocktail etc.) on three different days. The yield of total extracted DNA was determined by estimating its absorbance at 260 nm and the quality of DNA was checked by calculating the absorbance ratio of 260/280 nm (Nejad et al., 2014). The extracted DNA in this study showed the absorbance ratio of A260/A280 within 1.8 − 2.0 indicating that good quality DNA was obtained from all samples (Nejad et al., 2014). However, quantities of extracted DNA varied depending on nature of samples. The amount of DNA extracted from animal and fish tissues (20 mg) was 92−408 ng/µL, from plant tissues (100 mg), 75 − 172 ng/µL, from meat products (200 mg), 32 − 180 ng/µL, from thermally treated animal tissues, 41 − 160 ng/µL and from thermally treated meat products it was 32-110 ng/µL (Table 4.1). Hossain (2017) got DNA concentrations of 46-269 ng/µL for animal, fish and plant tissues whereas for meat products and thermally treated samples the concentrations were 33−147 ng/µL and 32-125 ng/µL, respectively.

Table 4.1: Concentration and purity of the extracted DNA.

Sample	Average Concentration (ng/µl)	Purity (A260/A280)
Animal tissue (raw)	150−408	1.95-2.0
Animal tissue (boiled)	90-160	1.86-1.97
Animal tissue (microwaved)	58-98	1.85-1.95
Animal tissue (autoclaved)	41-82	1.80-1.92
Fish tissue (raw)	92-192	1.89-1.98
Plant tissue (raw)	75 − 172	1.88-1.95
Meat products (raw)	95 − 180	1.86-1.95
Meat products (boiled)	72-110	1.83-1.92
Meat products (autoclaved)	32-68	1.80-1.90

4.2 Development of Biomarkers

In this study, seven pairs of species-specific primers were designed targeting cytb and ND5 genes of cow, buffalo, goat, sheep, chicken, duck and pig species to develop a heptaplex PCR assay with short amplicon lengths (Table 3.1). Our designed primers, in order to develop a successful heptaplex PCR assay, fulfilled all the required criteria including short length amplicons (73 -263 bp in this study), full matching with target DNA while mismatching with non-targets, acceptable GC content and Tm. The GC content of all the primers used in this study were between 37.5 and 50%. Tm values of all the primers were between 58.6 and 61.7ºC (Table 3.1).

4.2.1 *In silico* Analysis of Biomarkers Using Bioinformatics Tools

The designed primers were aligned *in silico* against the corresponding gene sequences of target 30 other non-target species (18 terrestrial animals, 9 aquatic and 3 plant species) as mentioned in Section 3.4.1. The sequences of each primer pair demonstrated full (100%) matching with only the respective target species, whereas with other species the level of mismatching was within the range of 13.63%−76.19% (3−18 nucleotides) (Tables

4.2 − 4.8). Pairwise distance for each target was carefully analyzed through the neighbor-joining technique (Tables 4.9 − 4.15). Cow biomarker showed the lowest distance (0.150) with Buffalo and the highest distance (1.403) with pepper. Similarly, buffalo, boat, sheep, chicken, duck and pork had the minimum distance with cow (0.187), sheep (0.214), buffalo (0.206), quail (0.175), pigeon (0.287) and cat (0.257), respectively. On the other hand, buffalo, goat, sheep, chicken, duck and pork demonstrated maximum distance with onion (2.214), pepper (1.419), wheat (1.647), onion (0.892), onion (1.699) and pepper (1.597), respectively (Tables 4.9 - 4.15). The phylogenetic tree analyses revealed findings supporting the observations from other *in silico* studies (Figure 4.1). Therefore, the bioinformatics study unveiled remarkable genetic distances among the species studied and thus ruled out the chances of any cross-target detection (Taboada et al., 2014).

Table 4.2: The mismatch comparison (intraspecies comparison shaded in grey) of cow ND5-specific primers against same gene of other 30 non-target species.

Species	Forward Primer																								Mis-match	Reversed Primer																						Mis-match
Cow Primers	G	G	T	T	T	C	A	T	T	T	T	A	G	C	A	A	T	A	G	C	A	T	G	G		C	T	C	A	A	C	A	T	A	C	C	C	T	T	G	A	T	T	G	G	A	C	
Cow (*Bos tauras*) (AF492351.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cow (*Bos taurus*) (AY526085.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cow (*Bos taurus*) (AB074965.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cow (*Bos javanicus*) (FJ997262.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cow (*Bos frontalis*) (MF959941.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	1
Cow (*Bos indicus*) (AY126697.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	1
Cow (*Bos indicus*) (GU256940.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	1
Cow (*Bos indicus*) (AF492350.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	1
Deer (*Cervus nippon*) (NC 006993.1)	.	.	.	.	.	T	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	4	.	.	.	C	.	.	.	.	.	.	.	C	.	A	.	.	G	.	.	C	.	5
Buffalo (*Babulus bubalis*) (NC 006295.1)	.	.	A	.	.	T	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	5	.	.	.	T	.	.	.	G	.	.	.	.	C	.	A	.	.	A	.	.	.	.	5
Sheep (*Ovis aries*) (KR868678.1)	.	.	A	.	.	T	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	A	5	.	.	.	.	.	.	.	C	.	.	.	.	C	.	A	.	.	A	.	.	.	.	4
Dog (*Canis lupus*) (NC 002008.4)	.	.	C	.	.	.	.	.	.	A	.	.	A	.	C	.	.	.	.	.	.	.	.	A	5	T	.	T	T	.	.	T	C	.	G	.	.	A	C	.	A	C	.	.	.	.	C	.	11
Cat (*Felis catus*) (NC 001700.1)	.	.	C	.	.	.	.	.	C	A	.	.	.	.	C	.	.	.	.	.	.	.	.	A	5	.	C	T	.	.	.	T	.	.	T	.	.	A	.	.	A	C	.	A	.	.	G	.	10
Donkey (*Equus africanus*) (KT182635.1)	.	.	.	.	.	.	.	.	C	A	.	.	.	.	C	.	.	.	.	.	C	.	.	A	5	.	A	.	C	.	.	.	C	.	C	.	.	A	C	.	C	T	.	A	.	.	C	.	10
Tuna (Thunnus orientalis) (KF906721.1)	.	.	A	C	.	.	.	.	.	C	.	T	.	.	C	.	.	.	.	.	.	.	.	A	6	T	C	T	.	.	C	T	C	.	C	.	.	.	C	.	C	C	.	A	.	.	.	.	11
Goat (Capra hircus) (KP271023.1)	.	.	G	.	.	T	.	.	C	C	.	.	A	.	.	.	.	.	.	.	.	.	.	A	6	.	.	.	.	.	.	.	C	.	.	.	.	T	C	.	.	.	A	.	.	.	.	4
Rat (Rattus rattus) (AC 000022.2)	.	.	.	.	.	.	.	.	C	C	.	.	.	.	T	.	.	.	A	.	C	.	.	A	6	.	A	A	T	C	T	A	G	.	C	.	.	T	C	.	C	.	C	A	.	.	.	.	13
Cod (Gadus morhua) (NC 002081.1)	.	.	.	.	.	A	.	.	C	C	.	.	.	G	T	.	.	G	.	.	.	.	.	A	7	.	A	T	.	.	C	T	C	.	C	.	.	T	C	.	A	C	.	C	.	.	T	T	13
Tilapia (Oreochromis niloticus) (GU238433.1)	.	.	C	C	.	A	.	.	.	.	.	T	.	.	T	.	.	.	.	.	T	.	.	A	7	T	.	T	.	.	C	T	T	A	C	.	.	.	C	.	T	G	.	.	.	.	C	.	11
Rohu (Labeo rohita) (NC 017608.1)	.	.	A	C	.	A	.	.	.	A	.	.	.	.	C	.	.	.	.	.	C	.	.	A	7	.	A	T	G	.	C	.	.	.	C	.	.	.	C	.	A	.	.	A	.	.	.	.	8
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	.	.	C	.	.	T	.	.	.	A	.	.	.	.	C	T	.	.	.	.	C	.	.	A	7	T	A	.	C	.	T	.	C	.	C	.	.	A	C	.	.	.	A	.	.	.	T	10
Horse (*Equus caballus*) (KU575247.1)	.	.	C	.	.	.	.	.	C	A	.	G	.	.	C	.	.	.	.	.	C	.	.	A	7	T	A	.	C	.	.	.	C	.	C	.	.	G	C	.	C	C	.	A	.	.	C	.	11
Pig (Sus scrofa) (KJ782448.1)	.	.	A	.	.	T	G	.	C	C	.	.	T	.	C	.	.	.	.	.	.	.	.	A	8	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	A	G	.	C	.	.	C	T	7
Chicken (Gallus gallus) (AP003580.1)	.	.	C	C	.	.	.	.	C	C	.	.	A	G	C	.	.	.	.	.	.	.	.	A	8	A	C	.	C	.	C	.	C	.	C	.	.	.	C	.	C	C	.	.	.	.	C	.	10
Monkey (Macaca fascicularis) (NC 012670.1)	.	.	.	C	.	T	.	.	C	C	.	G	.	.	C	.	.	.	A	.	.	.	.	A	8	A	A	G	C	T	C	.	C	.	T	.	.	A	.	.	.	G	.	A	A	.	C	.	14
Pigeon (Columba livia) (KJ722068.1)	.	.	.	C	.	A	.	.	C	C	.	.	A	G	T	.	.	.	.	.	C	.	.	A	9	A	C	.	T	.	T	T	C	.	C	.	.	T	C	.	A	C	.	A	.	.	C	.	13
Quail (Coturnix coturnix) (KX712089.1)	.	.	C	.	.	G	.	.	C	C	.	.	A	G	C	C	.	.	.	.	.	.	.	A	9	A	C	.	.	.	C	T	C	.	T	.	.	.	C	.	C	C	.	.	.	.	C	.	10
Salmon (Salmo salar) (KF792729.1)	.	.	A	C	.	T	.	.	C	.	.	.	A	G	C	.	.	.	.	.	.	C	.	.	A	9	.	C	T	C	.	C	A	C	.	C	.	.	.	C	.	C	.	.	A	.	.	C	.	11
Turkey (*Meleagris gallopavo*) (NC 010195.2)	.	.	C	C	.	T	.	.	C	C	.	.	A	G	C	.	.	.	.	.	.	.	.	A	9	A	C	.	T	.	C	T	C	.	T	.	.	.	C	.	T	C	.	A	.	.	T	T	13
Duck (Anas platyrhynchos) (EU009397.1)	.	.	C	C	.	A	.	.	C	C	.	G	A	G	C	.	.	.	.	.	.	.	.	A	10	.	C	.	C	.	T	.	C	.	C	.	.	.	C	.	C	C	.	A	.	.	.	.	9
Ostrich (*Struthio camelus*)(NC 002785.1)	.	.	C	C	.	.	.	.	C	C	.	.	A	G	C	.	.	G	.	.	C	.	.	A	10	.	C	.	.	.	T	T	C	.	.	.	.	C	.	C	C	.	A	.	.	C	.	9
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	.	.	C	C	.	A	G	.	.	G	.	C	.	.	C	C	.	G	.	.	.	.	.	A	10	.	C	.	.	.	T	.	.	.	C	.	.	T	G	C	A	C	.	A	.	.	G	.	10
Turtle (Cuora amboinensis) (NC 014769.1)	.	.	A	.	.	A	.	.	.	C	.	T	A	G	C	.	.	.	T	.	C	.	.	A	10	T	C	.	.	C	C	.	C	.	C	.	.	A	C	.	C	C	.	.	.	.	T	T	12
Pangas (Pangasius pangasius) (NC 023924.1)	.	.	C	C	.	A	.	.	.	C	.	T	A	.	T	.	.	T	.	.	C	.	.	A	10	.	A	T	.	.	C	A	C	.	T	.	.	.	C	.	A	.	.	G	.	.	C	A	11
Frog (Rana kunyuensis) (NC 024548.1)	.	.	C	.	.	.	C	.	A	C	.	.	T	.	C	T	.	T	T	G	C	.	.	A	12	.	.	.	C	C	.	.	T	G	.	.	.	C	.	T	C	.	.	.	.	G	T	9
Onion (Allium cepa) (NC 030100.1)	.	.	A	.	.	A	G	C	.	C	.	T	.	G	G	.	.	T	T	.	G	G	.	T	13	.	A	T	.	.	C	T	C	.	G	A	.	T	.	G	T	.	.	.	T	T	.	.	12
Wheat (*Triticum aestivum*) (GU985444.1)	.	.	A	.	.	A	G	C	.	C	.	T	.	G	G	.	.	T	T	T	T	G	.	T	14	.	A	T	.	.	C	T	C	.	G	A	.	T	.	G	T	.	.	.	T	T	.	.	12
Pepper (Capsicum annuum) (KJ865410.1)	.	.	A	.	.	A	G	C	.	C	C	T	.	G	G	.	.	T	T	T	G	G	.	T	15	.	A	T	.	.	C	T	C	.	T	A	.	T	.	G	T	.	.	.	T	T	.	.	12

Table 4.3: The mismatch comparison (intraspecies comparison shaded in grey) of buffalo ND5-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																							Mis-	Reversed Primer																			Mis-
Buffalo Primers	T	C	G	C	C	T	A	G	C	T	T	C	T	T	A	C	A	C	A	A	A	C	match	A	T	C	C	A	T	C	A	C	A	G	T	C	A	C	A	A	A	C	C	A	match
Buffalo (*Bubalus bubalis*) (NC 006295.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (*Bubalus bubalis*) (NC 006295.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (Bubalus bubalis) (AF547270.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (Bubalus bubalis) (KX758347.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (*Bubalus depressicornis*) (EF536351.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (Bubalus depressicornis) (NC 020615.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (Bubalus depressicornis) (EF536351.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (Bubalus carabanensis) (NC 006295.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (breed Haikou) (Bubalus carabanensis) (AY702618.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Buffalo (*Bubalus carabanensis*) (AY702618.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cow (Bos tauras) (AF492351.1)	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	T	.	.	.	T	4	.	.	.	T	.	C	.	C	T	G	.	.	.	.	.	.	.	.	.	.	.	5
Deer (*Cervus nippon*) (NC 006993.1)	C	.	.	.	.	.	G	.	.	C	C	.	C	.	.	.	.	T	.	.	.	T	7	.	.	.	G	.	.	.	.	T	G	.	.	T	.	.	.	.	.	.	.	.	4
Goat (Capra hircus) (KP271023.1)	C	.	.	.	T	.	.	A	.	C	C	.	C	.	.	.	.	T	.	.	.	.	7	.	G	.	T	.	C	.	.	.	.	A	C	A	.	.	.	.	.	.	.	.	6
Pig (Sus scrofa) (KJ782448.1)	C	.	.	.	.	.	.	C	.	A	A	.	A	.	.	.	C	A	C	.	.	.	8	.	.	.	A	.	.	.	.	T	.	.	.	A	T	.	.	.	.	T	.	.	5
Sheep (*Ovis aries*) (KR868678.1)	C	.	.	.	.	.	G	A	.	C	C	.	C	.	.	T	.	T	.	.	.	.	8	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	G	.	.	.	3
Dog (*Canis lupus*) (NC 002008.4)	C	.	.	.	.	.	C	A	.	A	C	.	.	A	.	A	.	.	.	.	G	T	9	T	.	.	T	.	C	.	C	T	T	A	.	T	T	.	.	.	T	.	.	10
Salmon (Salmo salar) (KF792729.1)	C	.	.	A	T	.	.	.	.	C	C	.	C	A	.	A	C	T	.	.	.	.	10	G	G	T	.	.	C	A	.	.	.	A	C	A	.	G	C	.	.	.	.	.	10
Cat (*Felis catus*) (NC 001700.1)	C	.	.	.	.	.	C	C	.	A	.	.	A	A	.	A	.	T	G	.	G	.	10	G	.	.	A	.	C	T	.	.	T	.	.	A	T	.	T	.	.	T	.	.	9
Donkey (*Equus africanus*) (KT182635.1)	.	.	.	A	.	.	C	C	.	A	C	.	A	C	T	.	G	.	.	.	G	.	10	C	.	.	A	.	.	.	C	T	.	A	.	T	T	.	C	.	C	.	.	.	9
Horse (*Equus caballus*) (KU575247.1)	C	.	.	A	.	.	C	C	.	A	C	.	G	C	T	.	G	.	T	.	.	.	11	C	.	.	G	.	.	.	C	T	.	A	.	T	T	.	C	.	C	.	.	.	9
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	.	.	.	T	A	.	T	C	T	A	C	.	C	.	.	T	C	T	.	.	.	.	11	.	.	.	A	.	C	.	T	.	C	.	.	A	T	.	C	T	C	A	.	.	10
Monkey (Macaca fascicularis) (NC 012670.1)	C	.	.	.	A	C	.	A	T	C	C	.	C	.	.	T	C	A	.	.	.	.	11	T	.	.	.	.	.	A	.	.	T	A	.	A	.	.	C	.	C	.	.	.	7
Cod (Gadus morhua) (NC 002081.1)	C	.	.	.	A	.	A	T	.	C	.	.	C	.	A	C	T	C	.	G	.	11	.	G	.	.	T	C	T	G	.	.	A	C	A	.	G	C	.	.	A	.	.	11
Tuna (Thunnus orientalis) (KF906721.1)	C	.	.	T	T	.	C	A	.	A	C	.	.	A	.	A	C	T	C	.	.	.	12	.	A	T	T	.	C	.	.	.	A	C	A	.	G	C	.	.	.	.	.	9
Tilapia (Oreochromis niloticus) (GU238433.1)	.	.	.	.	.	.	T	.	.	.	C	.	C	A	.	A	C	T	T	.	.	.	12	.	A	T	T	.	C	A	.	.	A	C	.	.	A	C	.	.	T	.	.	10
Rohu (Labeo rohita) (NC 017608.1)	C	.	.	A	.	.	C	C	T	A	C	.	A	A	.	A	C	T	.	.	.	.	12	.	G	.	A	.	A	A	.	T	T	A	C	A	.	A	C	G	.	.	A	C	14
Rat (Rattus rattus) (AC 000022.2)	C	.	.	A	A	.	T	A	T	.	C	.	.	C	.	A	.	A	.	.	C	T	12	.	.	.	T	.	A	A	.	T	.	A	.	A	T	.	C	.	.	.	.	.	8
Pangas (Pangasius pangasius) (NC 023924.1)	C	.	.	A	.	.	C	A	T	C	C	.	A	A	.	A	C	T	C	.	.	.	13	G	.	.	.	T	C	A	.	T	.	T	.	T	G	A	C	.	.	T	G	.	12
Turtle (Cuora amboinensis) (NC 014769.1)	C	.	.	T	T	.	.	T	T	C	C	.	A	A	C	G	.	T	C	.	.	.	13	.	A	G	.	.	C	A	C	T	.	A	.	.	T	.	.	T	C	A	.	.	11
Ostrich (*Struthio camelus*)(NC 002785.1)	.	.	.	A	.	.	C	A	G	C	.	.	C	A	T	A	.	A	.	C	T	A	13	.	G	.	T	.	A	A	.	.	C	T	C	A	.	.	T	C	C	A	.	.	12
Duck (Anas platyrhynchos) (EU009397.1)	C	.	.	A	T	.	C	T	G	C	.	.	C	A	.	A	.	.	C	C	T	G	14	.	A	G	.	.	A	A	G	.	C	.	C	A	.	.	C	C	T	A	.	.	12
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	.	.	A	T	A	C	C	C	T	G	C	.	A	.	C	T	.	T	.	.	T	.	14	.	G	.	.	.	A	A	C	T	G	C	.	A	.	.	T	.	.	.	.	.	9
Turkey (*Meleagris gallopavo*) (NC 010195.2)	.	.	.	G	A	.	C	A	G	C	C	.	.	G	C	A	.	T	C	T	C	.	14	.	A	G	.	.	A	A	.	.	C	T	C	A	.	.	C	.	C	.	.	.	10
Pigeon (Columba livia) (KJ722068.1)	C	.	.	A	.	.	T	A	G	C	.	.	A	A	C	A	G	G	C	C	T	.	15	.	G	.	.	.	A	A	.	.	C	T	C	A	.	T	C	.	.	T	.	.	10
Chicken (Gallus gallus) (AP003580.1)	C	.	.	A	A	.	T	A	G	C	C	.	C	.	C	A	.	T	C	C	T	.	15	.	A	.	.	.	A	A	.	T	C	T	C	A	.	.	C	.	C	.	.	.	10
Quail (Coturnix coturnix) (KX712089.1)	.	.	.	A	A	.	C	A	G	C	C	.	C	A	C	A	.	T	C	T	C	A	16	.	A	.	.	.	A	A	.	.	C	C	.	A	.	.	T	.	C	.	.	.	8
Wheat (*Triticum aestivum*) (GU985444.1)	.	G	A	.	T	.	T	C	T	A	G	T	C	A	G	A	T	.	G	T	T	.	17	G	G	.	.	G	A	G	C	G	.	A	.	A	.	G	T	C	.	T	.	.	13
Pepper (Capsicum annuum) (KJ865410.1)	.	G	A	.	T	.	T	C	T	A	G	T	C	A	G	A	T	.	G	T	T	.	17	G	G	.	.	G	A	G	C	G	.	A	.	A	.	G	T	C	.	T	.	.	13
Frog (Rana kunyuensis) (NC 024548.1)	C	.	.	A	A	C	C	.	T	C	G	T	C	A	C	G	G	T	C	.	C	T	18	C	A	T	T	G	A	G	C	T	.	.	.	A	C	G	T	G	T	.	.	.	15
Onion (Allium cepa) (NC 030100.1)	.	G	A	.	T	.	T	C	T	A	G	T	C	A	G	A	T	T	G	T	T	.	18	G	G	.	.	G	A	G	C	G	.	A	.	A	.	G	T	C	.	T	.	.	13

Table 4.4: The mismatch comparison (intraspecies comparison shaded in grey) of chicken cytb-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																									Mis-	Reversed Primer																					Mis-
Chicken Primers	C	T	T	T	G	C	A	A	T	C	G	C	A	G	G	T	A	T	T	A	C	T	A	T		match	A	C	T	C	T	C	A	T	A	C	T	C	A	C	C	C	C	A	T	T	C	C		match
Chicken (*Gallus gallus*) (NC 001323.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (*Gallus gallus)* (AY235571.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (Gallus gallus) (AY235570.1:1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (White Leghorn) (Gallus gallus) (AP003317.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (Gallus sonneratii) (AP003320.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (Gallus sonneratii) (AP006746.1:1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (Gallus gallus spadiceus) (AP003321.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0
Chicken (Gallus gallus bankiva) (AP003323.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.		0
Turkey (*Meleagris gallopavo*) (NC 010195.2)	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.		5	.	.	A	A	.	.	.	.	.	.	A	.	.	.	.	C	.	.	A	.				5
Duck (Anas platyrhynchos) (EU009397.1)	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	C	C	.		6	.	T	C	.	.	.	.	.	G	.	.	T	.	.	.	.	C	C	.	.	A				7
Pigeon (Columba livia) (KJ722068.1)	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	C	C	.	C	.	.	C	.	.		6	.	T	G	A	.	A	.	.	.	.	.	C	T	.	.	.	C	C	.	A	A				10
Quail (Coturnix coturnix) (KX712089.1)	A	.	.	C	T	T	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.		6	.	.	A	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.				3
Ostrich (*Struthio camelus*)(NC 002785.1)	A	.	.	.	.	T	.	.	.	.	.	T	.	.	C	.	.	C	.	.	C	C	.		7	.	.	G	.	.	A	.	.	.	T	.	.	.	T	.	.	.	C	C	.	A	.				7
Deer (*Cervus nippon*) (NC 006993.1)	A	.	.	.	A	T	C	.	.	.	.	.	.	C	A	C	.	.	G	.	.	.	.		8	T	T	A	.	.	T	C	.	.	G	.	A	C	T	.	T	T	C	C	.	A	A				15
Cod (Gadus morhua) (NC 002081.1)	.	.	.	.	.	T	T	G	.	T	.	.	T	.	C	.	T	.	.	.	.	A	.	.		8	G	.	.	G	.	G	.	.	G	.	.	T	C	T	G	G	G	C	.	.	A	A				13
Tuna (Thunnus orientalis) (KF906721.1)	A	.	.	C	.	T	C	.	.	.	.	.	.	C	C	.	.	G	.	.	A	.	.		8	G	T	G	A	.	.	C	.	G	.	.	A	G	T	G	G	.	.	C	.	T	G				14
Turtle (Cuora amboinensis) (NC 014769.1)	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	C	.	A	G	.	A	G	C		9	.	T	C	.	.	A	.	.	.	.	.	.	.	.	.	T	C	C	.	A	T				8
Cow (*Bos tauras*) (AF492351.1)	A	.	.	.	A	T	C	.	.	.	A	T	.	.	C	A	.	.	.	G	.	C	.	.		10	C	T	C	T	.	A	C	.	.	A	.	T	C	T	A	G	.	T	C	.	A	A				16
Dog (*Canis lupus*) (NC 002008.4)	T	.	.	C	A	T	C	.	.	.	.	.	.	C	.	C	.	A	G	.	A	.	.		10	C	T	.	A	C	T	C	.	.	.	.	.	C	T	A	A	T	C	C	.	A	A				15
Buffalo (*Babulus bubalis*) (NC 006295.1)	A	.	.	C	A	T	T	.	.	.	.	.	.	C	A	C	.	.	G	.	A	.	.		10	C	T	A	.	.	A	T	.	.	A	.	.	C	T	A	G	.	C	C	.	A	A				14
Tilapia (Oreochromis niloticus) (GU238433.1)	.	.	.	C	A	T	C	.	.	T	.	.	.	C	.	G	C	A	.	.	A	.	.		10	G	.	A	A	.	T	C	.	T	T	.	A	.	T	T	G	.	C	C	.	T	A				15
Frog (Rana kunyuensis) (NC 024548.1)	A	.	.	.	A	T	T	.	.	T	A	.	C	.	C	.	G	.	.	.	G	.	C	.		11	G	T	.	A	.	.	C	.	.	.	.	.	G	G	G	G	.	.	C	.	.	G				10
Rat (Rattus rattus) (AC 000022.2)	A	.	.	C	A	T	C	.	.	.	.	.	C	.	C	C	C	.	.	G	.	A	.	.		11	T	T	C	A	.	G	T	.	.	.	.	.	C	T	A	T	T	T	C	.	A	A				15
Salmon (Salmo salar) (KF792729.1)	A	.	.	C	.	T	T	.	.	T	.	.	.	.	C	.	G	C	C	.	.	A	G	.		11	G	T	A	G	C	.	.	.	.	.	.	A	C	T	T	G	G	C	C	.	A	A				15
Sheep (*Ovis aries)* (KR868678.1)	A	.	.	C	A	T	C	.	.	.	.	.	.	C	C	C	.	C	G	.	C	.	.		11	.	T	C	.	.	A	C	.	.	A	.	.	C	T	.	A	T	C	C	.	.	A				12
Donkey (*Equus africanus)* (KT182635.1)	.	.	.	.	A	T	C	.	.	.	A	.	G	.	C	C	C	.	G	G	T	A	.	.		12	C	T	.	.	.	.	.	C	.	.	G	.	.	C	T	A	.	T	C	C	.	A	.				11
Goat (Capra hircus) (KP271023.1)	A	.	.	C	A	T	C	.	.	.	A	.	.	.	C	C	C	.	C	G	.	C	.	.		12	.	T	G	.	.	A	C	.	.	A	.	T	C	T	T	G	T	T	C	.	A	A				15
Pepper (Capsicum annuum) (KJ865410.1)	.	.	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	C	.	G	.	C	.	12	G	T	A	G	C	T	T	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.				15
Rohu (Labeo rohita) (NC 017608.1)	A	.	.	.	A	T	C	.	.	T	.	.	C	.	C	C	G	C	A	.	.	C	C	.		13	G	T	A	A	.	T	.	.	.	.	.	A	C	T	A	G	.	C	C	.	.	A				13
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	A	.	.	.	A	T	C	.	.	T	.	.	.	A	C	.	T	.	A	G	T	C	T	.		13	C	T	.	G	.	A	C	C	.	T	.	.	T	.	.	C	.	C	.	T				13
Horse (*Equus caballus*) (KU575247.1)	.	.	.	C	A	T	C	.	.	.	A	.	.	.	C	C	C	.	G	G	T	A	G	.		13	C	T	C	.	.	.	.	C	.	G	A	.	.	T	T	G	.	T	C	C	.	A	.				13
Onion (Allium cepa) (NC 030100.1)	.	C	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	.	G	.	C	.		13	G	T	A	G	C	T	C	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.				15
Wheat (*Triticum aestivum*) (GU985444.1)	.	C	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	.	G	.	C	.		13	G	T	A	G	C	T	C	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.				15
Pig (Sus scrofa) (KJ782448.1)	.	.	.	C	.	T	C	.	.	T	A	.	C	.	C	C	C	.	C	G	.	A	G	C		14	T	T	A	T	.	T	.	.	.	A	.	A	C	T	A	A	T	C	C	.	A	.				15
Pangas (Pangasius pangasius) (NC 023924.1)	A	.	.	C	.	T	.	G	.	T	A	T	C	.	C	A	G	C	.	.	.	A	C	.		14	.	T	A	A	.	.	C	.	C	.	.	T	.	.	A	G	.	C	C	.	.	G				11
Monkey (Macaca fascicularis) (NC 012670.1)	.	.	.	C	A	T	C	.	.	.	A	T	C	.	C	C	C	.	C	G	.	A	G	.		14	.	T	C	.	.	.	C	.	T	.	.	.	T	T	.	A	T	C	C	.	A	G				12
Cat (*Felis catus*) (NC 001700.1)	A	.	.	C	A	T	T	.	.	.	T	.	.	.	C	C	T	.	A	G	.	A	G	G		14	C	T	A	G	.	A	C	.	.	G	.	T	T	T	A	A	.	.	C	.	.	A				14
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	A	.	.	.	A	T	C	C	.	.	A	T	.	.	C	C	.	C	C	.	T	C	C	.		14	G	.	C	.	.	G	G	C	C	A	C	.	G	T	.	.	T	.	.	.	A	A				13

122

Table 4.5: The mismatch comparison (intraspecies comparison shaded in grey) of duck ND5-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																								Mis-	Reversed Primer																		Mis-
Duck Primers	C	A	C	C	G	T	A	A	A	A	G	C	T	G	C	A	T	T	T	C	T	A	A	match	C	C	A	T	T	C	T	A	C	A	A	T	T	C	G	C	C	A	T	G	T	match
Duck (Anas platyrhynchos) (EU009397.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Duck (Anas platyrhynchos) (KJ637997.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Duck (breed mallard) (Anas platyrhynchos) (EU755253.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Duck (breed Beijing duck) (Anas platyrhynchos) (EU755252.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Duck (*Anas acuta*) (KF312717.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Duck (*Anas poecilorhyncha*) (KC466567.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Duck (Anas formosa) (JF730435.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Pigeon (Columba livia) (KJ722068.1)	.	.	.	T	.	.	C	.	.	.	A	T	C	.	.	.	.	.	.	.	.	.	.	5	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	.	C	A	.	5
Turkey (*Meleagris gallopavo*) (NC 010195.2)	A	.	.	.	.	.	T	.	.	.	A	.	.	.	C	.	.	C	.	.	.	C	6	.	A	.	.	C	.	.	.	G	.	G	.	.	.	.	.	.	C	A	.	6
Quail (Coturnix coturnix) (KX712089.1)	A	.	.	.	.	.	T	.	.	.	A	.	C	.	.	C	.	.	C	A	.	.	.	7	.	A	.	.	C	.	.	.	G	.	.	.	T	.	.	A	.	C	A	.	7
Chicken (Gallus gallus) (AP003580.1)	A	.	.	.	A	.	T	.	.	.	A	.	C	.	.	C	.	.	C	.	.	.	.	7	.	.	.	.	C	T	.	.	G	.	G	.	.	.	.	A	.	C	A	.	7
Pangas (Pangasius pangasius) (NC 023924.1)	.	G	.	T	.	.	.	.	G	C	A	.	.	.	.	.	.	.	.	T	.	T	.	7	.	A	.	.	.	.	.	G	G	.	.	.	T	.	.	A	T	C	A	.	8
Turtle (Cuora amboinensis) (NC 014769.1)	A	.	.	T	.	.	.	.	.	.	A	.	A	.	.	.	.	.	C	T	.	C	.	7	.	.	.	.	C	.	.	.	G	.	.	.	T	A	.	.	T	A	T	.	7
Monkey (Macaca fascicularis) (NC 012670.1)	A	.	.	.	.	.	.	T	.	T	G	.	.	.	T	.	.	.	.	A	.	T	.	7	.	.	.	.	.	A	.	.	G	.	.	.	.	T	.	G	C	.	A	.	6
Cod (Gadus morhua) (NC 002081.1)	A	G	.	A	.	.	.	.	.	G	T	.	.	.	.	T	.	.	C	T	.	.	.	8	.	T	.	.	.	T	.	.	G	.	.	.	.	T	.	.	T	.	C	A	.	7
Rohu (Labeo rohita) (NC 017608.1)	.	G	.	.	.	.	C	.	G	C	T	.	C	.	.	.	.	.	C	T	.	T	.	9	.	A	.	.	C	T	.	.	G	.	.	.	.	T	.	.	A	C	.	A	.	8
Frog (Rana kunyuensis) (NC 024548.1)	A	G	.	T	.	.	.	.	.	.	A	.	C	T	.	.	.	.	.	T	.	C	C	9	G	T	.	.	C	T	.	.	G	.	.	.	.	T	.	.	T	C	C	.	9
Tuna (Thunnus orientalis) (KF906721.1)	.	G	.	A	.	.	T	.	.	.	C	T	A	.	.	.	.	.	.	T	.	T	G	9	.	A	.	.	C	.	.	C	G	.	.	.	.	.	.	A	T	C	A	.	8
Ostrich (*Struthio camelus*)(NC 002785.1)	.	.	.	T	.	.	C	.	.	.	A	T	A	.	.	C	.	.	C	T	.	T	.	9	.	T	.	.	C	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	4
Tilapia (Oreochromis niloticus) (GU238433.1)	A	G	.	A	.	.	.	.	.	.	T	G	A	.	.	C	.	.	C	T	.	T	.	10	.	.	.	.	C	.	.	.	G	.	G	.	.	T	.	.	.	T	C	C	.	7
Goat (Capra hircus) (KP271023.1)	G	G	T	.	.	.	T	T	C	.	T	G	.	.	.	C	.	.	.	A	.	C	.	11	.	.	.	.	.	A	.	.	G	.	.	.	.	T	.	A	.	.	.	.	4
Donkey (*Equus africanus*) (KT182635.1)	.	.	T	.	A	.	T	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	11	.	T	.	.	C	A	.	.	G	.	.	.	.	T	.	.	C	.	A	.	7
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	G	G	.	A	.	C	T	.	.	.	C	T	G	.	.	C	.	.	C	.	.	T	.	11	.	T	.	.	C	A	.	.	G	.	G	.	.	T	A	.	.	G	.	A	C	10
Horse (*Equus caballus*) (KU575247.1)	.	.	.	T	A	.	C	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	11	.	T	.	.	.	A	.	G	G	.	.	.	.	T	.	.	C	.	A	.	7
Pig (Sus scrofa) (KJ782448.1)	.	.	.	.	.	.	.	T	C	C	T	A	C	.	.	C	.	.	C	A	A	C	T	C	12	.	A	.	.	.	A	.	.	G	.	.	.	.	T	.	.	.	.	A	.	5
Deer (*Cervus nippon*) (NC 006993.1)	A	.	.	T	A	.	C	T	C	.	T	A	.	.	.	T	.	.	C	A	.	C	.	12	.	.	.	.	.	A	.	.	G	.	.	.	.	T	.	A	.	.	.	.	4
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	T	T	.	A	.	.	.	T	C	T	T	A	.	.	.	.	.	.	C	A	A	C	T	.	12	.	T	.	.	C	A	.	.	G	.	.	.	.	T	T	.	.	.	A	.	7
Salmon (Salmo salar) (KF792729.1)	T	G	.	T	A	.	C	.	.	.	A	T	A	.	.	T	.	.	C	T	.	.	G	12	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	T	C	C	.	6
Sheep (*Ovis aries*) (KR868678.1)	A	.	.	.	A	.	T	T	C	.	T	G	C	.	.	T	.	.	C	A	.	C	.	12	.	T	.	.	.	A	.	.	G	.	.	.	.	T	.	A	.	.	A	.	6
Rat (Rattus rattus) (AC 000022.2)	A	T	.	A	A	.	T	.	.	.	T	T	C	T	.	.	.	.	C	.	.	C	C	12	.	A	.	.	.	A	.	.	.	.	.	.	.	T	.	T	T	C	A	.	7
Cow (*Bos tauras*) (AF492351.1)	A	G	.	T	A	.	C	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	13	.	T	.	.	.	A	.	.	G	.	.	.	.	T	.	A	.	.	A	.	6
Buffalo (*Babulus bubalis*) (NC 006295.1)	A	G	.	.	A	.	T	T	C	.	T	G	C	.	.	C	.	.	C	A	.	T	.	13	.	T	.	.	.	A	.	.	G	.	.	.	.	T	.	A	.	.	A	.	6
Cat (*Felis catus*) (NC 001700.1)	.	.	.	A	A	.	C	T	C	T	T	A	C	.	.	C	.	.	C	A	.	C	.	13	.	.	.	.	C	A	.	.	G	.	.	.	.	T	.	A	.	.	.	.	5
Dog (*Canis lupus*) (NC 002008.4)	.	G	.	T	A	C	T	T	C	T	T	A	C	.	.	G	.	.	C	A	.	.	.	14	.	T	.	.	C	A	.	.	G	.	.	.	.	T	.	T	.	.	A	.	7
Wheat (*Triticum aestivum*) (GU985444.1)	G	T	G	.	.	.	T	T	C	.	T	T	.	T	.	T	.	C	G	A	.	C	T	15	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	.	.	T	.	12
Onion (Allium cepa) (NC 030100.1)	G	T	G	.	.	.	T	T	C	.	T	T	.	T	.	T	.	C	A	A	.	C	T	15	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	A	.	.	T	.	13
Pepper (Capsicum annuum) (KJ865410.1)	G	T	G	.	.	.	T	T	C	.	T	T	C	T	.	T	.	C	G	A	.	C	T	16	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	.	.	T	.	12

Table 4.6: The mismatch comparison (intraspecies comparison shaded in grey) of goat ND5-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																										Mis-match	Reversed Primer																						Mis-match
Goat Primers	T	G	T	T	T	T	C	T	T	C	T	C	T	T	G	C	A	C	T	A	A	C	C	A	C	C	A	G	C	T	T	C	A	G	C	T	T	C	A	A	G
Goat (*Capra hircus*) (KP271023.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Goat (*Capra hircus*) (KM360063.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Goat (*Capra hircus*) (KM670319.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Goat (*Capra hircus*) (KP662713.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Goat (breed Boer goat) (*Capra hircus*) (KM233163.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Goat (Capra aegagrus) (KT290893.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Goat (Capra ibex) (FJ207526.1)	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
Goat (*Capra pyrenaica*) (FJ207528.1)	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	2
Goat (*Capra nubiana*) (FJ207527.1)	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1
Buffalo (*Babulus bubalis*) (NC 006295.1)	.	A	.	.	C	C	.	.	.	.	A	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	6	T	.	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	A	5
Sheep (*Ovis aries*) (KR868678.1)	.	A	.	.	.	.	.	C	.	.	C	.	.	C	A	.	.	.	.	.	G	T	.	.	.	.	7	.	.	.	C	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	A	4
Pig (Sus scrofa) (KJ782448.1)	C	A	.	.	C	G	.	C	.	.	A	.	.	C	A	.	.	T	.	.	.	.	.	.	.	.	9	.	G	.	A	G	.	.	C	.	C	.	.	T	.	.	T	.	.	.	.	T	.	.	A	9
Cow (*Bos tauras*) (AF492351.1)	.	A	.	.	C	.	.	C	.	.	A	.	.	C	T	.	.	.	.	.	G	T	T	.	.	.	9	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	A	4
Cat (*Felis catus*) (NC 001700.1)	.	A	.	.	.	A	.	C	C	.	A	.	.	C	A	T	.	.	.	.	.	.	T	G	.		10	T	.	.	C	.	.	G	C	.	.	.	A	.	.	A	.	.	.	.	T	.	.	A	8
Horse (*Equus caballus*) (KU575247.1)	.	.	.	.	C	.	.	C	.	.	C	.	.	C	A	T	.	.	.	.	G	T	T	T	.		10	.	.	.	C	.	.	.	C	.	.	.	C	.	.	A	.	.	.	.	.	.	.	A	5
Duck (Anas platyrhynchos) (EU009397.1)	.	C	A	.	.	A	A	C	.	.	C	.	.	C	A	.	.	.	.	C	C	T	.	.	.		11	.	T	.	C	.	.	.	A	.	C	C	.	C	.	.	A	.	.	C	.	A	.	.	A	10
Dog (*Canis lupus*) (NC 002008.4)	.	A	.	.	.	.	.	.	.	.	A	T	G	C	A	T	.	A	.	C	.	.	A	G	.		11	T	A	.	A	.	.	.	C	.	.	.	.	C	A	.	G	.	.	T	.	.	.	.	A	9
Deer (*Cervus nippon*) (NC 006993.1)	.	A	.	.	C	.	.	C	.	.	C	T	.	C	A	.	G	.	.	.	G	T	T	.	.		11	T	A	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	5
Donkey (*Equus africanus*) (KT182635.1)	.	A	.	.	C	.	.	C	.	.	C	.	.	C	A	T	G	.	.	.	G	.	T	T	.		11	.	.	.	C	.	.	.	C	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	A	5
Pepper (Capsicum annuum) (KJ865410.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	.	G	T		11	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	.	.	T	T	.	14
Wheat (*Triticum aestivum*) (GU985444.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	T	G	T		12	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	.	.	T	T	T	15
Onion (Allium cepa) (NC 030100.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	T	G	T		12	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	.	C	T	G	T	.	16
Frog (Rana kunyuensis) (NC 024548.1)	.	.	.	.	A	.	A	.	C	.	C	.	.	C	C	.	C	.	.	T	T	G	.	C	T		12	.	A	.	A	T	.	T	C	.	C	C	A	T	T	.	T	G	.	T	C	.	.	C	.	A	16
Monkey (Macaca fascicularis) (NC 012670.1)	.	.	C	A	C	A	.	C	C	.	C	A	.	C	A	T	.	A	.	.	.	.	A	.	.		13	A	T	.	A	G	.	C	C	.	.	A	.	A	.	.	A	.	.	.	.	T	.	.	A	11
Quail (Coturnix coturnix) (KX712089.1)	.	C	C	.	A	A	A	.	A	.	C	.	.	C	A	.	.	.	C	.	T	T	A	.	.		13	.	T	.	C	.	.	.	A	.	C	C	.	A	.	.	A	.	C	.	C	.	.	.	A	10
Chicken (Gallus gallus) (AP003580.1)	.	C	C	.	.	A	A	C	A	.	C	.	.	C	A	.	.	A	C	.	T	T	A	.	.		14	.	T	.	C	.	.	G	A	.	C	C	.	C	A	.	.	T	C	.	C	.	A	.	.	A	13
Cod (Gadus morhua) (NC 002081.1)	.	A	.	A	.	A	.	.	.	.	A	A	G	C	C	T	T	T	.	.	T	T	A	.	T		15	.	.	.	A	G	.	T	A	.	T	A	.	C	T	.	A	.	.	.	.	T	.	.	.	10
Pangas (Pangasius pangasius) (NC 023924.1)	.	T	A	.	G	A	.	.	A	.	.	A	C	C	C	T	G	.	.	C	C	T	A	.	.		15	.	T	.	C	G	.	C	A	.	C	A	A	T	A	.	.	.	.	C	.	.	.	.	A	12
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	.	A	.	.	C	.	.	C	A	.	C	T	C	.	.	T	.	G	C	T	G	T	A	T	.		15	.	T	.	A	.	.	.	.	.	.	A	.	A	A	C	.	.	.	.	.	T	.	.	A	8
Turkey (*Meleagris gallopavo*) (NC 010195.2)	.	A	C	.	A	A	A	C	A	.	C	T	.	C	A	.	T	.	.	.	C	T	A	.	.		15	.	T	.	C	.	.	.	A	.	T	C	.	A	A	.	.	T	C	T	C	.	A	.	.	.	12
Tuna (Thunnus orientalis) (KF906721.1)	.	A	A	.	A	A	.	A	A	.	A	A	G	C	C	T	.	A	.	C	.	T	.	.	T		16	.	T	.	.	G	.	T	A	.	C	A	A	T	A	.	T	.	.	.	C	.	G	.	.	A	13
Tilapia (Oreochromis niloticus) (GU238433.1)	.	A	A	.	A	G	.	.	A	.	C	A	G	C	C	T	.	A	.	C	.	T	.	.	T		16	.	T	.	.	G	.	T	G	.	.	A	A	T	A	.	.	.	.	.	C	.	.	.	.	A	10
Rat (Rattus rattus) (AC 000022.2)	.	A	A	.	A	A	.	A	A	T	C	.	.	A	A	T	C	.	.	C	.	T	A	.	T		16	T	A	.	.	.	.	.	C	.	T	A	.	T	A	.	A	.	.	T	.	.	.	.	A	10
Ostrich (*Struthio camelus*)(NC 002785.1)	.	T	.	.	C	A	A	C	A	.	C	T	C	C	C	T	T	T	.	.	C	T	.	.	.		16	T	.	.	C	.	.	.	A	.	C	C	.	A	.	.	.	.	.	.	.	.	.	.	A	6
Pigeon (Columba livia) (KJ722068.1)	.	C	C	.	.	A	A	C	A	.	C	T	C	C	A	T	C	.	.	C	C	T	A	.	.		17	.	T	.	C	.	.	.	A	.	C	C	.	A	A	.	T	.	.	.	C	.	T	.	.	A	11
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	C	A	.	.	A	G	.	C	.	T	A	A	.	.	A	T	T	A	.	.	C	T	G	T	T		17	A	A	C	C	.	C	.	A	.	T	C	G	C	G	.	T	.	.	.	.	.	.	C	A	14
Salmon (Salmo salar) (KF792729.1)	.	C	C	.	A	A	G	C	.	.	A	A	C	C	C	T	T	T	.	.	.	T	A	.	T		17	.	T	.	C	G	.	C	A	.	T	A	A	C	.	.	.	.	.	.	T	.	.	A	11
Rohu (Labeo rohita) (NC 017608.1)	.	-	A	C	A	C	T	C	A	T	C	A	C	.	T	-	-	.	.	C	C	T	A	.	T		17	A	T	.	.	G	.	T	A	.	C	A	A	T	A	.	T	.	.	.	.	.	.	.	A	12
Turtle (Cuora amboinensis) (NC 014769.1)	C	A	A	.	A	A	A	C	.	.	C	A	C	C	C	T	T	.	.	C	T	T	A	.	.		18	A	T	.	.	.	.	C	A	.	T	A	A	C	A	.	A	.	.	.	.	T	.	.	A	12

Table 4.7: The mismatch comparison (intraspecies comparison shaded in grey) of sheep ND5-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																							Mis-	Reversed Primer																							Mis-
Sheep Primers	T	T	C	C	T	C	C	C	T	C	A	C	A	C	T	A	G	T	C	A	C	C		match	G	A	C	T	T	C	T	T	C	T	C	A	A	T	A	A	T	A	A	T	T	C	G	T	T	C	C	A	G	match
Sheep (*Ovis aries*) (AY858379.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (Ovis aries) (KP702285.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (Ovis aries) (KJ954145.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis aries*) (KR868678.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis aries* isolate kk1) (HM236176.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis aries* isolate GT1) (KU681175.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis aries* breed Altay) (KF938320.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis aries* breed Duolang) (KF938332.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (Ovis canadensis) (JN181255.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Sheep (*Ovis orientalis*) (KF938360.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (*Ovis ammon*) (KT781689.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.		1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Deer (*Cervus nippon*) (NC 006993.1)	C	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	T	.	.	.		4	.	.	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	5
Cow (*Bos tauras*) (AF492351.1)	C	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	T	.	.	T		5	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	C	.	.	.	.	5
Horse (*Equus caballus*) (KU575247.1)	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	T	.	A		5	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	4
Goat (Capra hircus) (KP271023.1)	.	.	.	T	.	.	T	.	.	T	G	.	.	.	.	.	A	C	.	.	.	.		6	.	.	.	.	A	T	.	.	T	.	.	.	G	.	.	.	.	.	.	T	.	.	C	.	.	.	.	6
Pig (Sus scrofa) (KJ782448.1)	C	G	.	.	.	.	A	.	.	.	.	.	.	T	.	.	A	C	.	.	.	A		7	.	.	.	.	A	T	.	.	.	.	.	.	G	.	.	.	.	.	.	T	A	.	.	.	.	T	.	6
Donkey (*Equus africanus*) (KT182635.1)	C	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	C	T	T	.	A		7	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	T	.	.	A	.	.	.	.	5
Buffalo (*Babulus bubalis*) (NC 006295.1)	C	C	.	T	.	.	A	.	.	T	.	T	.	.	.	.	A	C	.	.	.	.		8	.	.	T	.	A	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	5
Monkey (Macaca fascicularis) (NC 012670.1)	C	A	.	.	C	.	.	A	.	.	.	T	.	A	.	.	A	C	A	.	.	.		9	.	.	T	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	A	.	.	.	.	.	A	6
Cat (*Felis catus*) (NC 001700.1)	.	A	.	.	C	.	A	.	.	.	.	T	.	.	.	.	A	C	T	G	.	A		9	.	.	T	.	A	T	.	.	.	.	.	.	.	C	C	.	.	C	.	.	T	A	.	C	.	.	T	.	10
Salmon (Salmo salar) (KF792729.1)	C	.	.	A	A	.	.	.	.	T	T	T	.	A	.	.	A	.	.	T	T	.		10	.	.	.	C	A	.	.	A	.	.	.	C	.	.	C	.	.	T	.	.	T	A	C	C	.	.	.	A	11
Rohu (Labeo rohita) (NC 017608.1)	C	.	.	A	.	.	A	.	.	T	C	T	C	.	.	.	A	.	.	T	T	.		10	.	.	.	C	A	.	.	A	.	.	.	T	C	.	.	.	.	C	.	.	.	.	C	.	.	.	.	A	8
Turkey (*Meleagris gallopavo*) (NC 010195.2)	C	A	.	.	.	T	.	A	C	T	C	T	.	.	.	.	A	C	.	.	T	A		10	.	.	.	C	.	A	.	A	.	.	.	C	.	.	.	.	.	.	.	.	C	.	C	.	.	C	A	8
Dog (*Canis lupus*) (NC 002008.4)	.	.	.	T	.	.	A	T	G	.	.	T	.	A	.	C	A	C	A	G	.	.		11	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	A	.	.	T	.	5
Rat (Rattus rattus) (AC 000022.2)	A	A	.	A	A	T	.	.	.	A	.	T	C	.	.	C	A	.	A	.	T	T		11	.	.	.	.	A	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	C	.	A	T	.	.	.	.	7
Pigeon (Columba livia) (KJ722068.1)	C	A	.	.	.	.	.	A	.	.	C	T	C	.	.	.	A	C	A	T	T	T		12	.	.	T	C	A	A	.	A	.	.	.	C	C	.	.	.	.	.	.	T	C	.	C	.	.	C	.	11
Cod (Gadus morhua) (NC 002081.1)	.	.	.	A	A	G	.	.	.	T	T	T	.	T	.	.	A	.	.	A	T	T	T	12	.	.	.	C	A	.	.	A	T	.	.	T	.	.	T	.	.	T	.	.	T	A	.	.	.	.	T	A	11
Pangas (Pangasius pangasius) (NC 023924.1)	.	A	.	T	A	.	.	.	.	G	C	T	C	.	.	.	A	C	.	C	T	A		12	.	.	.	C	A	.	.	A	.	.	.	.	C	.	.	.	.	C	.	.	T	A	C	C	.	.	.	A	10
Ostrich (*Struthio camelus*)(NC 002785.1)	C	A	.	.	.	.	.	.	.	T	T	T	.	.	.	C	A	C	A	C	T	A		12	.	.	.	C	A	A	.	A	T	.	.	.	.	.	C	.	.	.	.	.	C	.	C	.	.	T	.	9
Wheat (*Triticum aestivum*) (GU985444.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	T	G	T	.		13	.	.	T	A	G	.	C	.	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	17
Onion (Allium cepa) (NC 030100.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	T	G	T	.		13	.	.	T	A	G	.	C	C	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	18
Pepper (Capsicum annuum) (KJ865410.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	.	G	T	A		13	.	.	T	A	G	.	C	C	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	18
Tuna (Thunnus orientalis) (KF906721.1)	A	A	.	A	A	G	.	.	.	A	.	T	C	A	.	C	A	.	.	T	T	.		13	.	.	.	C	A	.	.	A	T	.	.	.	.	C	.	.	T	.	.	.	A	C	C	.	.	.	A	10
Turtle (Cuora amboinensis) (NC 014769.1)	C	.	.	.	A	.	.	.	.	T	C	T	C	T	.	.	A	C	A	C	T	A		13	.	.	T	C	A	G	.	A	.	.	.	C	.	.	T	.	.	.	.	.	T	A	.	A	.	.	C	A	12
Tilapia (Oreochromis niloticus) (GU238433.1)	.	A	.	.	A	G	.	.	.	A	.	T	C	A	.	C	A	.	T	T	T	T		13	.	.	T	A	.	T	.	A	.	.	.	.	.	.	T	.	.	C	.	.	.	A	C	C	.	.	C	.	10
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	C	.	.	.	A	.	.	T	C	T	G	T	.	G	C	T	.	.	A	T	.	A		13	.	.	T	.	A	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.	A	C	C	.	.	.	A	8
Quail (Coturnix coturnix) (KX712089.1)	.	A	.	.	C	T	.	A	C	A	C	.	.	T	.	.	A	C	A	C	T	T		14	.	.	.	.	.	A	.	A	T	.	.	C	.	.	.	.	.	.	T	T	.	C	.	.	.	.	A	8
Chicken (Gallus gallus) (AP003580.1)	C	A	.	.	C	T	.	A	C	A	.	.	.	T	.	.	A	C	A	C	T	A		14	.	.	.	A	.	A	.	A	.	.	.	C	.	.	.	.	.	.	.	T	.	C	.	.	C	A	8
Frog (Rana kunyuensis) (NC 024548.1)	.	C	.	.	C	T	.	.	C	.	C	T	T	T	G	C	C	.	T	G	.	G		14	.	.	.	C	.	A	.	A	.	A	.	T	G	.	T	T	.	G	.	.	T	C	.	C	.	.	C	A	14
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	C	.	T	A	A	T	T	A	.	T	.	T	.	.	.	G	T	.	.	C	T	G		14	.	.	.	A	.	T	.	A	.	.	.	C	.	C	C	T	.	C	.	.	.	T	.	A	.	.	C	A	12
Duck (Anas platyrhynchos) (EU009397.1)	C	.	.	.	C	T	.	A	C	A	C	T	C	.	.	C	A	C	A	C	T	A		16	.	.	.	C	A	A	.	A	T	.	.	.	.	.	.	C	.	.	.	.	C	.	.	C	.	.	C	A	10

Table 4.8: The mismatch comparison (intraspecies comparison shaded in grey) of pig ND5-specific primers against the same gene of other 30 non-target species.

Species	Forward Primer																						Mis-	Reversed Primer																						Mis-
Pig Primers	G	A	T	T	C	C	T	A	A	C	C	C	A	C	T	C	A	A	A	C	G	match	C	A	A	T	G	A	A	T	G	C	C	C	A	A	A	C	A	T	A	C	C	match
Pig (Sus scrofa) (KJ782448.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa domestica) (AP003428.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa isolate JNP-9) (AY334492.2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa breed Hampshire) (AY574046.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa breed Duroc) (AY337045.1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa breed Berkshire) (AY574045.1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa isolate KWB1) (AY574047.1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa isolate KS17) (DQ207753.1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus scrofa breed Large White) (AY574048.1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (Sus celebensis) (NC 024860.1)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Pig (Sus cebifrons) (KF952600.1)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Cat (*Felis catus*) (NC 001700.1)	.	.	.	.	T	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	3	.	.	.	C	.	.	G	A	A	.	.	T	.	.	.	T	.	.	T	.	.	7
Horse (*Equus caballus*) (KU575247.1)	.	.	.	.	.	.	.	.	T	T	.	A	.	.	A	.	C	.	.	.	A	6	.	C	C	C	A	.	C	C	T	T	A	.	C	.	.	.	C	.	C	.	.	12
Donkey (*Equus africanus*) (KT182635.1)	.	.	.	.	.	.	.	.	T	T	.	A	.	.	G	.	C	.	.	.	A	6	.	C	C	.	A	.	C	C	T	.	A	.	C	.	.	.	C	.	C	.	.	10
Deer (*Cervus nippon*) (NC 006993.1)	.	.	.	.	.	.	.	C	.	.	A	A	.	T	C	T	T	.	.	.	.	7	.	C	C	A	A	.	C	A	A	.	T	.	C	.	.	.	.	.	.	.	.	9
Dog (*Canis lupus*) (NC 002008.4)	.	.	.	.	T	.	.	.	C	T	A	A	.	.	.	T	.	.	.	.	A	7	A	.	-	C	.	.	T	A	A	T	T	T	T	.	.	T	C	.	G	.	.	12
Goat (Capra hircus) (KP271023.1)	.	.	.	.	T	.	.	.	.	T	.	A	.	.	C	T	T	.	.	.	A	7	G	C	C	A	.	.	.	A	A	.	T	.	.	.	.	.	C	.	.	.	.	8
Buffalo (*Babulus bubalis*) (NC 006295.1)	.	.	.	.	.	.	.	.	G	.	A	A	.	T	C	T	T	.	.	.	A	8	.	C	C	A	.	.	C	A	A	.	T	.	T	.	.	.	G	.	.	.	.	9
Sheep (*Ovis aries*) (KR868678.1)	.	.	.	.	T	.	.	.	.	T	T	A	.	.	C	T	C	.	.	.	A	8	T	C	C	A	A	.	C	G	A	.	T	.	.	.	.	.	C	.	.	.	.	10
Pigeon (Columba livia) (KJ722068.1)	.	.	C	.	T	G	C	T	.	T	.	.	.	.	A	.	.	.	.	.	A	8	.	.	.	.	-	.	.	A	C	A	.	.	T	.	T	T	C	.	C	.	.	8
Salmon (Salmo salar) (KF792729.1)	.	.	.	.	.	G	C	.	.	.	A	A	.	.	C	T	G	.	.	.	T	8	A	G	.	A	C	T	C	G	A	.	.	T	C	.	C	A	C	.	C	.	.	14
Tuna (Thunnus orientalis) (KF906721.1)	.	.	A	.	A	G	C	.	.	.	T	A	.	.	C	T	.	.	.	.	T	9	A	.	.	C	T	T	C	G	A	T	.	T	.	.	C	T	C	.	C	.	.	13
Quail (Coturnix coturnix) (KX712089.1)	.	.	C	.	A	G	C	C	-	T	.	T	.	.	.	T	.	.	.	.	A	9	.	.	.	.	.	.	.	A	C	A	.	.	.	.	C	T	C	.	T	.	.	7
Cow (*Bos tauras*) (AF492351.1)	.	G	.	.	.	.	.	.	.	.	A	A	.	T	C	T	C	.	.	T	A	9	.	C	C	A	A	G	C	G	A	.	T	.	.	.	.	.	.	.	.	.	.	9
Monkey (Macaca fascicularis) (NC 012670.1)	.	.	.	.	T	.	.	C	C	T	A	.	.	T	.	A	C	.	.	.	T	9	.	T	C	C	A	.	C	.	C	A	A	G	C	T	C	.	C	.	T	.	.	14
Duck (Anas platyrhynchos) (EU009397.1)	.	.	C	.	A	G	C	.	-	T	.	A	.	.	.	T	C	.	.	.	A	9	.	.	.	.	.	.	.	A	C	.	.	.	C	.	T	.	C	.	C	.	.	6
Pangas (Pangasius pangasius) (NC 023924.1)	.	.	A	.	T	G	C	.	.	.	A	A	.	.	C	T	C	.	.	.	T	10	A	G	.	A	T	T	C	G	A	.	A	T	.	.	C	A	C	.	T	.	.	14
Tilapia (Oreochromis niloticus) (GU238433.1)	.	.	A	.	.	G	C	.	.	.	T	T	C	.	C	T	C	.	.	.	T	10	A	G	.	.	T	T	T	G	A	T	T	T	.	.	C	T	T	A	C	.	.	15
Frog (Rana kunyuensis) (NC 024548.1)	.	.	.	.	T	T	.	T	.	A	A	A	.	T	C	.	C	.	.	-	A	10	A	.	.	.	.	.	.	A	C	T	G	G	C	C	.	.	T	G	.	.	.	10
Turtle (Cuora amboinensis) (NC 014769.1)	.	.	C	.	G	G	C	.	.	T	A	A	.	.	A	.	.	.	.	T	A	10	.	.	.	.	.	C	C	A	.	T	T	.	.	.	C	C	.	C	.	C	.	.	9
Rat (Rattus rattus) (AC 000022.2)	.	.	.	.	.	T	G	C	C	T	A	A	.	.	A	T	.	.	.	.	T	10	.	.	C	C	A	.	C	A	A	.	A	A	T	C	T	A	G	.	C	.	.	14
Chicken (Gallus gallus) (AP003580.1)	.	.	C	.	A	G	C	C	-	T	.	T	T	.	C	T	.	.	.	.	A	11	.	.	.	.	.	.	.	A	C	A	.	.	C	.	C	.	C	.	C	.	.	7
Rohu (Labeo rohita) (NC 017608.1)	.	.	C	.	T	G	C	.	.	T	A	A	.	.	C	T	C	.	.	.	T	11	A	.	.	C	T	T	T	G	A	.	A	T	G	.	C	.	.	.	C	.	.	12
Ostrich (*Struthio camelus*)(NC 002785.1)	.	.	C	.	T	G	C	T	.	T	.	A	.	.	C	T	C	.	.	.	A	11	.	.	.	.	.	.	.	A	C	.	.	.	.	T	T	C	.	.	.	.	5
Rabbit (*Oryctolagus cuniculus*) (AJ001588.1)	.	.	.	.	T	G	C	T	.	T	T	A	.	.	C	T	C	.	.	.	A	11	.	.	.	.	.	A	.	C	A	T	T	A	.	C	.	T	.	C	.	C	.	.	10
Onion (Allium cepa) (NC 030100.1)	.	T	C	G	T	T	.	T	.	.	.	.	T	.	.	T	C	.	.	A	C	11	A	T	T	C	A	.	T	G	C	.	A	T	.	.	C	T	C	.	G	A	T	16
Cod (Gadus morhua) (NC 002081.1)	.	.	C	.	A	G	C	T	.	.	T	A	.	.	G	T	T	.	.	.	A	11	G	.	.	.	T	T	G	G	A	.	A	T	.	.	C	T	C	.	C	.	.	12
Turkey (*Meleagris gallopavo*) (NC 010195.2)	.	.	C	.	A	G	C	C	.	T	.	A	.	.	C	T	.	.	.	T	A	11	.	.	.	.	.	.	.	A	C	A	.	.	T	.	C	T	C	.	T	.	.	8
Wheat (*Triticum aestivum*) (GU985444.1)	.	T	.	G	T	T	.	T	.	.	T	.	T	.	.	T	C	.	.	A	C	11	A	T	T	G	A	.	T	G	C	.	A	T	.	.	C	T	C	.	G	A	T	16
Crocodyle (*Crocodylus porosus*) (DQ273698.1)	.	.	A	.	A	G	.	T	.	T	.	A	.	.	A	.	C	T	G	T	C	12	.	.	.	.	.	.	.	T	A	.	.	.	.	.	T	.	.	.	C	.	.	4
Pepper (Capsicum annuum) (KJ865410.1)	.	T	.	G	T	T	.	T	.	.	T	.	T	A	.	T	C	.	.	A	C	12	A	T	T	G	A	.	T	G	C	.	A	T	.	.	C	T	C	.	T	A	T	16

Table 4.9: Pairwise distances of cow ND5-specific 106 bp site against other 30 non-target species

Species	Cow	Pig	Goat	Buffa	Duck	Chic	Shee	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Cow_(V00654.1)	0.000																														
Pig_(KJ782448.1)	0.448																														
Goat_(KP271023.1)	0.258	0.362																													
Buffalo_(NC_006295.1)	0.150	0.327	0.137																												
Duck_(EU009397.1)	0.631	0.736	0.500	0.544																											
Chicken_(AP003580.1)	0.562	0.604	0.502	0.527	0.207																										
Sheep_(KR868678.1)	0.203	0.356	0.125	0.125	0.457	0.477																									
Dog_(NC_002008.4)	0.454	0.388	0.365	0.399	0.582	0.485	0.349																								
Pigeon_(KJ722068.1)	0.793	0.738	0.592	0.663	0.344	0.307	0.603	0.521																							
Quail_(KX712089.1)	0.714	0.634	0.556	0.650	0.220	0.176	0.548	0.509	0.338																						
Cod_(NC_002081.1)	0.648	0.545	0.544	0.523	0.526	0.479	0.492	0.539	0.426	0.524																					
Salmon_(KF792729.1)	0.634	0.588	0.498	0.458	0.419	0.422	0.457	0.479	0.447	0.476	0.493																				
Pangas_(NC_023924.1)	0.572	0.572	0.506	0.465	0.529	0.514	0.464	0.448	0.583	0.597	0.523	0.361																			
Tuna_(KF906721.1)	0.575	0.631	0.543	0.484	0.422	0.427	0.386	0.411	0.476	0.462	0.460	0.283	0.336																		
Tilapia_(GU238433.1)	0.640	0.676	0.636	0.616	0.669	0.572	0.543	0.571	0.614	0.647	0.489	0.329	0.363	0.331																	
Rohu_(NC_017608.1)	0.456	0.598	0.455	0.395	0.460	0.493	0.315	0.416	0.480	0.547	0.493	0.280	0.313	0.303	0.331																
Frog_(NC_024548.1)	0.638	0.691	0.713	0.645	1.103	0.929	0.666	0.663	1.153	0.987	1.073	1.008	0.739	1.028	1.015	0.773															
Turtle_(NC_014769.1)	0.706	0.546	0.589	0.629	0.466	0.465	0.539	0.397	0.417	0.443	0.501	0.437	0.528	0.441	0.611	0.411	0.812														
Deer_(NC_006993.1)	0.230	0.345	0.261	0.151	0.663	0.540	0.231	0.505	0.604	0.575	0.516	0.442	0.497	0.547	0.591	0.417	0.719	0.706													
Rabbit_(AJ001588.1)	0.435	0.451	0.332	0.399	0.519	0.556	0.253	0.379	0.579	0.554	0.417	0.475	0.441	0.441	0.498	0.280	0.615	0.410	0.510												
Monkey_(NC_012670.1)	0.494	0.473	0.458	0.455	0.615	0.606	0.416	0.555	0.653	0.615	0.740	0.603	0.662	0.634	0.719	0.522	0.822	0.671	0.397	0.499											
Cat_(NC_001700.1)	0.516	0.314	0.419	0.435	0.589	0.611	0.415	0.263	0.602	0.464	0.606	0.589	0.706	0.502	0.748	0.506	0.834	0.591	0.453	0.458	0.514										
Horse_(KU575247.1)	0.349	0.418	0.299	0.300	0.425	0.487	0.265	0.331	0.519	0.504	0.625	0.541	0.526	0.441	0.655	0.441	0.640	0.442	0.414	0.309	0.414	0.327									
Rat_(AC_000022.2)	0.455	0.626	0.442	0.365	0.619	0.592	0.416	0.484	0.461	0.623	0.596	0.471	0.605	0.443	0.769	0.479	0.828	0.545	0.416	0.452	0.512	0.513	0.348								
Ostrich_(NC_002785.1)	0.609	0.686	0.462	0.544	0.281	0.236	0.439	0.504	0.282	0.312	0.469	0.440	0.465	0.467	0.634	0.410	0.813	0.443	0.557	0.418	0.603	0.568	0.442	0.592							
Donkey_(KT182635.1)	0.333	0.367	0.300	0.300	0.503	0.508	0.265	0.346	0.562	0.565	0.581	0.542	0.549	0.456	0.650	0.442	0.642	0.481	0.398	0.293	0.434	0.374	0.089	0.383	0.480						
Crocodyle_(DQ273698.1)	1.157	1.114	1.169	1.253	0.687	0.832	0.954	1.073	0.701	0.821	0.856	1.136	1.243	0.839	1.114	0.865	1.509	1.045	1.101	0.916	1.178	0.727	0.909	1.107	0.809	0.970					
Turkey_(NC_010195.2)	0.648	0.658	0.460	0.521	0.233	0.202	0.472	0.594	0.322	0.192	0.462	0.469	0.487	0.381	0.630	0.511	0.925	0.529	0.556	0.495	0.607	0.588	0.516	0.628	0.309	0.556	0.876				
Wheat_(GU985444.1)	1.267	1.202	1.147	1.247	1.680	1.505	1.037	1.122	1.465	1.589	1.068	1.328	1.223	1.266	1.098	1.225	1.481	1.434	1.303	1.285	1.390	1.217	1.789	1.347	1.383	1.831	1.538	1.893			
Onion_(NC_030100.1)	1.325	1.211	1.156	1.303	1.448	1.313	1.119	1.215	1.513	1.376	1.114	1.367	1.101	1.325	1.247	1.172	1.645	1.372	1.362	1.481	1.403	1.183	1.874	1.501	1.325	1.900	1.378	1.685	0.077		
Pepper_(KJ865410.1)	1.403	1.380	1.306	1.377	1.850	1.638	1.177	1.345	1.771	1.673	1.196	1.534	1.181	1.429	1.292	1.399	1.693	1.667	1.443	1.481	1.456	1.321	1.955	1.626	1.701	1.990	1.693	1.976	0.066	0.077	0.000

Table 4.10: Pairwise distances of buffalo ND5-specific 138 bp site against other 30 non-target species

Species	Buffa	Pig	Goat	Cow	Duck	Chick	Shee	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Buffalo_(NC_006295.1)	0.000																														
Pig_(KJ782448.1)	0.303																														
Goat_(KP271023.1)	0.247	0.335																													
Cow_(AF492351.1)	0.187	0.301	0.219																												
Duck_(EU009397.1)	1.161	1.384	1.116	1.368																											
Chicken_(AP003580.1)	1.139	1.052	0.914	1.206	0.474																										
Sheep_(KR868678.1)	0.236	0.338	0.110	0.207	1.284	0.931																									
Dog_(NC_002008.4)	0.428	0.427	0.455	0.380	1.368	1.266	0.452																								
Pigeon_(KJ722068.1)	0.986	0.931	0.977	1.090	0.510	0.301	1.057	0.978																							
Quail_(KX712089.1)	1.192	1.027	1.052	1.267	0.513	0.217	1.075	1.201	0.363																						
Cod_(NC_002081.1)	0.997	0.983	0.777	1.066	1.171	0.800	0.847	1.112	0.892	0.983																					
Salmon_(KF792729.1)	0.918	1.017	0.665	1.011	0.878	0.679	0.771	1.184	0.718	0.693	0.512																				
Pangas_(NC_023924.1)	1.181	1.000	1.108	1.162	1.181	0.767	0.954	1.007	0.717	0.835	0.799	0.641																			
Tuna_(KF906721.1)	1.008	1.014	0.750	1.045	0.823	0.817	0.815	1.004	0.772	0.851	0.461	0.413	0.701																		
Tilapia_(GU238433.1)	0.785	0.816	0.828	0.877	0.801	0.593	0.802	1.040	0.637	0.643	0.504	0.385	0.616	0.330																	
Rohu_(NC_017608.1)	1.184	0.952	0.819	0.995	1.076	0.819	0.825	1.188	0.783	0.800	0.776	0.504	0.669	0.680	0.782																
Frog_(NC_024548.1)	1.764	1.735	1.733	1.589	1.607	1.179	1.738	1.691	1.228	1.096	1.838	1.568	1.305	1.426	1.710	1.923															
Turtle_(NC_014769.1)	1.018	0.933	0.837	0.893	0.746	0.587	0.777	0.962	0.727	0.749	0.919	0.865	0.940	0.682	0.795	0.930	1.352														
Deer_(NC_006993.1)	0.216	0.291	0.272	0.248	1.362	0.946	0.217	0.412	0.969	1.091	1.084	0.896	0.979	1.052	0.864	0.935	1.476	1.018													
Rabbit_(AJ001588.1)	0.697	0.642	0.728	0.679	1.041	1.023	0.675	0.831	1.005	0.893	1.072	1.004	1.288	1.001	1.126	1.078	1.920	0.874	0.728												
Monkey_(NC_012670.1)	0.534	0.570	0.485	0.585	1.013	0.795	0.463	0.631	0.783	0.757	0.705	0.716	0.955	0.730	0.774	0.790	1.829	0.857	0.569	0.610											
Cat_(NC_001700.1)	0.450	0.329	0.463	0.493	1.106	0.962	0.409	0.349	0.953	0.988	1.029	1.034	0.956	0.883	0.868	0.873	1.801	0.906	0.459	0.765	0.692										
Horse_(KU575247.1)	0.435	0.314	0.457	0.405	1.051	1.004	0.414	0.348	0.980	0.998	1.095	1.145	1.064	0.880	0.957	0.981	1.276	0.829	0.408	0.679	0.571	0.429									
Rat_(AC_000022.2)	0.595	0.572	0.635	0.633	1.264	0.849	0.599	0.574	0.803	0.829	0.871	0.919	1.013	0.885	0.755	1.029	1.274	1.024	0.587	0.748	0.664	0.654	0.648								
Ostrich_(NC_002785.1)	1.091	1.224	1.052	1.237	0.397	0.379	1.134	1.025	0.290	0.290	1.131	0.835	0.887	0.962	0.799	0.795	1.490	0.814	1.150	1.060	0.932	1.083	1.189	0.977							
Donkey_(KT182635.1)	0.436	0.338	0.470	0.392	1.177	1.021	0.427	0.358	1.075	1.015	1.135	1.183	1.134	0.964	1.056	0.972	1.516	0.785	0.463	0.661	0.568	0.398	0.093	0.703	1.155						
Crocodyle_(DQ273698.1)	0.912	0.869	0.842	0.711	1.376	0.707	0.862	1.042	0.860	0.747	1.104	0.947	1.346	1.100	1.144	0.886	1.423	0.804	0.797	1.015	0.862	0.998	0.924	0.876	0.860	0.901					
Turkey_(NC_010195.2)	1.132	1.082	0.970	1.208	0.495	0.185	1.007	1.103	0.347	0.201	0.904	0.745	0.891	0.764	0.728	0.831	1.144	0.649	1.103	0.902	0.849	0.930	1.039	0.856	0.380	1.005	0.712				
Wheat_(GU985444.1)	2.156	1.742	1.837	2.134	2.103	1.369	2.098	1.742	1.300	1.515	1.273	1.095	1.528	1.372	1.279	1.361	1.404	1.928	2.214	1.825	2.032	1.532	2.115	1.529	1.736	2.104	1.236	1.594			
Onion_(NC_030100.1)	2.214	1.742	1.779	2.077	2.163	1.333	2.040	1.796	1.300	1.470	1.240	1.067	1.485	1.335	1.245	1.325	1.369	1.865	2.156	1.770	2.032	1.486	2.177	1.529	1.736	2.165	1.204	1.548	0.008		
Pepper_(KJ865410.1)	2.057	1.654	1.744	2.038	1.960	1.316	2.001	1.737	1.160	1.331	1.254	1.057	1.369	1.271	1.231	1.364	1.371	1.822	2.114	1.726	1.795	1.463	2.012	1.350	1.565	2.002	1.191	1.401	0.033	0.041	0.000

Table 4.11: Pairwise distances of chicken cytb-specific 161 bp site against other 30 non-target species

Species	Chic	Shee	Duck	Goat	Pig	Cow	Buffa	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Dog	Rabb	Rat	Monk	Cat	Horse	Donk	Croc	Ostr	Turk	Onio	Whea	Pepp
Chicken_(NC_001323.1)	0.000																														
Sheep_(KR868678.1)	0.459																														
Duck_(EU009397.1)	0.251	0.388																													
Goat_(KP271023.1)	0.482	0.128	0.431																												
Pig_(KJ782448.1)	0.499	0.291	0.574	0.281																											
Cow_(AF492351.1)	0.527	0.240	0.389	0.184	0.346																										
Buffalo_(NC_006295.1)	0.462	0.174	0.426	0.181	0.304	0.158																									
Pigeon_(KJ722068.1)	0.244	0.380	0.255	0.366	0.537	0.404	0.363																								
Quail_(KX712089.1)	0.175	0.430	0.271	0.418	0.464	0.448	0.387	0.291																							
Cod_(NC_002081.1)	0.460	0.465	0.515	0.484	0.508	0.466	0.383	0.480	0.567																						
Salmon_(KF792729.1)	0.586	0.513	0.538	0.499	0.482	0.529	0.429	0.435	0.526	0.339																					
Pangas_(NC_023924.1)	0.616	0.607	0.492	0.599	0.577	0.492	0.526	0.612	0.572	0.510	0.409																				
Tuna_(KF906721.1)	0.534	0.481	0.532	0.465	0.517	0.477	0.477	0.432	0.472	0.335	0.359	0.400																			
Tilapia_(GU238433.1)	0.535	0.512	0.467	0.437	0.495	0.456	0.408	0.486	0.591	0.399	0.345	0.388	0.344																		
Rohu_(NC_017608.1)	0.566	0.400	0.490	0.445	0.395	0.368	0.379	0.418	0.535	0.420	0.335	0.320	0.295	0.320																	
Frog_(NC_024548.1)	0.566	0.559	0.578	0.551	0.648	0.529	0.571	0.542	0.653	0.459	0.428	0.434	0.408	0.461	0.388																
Turtle_(NC_014769.1)	0.479	0.419	0.433	0.485	0.423	0.517	0.424	0.429	0.498	0.417	0.458	0.463	0.393	0.393	0.433	0.588															
Deer_(NC_006993.1)	0.503	0.190	0.457	0.200	0.345	0.241	0.198	0.424	0.442	0.510	0.520	0.542	0.516	0.440	0.400	0.602	0.442														
Dog_(MH891616.1)	0.467	0.255	0.497	0.255	0.243	0.298	0.219	0.433	0.437	0.472	0.424	0.566	0.519	0.428	0.408	0.563	0.454	0.265													
Rabbit_(AJ001588.1)	0.490	0.294	0.520	0.362	0.454	0.364	0.356	0.437	0.493	0.471	0.554	0.661	0.537	0.519	0.448	0.484	0.501	0.383	0.332												
Rat_(012374.1)	0.508	0.287	0.545	0.277	0.305	0.323	0.286	0.447	0.495	0.435	0.484	0.509	0.403	0.440	0.302	0.523	0.359	0.297	0.273	0.477											
Monkey_(NC_012670.1)	0.483	0.275	0.440	0.339	0.319	0.355	0.342	0.424	0.435	0.607	0.552	0.493	0.504	0.532	0.472	0.575	0.348	0.342	0.300	0.431	0.353										
Cat_(NC_001700.1)	0.501	0.281	0.504	0.308	0.338	0.312	0.261	0.482	0.400	0.487	0.454	0.557	0.467	0.480	0.418	0.552	0.497	0.347	0.267	0.327	0.349	0.305									
Horse_(KU575247.1)	0.537	0.289	0.492	0.316	0.267	0.344	0.269	0.507	0.472	0.518	0.491	0.656	0.508	0.494	0.534	0.673	0.418	0.326	0.302	0.448	0.357	0.236	0.268								
Donkey_(KT182635.1)	0.447	0.228	0.448	0.245	0.243	0.262	0.218	0.416	0.417	0.483	0.501	0.629	0.543	0.550	0.431	0.602	0.431	0.263	0.242	0.385	0.274	0.263	0.286	0.142							
Crocodyle_(DQ273698.1)	0.545	0.572	0.582	0.579	0.707	0.571	0.644	0.453	0.532	0.663	0.680	0.693	0.567	0.648	0.551	0.704	0.583	0.610	0.659	0.625	0.523	0.574	0.665	0.633	0.589						
Ostrich_(NC_002785.1)	0.309	0.435	0.284	0.455	0.489	0.522	0.464	0.220	0.309	0.519	0.527	0.535	0.510	0.572	0.459	0.572	0.426	0.472	0.519	0.532	0.487	0.450	0.458	0.516	0.429	0.463					
Turkey_(NC_010195.2)	0.209	0.476	0.309	0.481	0.455	0.431	0.338	0.254	0.178	0.474	0.519	0.509	0.433	0.450	0.510	0.677	0.415	0.449	0.409	0.473	0.498	0.377	0.463	0.461	0.406	0.585	0.359				
Onion_(NC_030100.1)	0.892	0.918	0.813	0.885	0.927	1.027	1.041	0.832	0.811	0.891	0.770	0.851	0.770	0.741	0.788	0.866	0.826	0.933	0.949	0.936	0.958	0.895	1.028	0.868	0.961	0.936	0.860	0.796			
Wheat_(GU985444.1)	0.838	0.869	0.819	0.874	0.897	1.033	1.046	0.798	0.762	0.889	0.739	0.858	0.762	0.771	0.779	0.829	0.859	0.918	0.936	0.924	0.945	0.873	0.998	0.846	0.918	0.879	0.824	0.803	0.026		
Pepper_(KJ865410.1)	0.853	0.850	0.793	0.860	0.857	0.986	0.961	0.796	0.806	0.851	0.714	0.804	0.751	0.716	0.730	0.844	0.837	0.898	0.894	0.867	0.933	0.924	1.026	0.869	0.942	0.912	0.839	0.807	0.039	0.039	0.000

Table 4.12: Pairwise distances of Duck ND5-specific 203 bp site against other 30 non-target species

Species	Duck	Pig	Goat	Cow	Buffa	Chic	Shee	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Duck_(EU009397.1)	0.000																														
Pig_(KJ782448.1)	0.547																														
Goat_(KP271023.1)	0.468	0.259																													
Cow_(AF492351.1)	0.522	0.212	0.170																												
Buffalo_(NC_006295.1)	0.544	0.239	0.139	0.106																											
Chicken_(AP003580.1)	0.306	0.578	0.530	0.475	0.484																										
Sheep_(KR868678.1)	0.498	0.219	0.151	0.129	0.094	0.413																									
Dog_(NC_002008.4)	0.593	0.317	0.302	0.232	0.273	0.576	0.246																								
Pigeon_(KJ722068.1)	0.287	0.532	0.531	0.523	0.542	0.311	0.525	0.581																							
Quail_(KX712089.1)	0.344	0.564	0.463	0.473	0.464	0.218	0.419	0.590	0.312																						
Cod_(NC_002081.1)	0.613	0.641	0.701	0.653	0.711	0.706	0.678	0.685	0.737	0.665																					
Salmon_(KF792729.1)	0.603	0.602	0.662	0.524	0.584	0.542	0.566	0.633	0.577	0.575	0.428																				
Pangas_(NC_023924.1)	0.559	0.588	0.607	0.605	0.627	0.583	0.640	0.643	0.541	0.520	0.553	0.335																			
Tuna_(KF906721.1)	0.541	0.622	0.653	0.622	0.651	0.595	0.631	0.664	0.532	0.574	0.492	0.296	0.325																		
Tilapia_(GU238433.1)	0.743	0.644	0.699	0.607	0.651	0.547	0.649	0.631	0.689	0.689	0.529	0.424	0.396	0.312																	
Rohu_(NC_017608.1)	0.640	0.490	0.565	0.506	0.506	0.523	0.489	0.543	0.554	0.565	0.527	0.397	0.314	0.336	0.414																
Frog_(NC_024548.1)	0.982	0.826	1.103	1.078	1.136	1.028	1.018	1.036	0.962	0.934	0.810	0.830	0.802	0.753	0.781	0.828															
Turtle_(NC_014769.1)	0.582	0.657	0.706	0.584	0.635	0.592	0.671	0.678	0.502	0.517	0.576	0.470	0.514	0.554	0.544	0.524	0.797														
Deer_(NC_006993.1)	0.518	0.245	0.164	0.123	0.164	0.547	0.135	0.252	0.536	0.522	0.792	0.613	0.635	0.743	0.644	0.561	1.067	0.602													
Rabbit_(AJ001588.1)	0.644	0.368	0.409	0.339	0.338	0.593	0.361	0.385	0.664	0.589	0.643	0.619	0.569	0.646	0.595	0.539	1.091	0.671	0.338												
Monkey_(NC_012670.1)	0.541	0.387	0.420	0.364	0.364	0.653	0.365	0.469	0.572	0.564	0.641	0.602	0.687	0.662	0.600	0.491	0.986	0.581	0.405	0.430											
Cat_(NC_001700.1)	0.518	0.284	0.269	0.192	0.230	0.512	0.218	0.238	0.549	0.557	0.753	0.635	0.740	0.666	0.622	0.584	1.112	0.666	0.200	0.394	0.440										
Horse_(KU575247.1)	0.464	0.254	0.272	0.162	0.199	0.544	0.150	0.230	0.542	0.540	0.645	0.515	0.581	0.569	0.640	0.503	1.059	0.612	0.180	0.364	0.391	0.233									
Rat_(AC_000022.2)	0.675	0.482	0.554	0.470	0.522	0.673	0.468	0.515	0.655	0.646	0.755	0.588	0.645	0.526	0.651	0.554	0.906	0.648	0.479	0.601	0.633	0.593	0.498								
Ostrich_(NC_002785.1)	0.312	0.557	0.432	0.385	0.392	0.358	0.401	0.479	0.359	0.314	0.632	0.527	0.549	0.521	0.584	0.517	1.095	0.542	0.473	0.583	0.598	0.412	0.423	0.657							
Donkey_(KT182635.1)	0.490	0.255	0.258	0.186	0.198	0.532	0.174	0.224	0.570	0.501	0.704	0.562	0.589	0.598	0.649	0.511	1.046	0.614	0.217	0.371	0.390	0.239	0.078	0.496	0.448						
Crocodyle_(DQ273698.1)	0.863	0.910	0.929	0.830	0.817	0.697	0.827	0.855	0.818	0.730	1.023	0.944	1.010	0.925	0.806	0.906	1.014	0.704	0.859	0.848	0.932	0.910	0.851	0.895	0.841	0.806					
Turkey_(NC_010195.2)	0.320	0.550	0.533	0.478	0.506	0.192	0.450	0.607	0.380	0.250	0.697	0.586	0.586	0.557	0.606	0.579	0.865	0.579	0.564	0.572	0.591	0.581	0.554	0.598	0.403	0.542	0.728				
Wheat_(GU985444.1)	1.560	1.179	1.212	1.271	1.383	1.997	1.248	1.279	1.731	1.865	1.419	1.585	1.825	1.558	1.586	1.455	1.694	1.612	1.234	1.495	1.403	1.351	1.127	1.457	1.469	1.133	1.828	1.540			
Onion_(NC_030100.1)	1.699	1.249	1.213	1.270	1.356	2.061	1.271	1.365	1.677	2.018	1.429	1.513	1.727	1.514	1.605	1.444	1.789	1.658	1.278	1.643	1.419	1.349	1.256	1.525	1.491	1.263	1.979	1.654	0.062		
Pepper_(KJ865410.1)	1.653	1.179	1.256	1.341	1.383	1.997	1.271	1.326	1.693	1.909	1.447	1.645	1.825	1.591	1.586	1.399	1.626	1.524	1.279	1.560	1.384	1.377	1.207	1.457	1.582	1.193	1.682	1.572	0.036	0.068	0.000

Table 4.13: Pairwise distances of goat ND5-specific 236 bp site against other 30 non-target species

Species	Goat	Pig	Cow	Buffa	Duck	Chic	Shee	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Goat_(KP271023.1)	0.000																														
Pig_(KJ782448.1)	0.376																														
Cow_(AF492351.1)	0.276	0.339																													
Buffalo_(NC_006295.1)	0.224	0.356	0.205																												
Duck_(EU009397.1)	0.728	0.753	0.805	0.816																											
Chicken_(AP003580.1)	0.801	0.752	0.691	0.760	0.358																										
Sheep_(KR868678.1)	0.214	0.338	0.223	0.186	0.707	0.669																									
Dog_(NC_002008.4)	0.455	0.396	0.389	0.402	0.835	0.741	0.388																								
Pigeon_(KJ722068.1)	0.815	0.790	0.811	0.782	0.382	0.471	0.752	0.775																							
Quail_(KX712089.1)	0.805	0.806	0.772	0.769	0.407	0.230	0.706	0.776	0.421																						
Cod_(NC_002081.1)	1.187	0.907	1.022	1.141	0.956	1.147	1.167	1.102	0.972	0.907																					
Salmon_(KF792729.1)	0.964	0.715	0.853	0.837	0.940	0.813	0.855	0.829	0.794	0.842	0.558																				
Pangas_(NC_023924.1)	0.869	0.818	0.948	0.933	0.836	0.841	0.879	0.915	0.690	0.771	0.746	0.498																			
Tuna_(KF906721.1)	1.047	0.900	1.026	0.975	0.993	1.100	1.059	1.015	0.896	1.130	0.712	0.471	0.618																		
Tilapia_(GU238433.1)	1.011	0.928	0.888	0.892	1.157	1.000	0.926	0.931	0.993	1.126	0.733	0.574	0.573	0.306																	
Rohu_(NC_017608.1)	0.945	0.790	0.858	0.776	0.956	0.808	0.853	0.937	0.768	0.879	0.871	0.571	0.465	0.538	0.572																
Frog_(NC_024548.1)	1.343	1.147	1.505	1.547	1.356	1.711	1.362	1.435	1.458	1.523	1.312	1.348	1.253	1.234	1.220	1.293															
Turtle_(NC_014769.1)	0.950	0.924	0.775	0.882	0.788	0.777	0.856	0.904	0.715	0.766	0.890	0.742	0.655	0.852	0.862	0.661	1.151														
Deer_(NC_006993.1)	0.283	0.345	0.157	0.262	0.779	0.727	0.204	0.368	0.780	0.729	1.225	0.877	0.915	1.056	0.860	0.868	1.336	0.873													
Rabbit_(AJ001588.1)	0.613	0.514	0.529	0.541	1.009	0.792	0.569	0.555	0.818	0.815	1.088	0.994	0.876	1.093	0.956	0.896	1.718	1.022	0.475												
Monkey_(NC_012670.1)	0.566	0.499	0.539	0.471	0.710	0.828	0.535	0.574	0.782	0.783	0.908	0.825	0.928	0.933	0.825	0.787	1.545	0.811	0.565	0.600											
Cat_(NC_001700.1)	0.407	0.367	0.348	0.322	0.717	0.646	0.310	0.315	0.752	0.705	1.004	0.833	1.011	1.088	0.964	0.919	1.313	0.868	0.342	0.531	0.522										
Horse_(KU575247.1)	0.344	0.338	0.246	0.313	0.637	0.681	0.245	0.378	0.765	0.694	0.950	0.786	0.920	0.946	0.867	0.806	1.230	0.788	0.243	0.542	0.493	0.280									
Rat_(AC_000022.2)	0.806	0.720	0.743	0.816	0.934	1.009	0.691	0.675	0.889	0.922	1.160	0.969	0.988	0.934	0.909	0.752	1.276	0.924	0.744	0.960	0.892	0.855	0.769								
Ostrich_(NC_002785.1)	0.734	0.843	0.670	0.691	0.441	0.452	0.644	0.743	0.418	0.408	0.941	0.804	0.768	1.000	0.855	0.774	1.429	0.669	0.693	0.852	0.783	0.632	0.619	0.943							
Donkey_(KT182635.1)	0.327	0.369	0.284	0.249	0.640	0.690	0.283	0.422	0.744	0.745	1.075	0.864	0.969	0.991	0.939	0.886	1.328	0.832	0.311	0.569	0.510	0.320	0.104	0.805	0.670						
Crocodyle_(DQ273698.1)	1.222	1.069	1.107	1.065	1.073	0.943	1.184	1.271	1.052	0.994	1.497	1.260	1.255	1.301	1.338	1.096	2.107	0.888	1.015	1.289	1.079	1.238	1.071	1.208	1.199	1.153					
Turkey_(NC_010195.2)	0.823	0.767	0.723	0.783	0.440	0.268	0.700	0.750	0.507	0.290	1.041	0.875	0.810	1.115	1.062	0.888	1.368	0.813	0.724	0.756	0.752	0.688	0.706	0.885	0.490	0.746	0.953				
Wheat_(GU985444.1)	1.327	1.516	1.467	1.501	2.020	2.395	1.412	1.462	1.985	2.464	1.581	1.531	1.617	1.414	1.397	1.383	1.610	2.328	1.369	1.624	1.633	1.399	1.264	1.842	1.883	1.292	2.199	2.008			
Onion_(NC_030100.1)	1.301	1.460	1.460	1.493	2.097	2.474	1.360	1.431	1.979	2.623	1.599	1.443	1.545	1.403	1.388	1.399	1.626	2.205	1.409	1.720	1.594	1.325	1.279	1.869	1.903	1.351	2.170	2.097	0.056		
Pepper_(KJ865410.1)	1.419	1.398	1.571	1.556	1.860	2.102	1.463	1.490	1.738	2.218	1.469	1.453	1.558	1.461	1.474	1.351	1.519	1.759	1.489	1.664	1.469	1.379	1.267	1.947	1.768	1.316	2.045	1.955	0.089	0.105	0.000

Table 4.14: Pairwise distances of sheep ND5-specific 263 bp site against other 30 non-target species

Species	Shee	Pig	Goat	Cow	Buffa	Duck	Chic	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Sheep_(KR868678.1)	0.000																														
Pig_(KJ782448.1)	0.346																														
Goat_(KP271023.1)	0.224	0.358																													
Cow_(AF492351.1)	0.222	0.328	0.260																												
Buffalo_(NC_006295.1)	0.206	0.367	0.222	0.206																											
Duck_(EU009397.1)	0.739	0.862	0.703	0.733	0.783																										
Chicken_(AP003580.1)	0.665	0.754	0.716	0.765	0.741	0.317																									
Dog_(NC_002008.4)	0.390	0.383	0.446	0.391	0.395	0.854	0.791																								
Pigeon_(KJ722068.1)	0.741	0.723	0.767	0.731	0.749	0.369	0.460	0.806																							
Quail_(KX712089.1)	0.665	0.830	0.639	0.737	0.720	0.368	0.212	0.837	0.424																						
Cod_(NC_002081.1)	1.062	0.946	1.116	1.025	1.141	0.822	1.015	0.977	0.862	0.880																					
Salmon_(KF792729.1)	0.806	0.800	0.924	0.793	0.849	0.894	0.804	0.910	0.776	0.831	0.517																				
Pangas_(NC_023924.1)	0.929	0.812	0.865	0.939	0.874	0.803	0.876	0.982	0.704	0.859	0.678	0.443																			
Tuna_(KF906721.1)	0.943	1.004	0.996	1.038	1.053	0.859	0.971	1.077	0.897	0.986	0.638	0.409	0.544																		
Tilapia_(GU238433.1)	0.933	0.942	1.052	0.922	1.009	1.033	0.906	1.009	0.926	1.102	0.704	0.532	0.574	0.310																	
Rohu_(NC_017608.1)	0.640	0.655	0.686	0.701	0.670	0.840	0.774	0.783	0.735	0.883	0.738	0.473	0.395	0.461	0.587																
Frog_(NC_024548.1)	1.430	1.230	1.469	1.514	1.580	1.356	1.510	1.461	1.293	1.369	1.180	1.166	1.110	1.145	1.191	1.189															
Turtle_(NC_014769.1)	0.969	0.892	0.905	0.893	0.815	0.782	0.745	1.002	0.683	0.775	0.808	0.664	0.592	0.772	0.778	0.638	1.110														
Deer_(NC_006993.1)	0.229	0.367	0.285	0.176	0.272	0.815	0.821	0.394	0.738	0.760	1.196	0.883	0.953	1.105	0.942	0.802	1.344	0.859													
Rabbit_(AJ001588.1)	0.569	0.544	0.649	0.551	0.553	0.915	0.875	0.572	0.925	0.860	0.872	0.911	0.830	1.063	0.909	0.926	1.609	0.964	0.511												
Monkey_(NC_012670.1)	0.513	0.515	0.578	0.540	0.482	0.667	0.738	0.563	0.800	0.706	0.936	0.803	1.002	1.027	0.936	0.732	1.431	0.759	0.571	0.547											
Cat_(NC_001700.1)	0.353	0.349	0.404	0.335	0.319	0.826	0.765	0.313	0.769	0.867	0.968	0.810	0.948	1.054	0.903	0.752	1.485	0.911	0.358	0.535	0.537										
Horse_(KU575247.1)	0.272	0.373	0.365	0.278	0.318	0.736	0.758	0.372	0.761	0.784	0.956	0.759	0.878	0.908	0.879	0.680	1.240	0.791	0.258	0.547	0.513	0.296									
Rat_(AC_000022.2)	0.671	0.678	0.763	0.685	0.742	0.949	0.914	0.651	0.874	0.942	0.973	0.851	0.918	0.831	0.859	0.710	1.253	0.898	0.690	0.937	0.889	0.800	0.727								
Ostrich_(NC_002785.1)	0.595	0.745	0.628	0.599	0.576	0.382	0.426	0.651	0.408	0.404	0.793	0.776	0.774	0.864	0.844	0.771	1.497	0.696	0.728	0.828	0.761	0.640	0.656	0.886							
Donkey_(KT182635.1)	0.318	0.394	0.351	0.312	0.287	0.758	0.770	0.403	0.833	0.785	1.092	0.803	0.844	0.934	0.892	0.690	1.395	0.792	0.311	0.577	0.529	0.342	0.115	0.790	0.710						
Crocodyle_(DQ273698.1)	1.041	1.204	1.136	1.115	1.023	1.012	0.803	1.264	0.995	0.902	1.388	1.186	1.183	1.138	1.110	0.957	1.738	0.838	1.137	1.253	1.264	1.246	1.110	1.155	1.016	1.101					
Turkey_(NC_010195.2)	0.680	0.780	0.690	0.754	0.726	0.415	0.272	0.821	0.502	0.302	0.949	0.847	0.865	1.058	1.037	0.801	1.293	0.804	0.849	0.822	0.774	0.851	0.792	0.938	0.485	0.809	0.877				
Wheat_(GU985444.1)	1.647	2.148	1.673	1.781	1.612	2.004	2.128	1.582	1.979	2.029	1.575	1.847	2.322	2.027	1.770	1.741	2.026	1.765	1.554	1.711	1.666	1.834	1.586	2.120	1.707	1.807	2.277	1.753			
Onion_(NC_030100.1)	1.635	2.088	1.684	1.714	1.600	1.883	1.922	1.573	1.763	1.867	1.492	1.746	2.243	2.006	1.755	1.699	2.089	1.720	1.544	1.639	1.533	1.664	1.551	2.132	1.541	1.844	2.327	1.630	0.052		
Pepper_(KJ865410.1)	1.472	1.810	1.517	1.740	1.577	1.825	2.049	1.524	2.057	2.037	1.540	1.558	1.859	1.904	1.648	1.644	1.944	1.644	1.566	1.852	1.631	1.636	1.503	2.042	1.665	1.699	2.252	1.608	0.084	0.093	0.000

Table 4.15: Pairwise distances of pig ND5-specific 73 bp site against other 30 non-target species

Species	Pig	Goat	Cow	Buffa	Duck	Chic	Shee	Dog	Pigeo	Quail	Cod	Salm	Pang	Tuna	Tilap	Rohu	Frog	Turtl	Deer	Rabb	Monk	Cat	Horse	Rat	Ostri	Donk	Croc	Turk	Whea	Onio	Pepp
Pig_(AF034253.1)	0.000																														
Goat_(KP271023.1)	0.283																														
Cow_(AF492351.1)	0.307	0.232																													
Buffalo_(NC_006295.1)	0.282	0.146	0.089																												
Duck_(EU009397.1)	0.674	0.560	0.727	0.635																											
Chicken_(AP003580.1)	0.704	0.624	0.816	0.671	0.290																										
Sheep_(KR868678.1)	0.303	0.088	0.166	0.165	0.556	0.656																									
Dog_(NC_002008.4)	0.357	0.233	0.355	0.278	0.667	0.667	0.257																								
Pigeon_(KJ722068.1)	0.717	0.674	0.915	0.761	0.372	0.279	0.705	0.697																							
Quail_(KX712089.1)	0.681	0.660	1.003	0.831	0.260	0.230	0.730	0.660	0.279																						
Cod_(NC_002081.1)	0.833	0.565	0.746	0.662	0.612	0.707	0.536	0.588	0.720	0.662																					
Salmon_(KF792729.1)	0.612	0.545	0.553	0.518	0.565	0.674	0.484	0.515	0.665	0.688	0.380																				
Pangas_(NC_023924.1)	0.717	0.573	0.582	0.577	0.582	0.758	0.508	0.446	0.903	0.667	0.447	0.263																			
Tuna_(KF906721.1)	0.688	0.637	0.707	0.662	0.641	0.641	0.568	0.443	0.656	0.568	0.378	0.217	0.243																		
Tilapia_(GU238433.1)	0.809	0.655	0.725	0.717	0.844	0.824	0.586	0.656	0.834	0.783	0.502	0.264	0.430	0.239																	
Rohu_(NC_017608.1)	0.606	0.447	0.442	0.469	0.573	0.641	0.392	0.417	0.620	0.620	0.466	0.261	0.237	0.319	0.345																
Frog_(NC_024548.1)	0.738	0.621	0.603	0.560	1.180	1.164	0.631	0.556	0.994	1.222	1.453	1.037	0.895	1.183	1.234	0.732															
Turtle_(NC_014769.1)	0.505	0.471	0.637	0.524	0.431	0.440	0.498	0.450	0.436	0.401	0.563	0.530	0.607	0.527	0.717	0.440	0.923														
Deer_(NC_006993.1)	0.359	0.280	0.232	0.167	0.712	0.692	0.301	0.494	0.742	0.692	0.794	0.498	0.783	0.798	0.792	0.511	0.631	0.597													
Rabbit_(AJ001588.1)	0.450	0.263	0.423	0.362	0.484	0.524	0.239	0.367	0.509	0.539	0.488	0.525	0.491	0.502	0.568	0.332	0.621	0.292	0.504												
Monkey_(NC_012670.1)	0.505	0.553	0.588	0.544	0.813	0.762	0.513	0.553	0.639	0.624	1.013	0.685	0.709	0.804	0.930	0.528	0.592	0.678	0.508	0.478											
Cat_(NC_001700.1)	0.257	0.359	0.468	0.408	0.683	0.818	0.408	0.334	0.676	0.495	0.573	0.590	0.549	0.561	0.763	0.541	0.896	0.518	0.379	0.490	0.616										
Horse_(KU575247.1)	0.288	0.263	0.317	0.289	0.528	0.683	0.260	0.317	0.605	0.639	0.665	0.669	0.563	0.610	0.761	0.480	0.641	0.292	0.518	0.235	0.462	0.384									
Rat_(AC_000022.2)	0.573	0.471	0.467	0.345	0.691	0.683	0.468	0.442	0.539	0.683	0.719	0.466	0.536	0.615	0.830	0.404	0.786	0.580	0.428	0.430	0.568	0.488	0.442								
Ostrich_(NC_002785.1)	0.645	0.436	0.647	0.563	0.311	0.317	0.462	0.565	0.299	0.367	0.612	0.667	0.582	0.626	0.857	0.475	0.824	0.371	0.583	0.313	0.697	0.607	0.572	0.646							
Donkey_(KT182635.1)	0.267	0.266	0.321	0.292	0.536	0.696	0.263	0.345	0.692	0.722	0.635	0.610	0.572	0.568	0.710	0.487	0.688	0.401	0.525	0.282	0.498	0.415	0.070	0.482	0.580						
Crocodyle_(DQ273698.1)	1.007	1.083	1.208	1.189	0.703	0.890	1.071	1.634	0.688	0.890	1.189	1.223	1.539	1.128	1.344	1.129	1.773	1.143	1.056	0.918	1.506	0.890	1.065	0.992	0.983	0.997					
Turkey_(NC_010195.2)	0.906	0.626	0.910	0.751	0.284	0.209	0.732	0.707	0.280	0.150	0.678	0.683	0.752	0.549	0.850	0.681	1.302	0.466	0.732	0.588	0.725	0.695	0.717	0.725	0.342	0.771	0.902				
Wheat_(GU985444.1)	1.257	1.268	1.317	1.592	1.765	1.641	1.089	1.133	1.739	1.484	0.913	1.209	1.116	1.355	1.268	1.145	1.416	1.803	1.340	1.079	1.236	1.129	1.411	1.459	1.177	1.386	1.756	1.851			
Onion_(NC_030100.1)	1.437	1.441	1.587	1.910	1.748	1.459	1.367	1.364	1.710	1.459	1.042	1.480	1.035	1.664	1.532	1.236	1.675	1.889	1.620	1.336	1.484	1.289	1.609	1.858	1.149	1.587	1.736	1.836	0.087		
Pepper_(KJ865410.1)	1.597	1.592	1.592	1.851	2.014	1.793	1.359	1.544	1.820	1.706	1.099	1.480	1.089	1.664	1.532	1.367	1.597	2.055	1.627	1.275	1.340	1.289	1.689	1.765	1.322	1.670	2.003	2.087	0.071	0.088	0.000

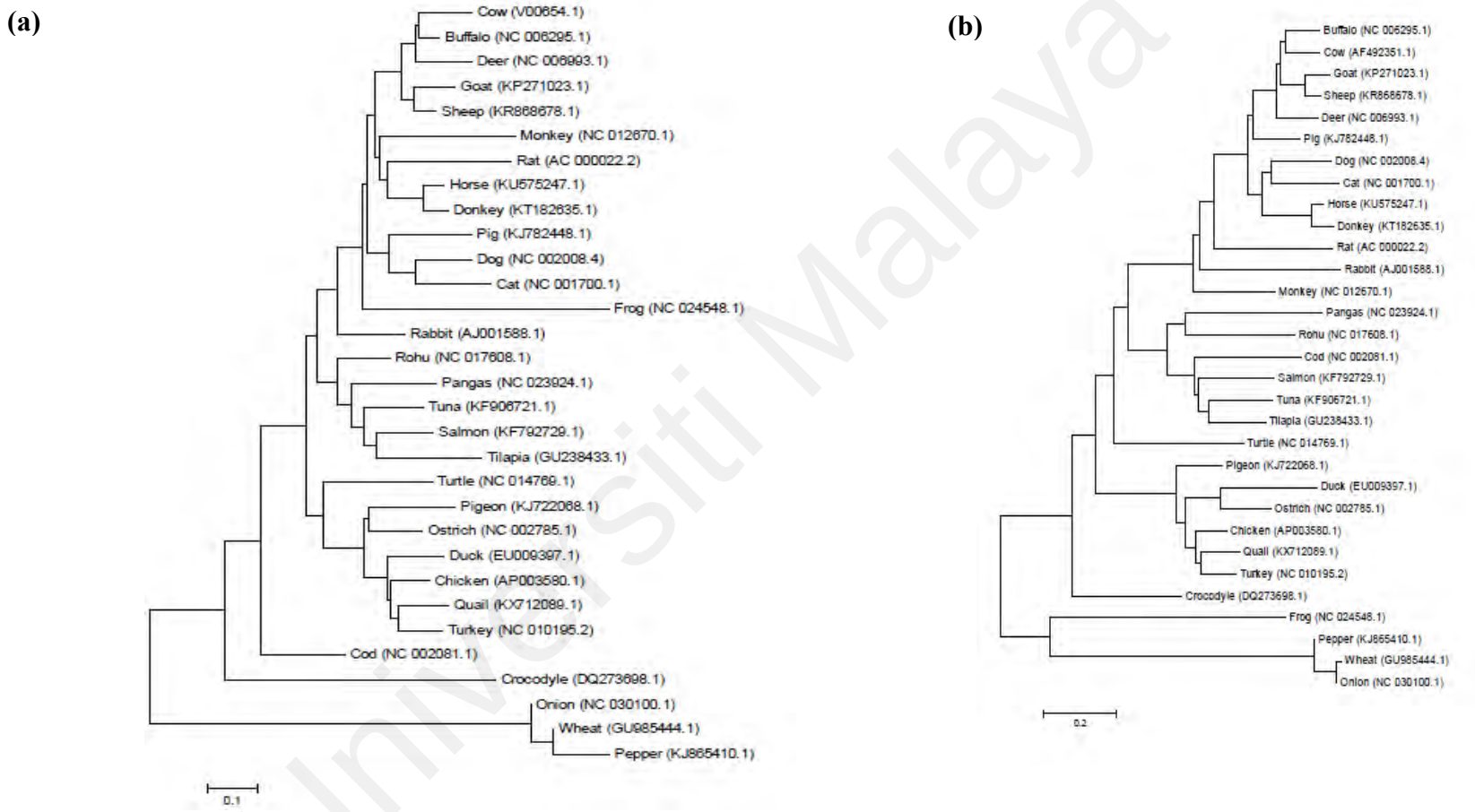
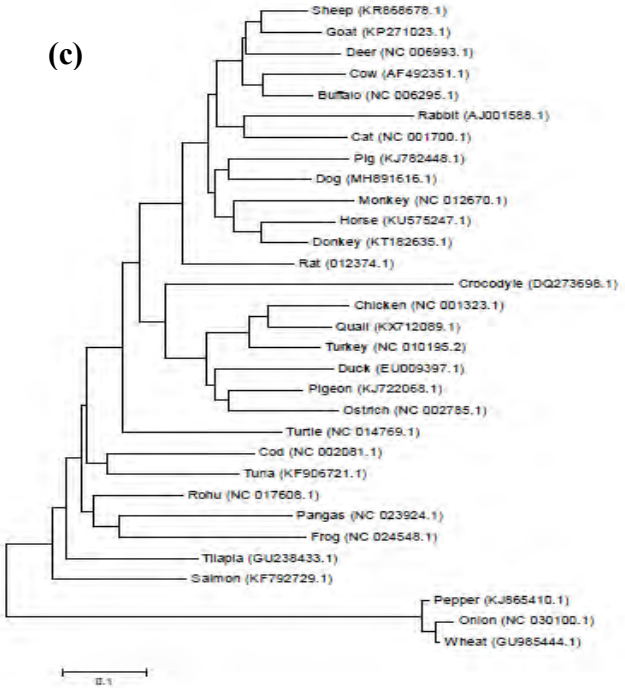


Figure 4.1: Phylogenetic tree generated from the amplicon sequences of each target gene and same gene sequences of other 30 non-target species using the neighbour-joining method. Phylogenetic tree of beef ND5-specific 106 bp site (a), buffalo ND5-specific 138 bp site (b), chicken cytb-specific 161 bp site (c), duck ND5-specific 203 bp site (d), goat ND5-specific 236 bp site (e), sheep ND5-specific 263 bp site (f) and Pig ND5-specific 73 bp site (g), respectively

(c)



(d)

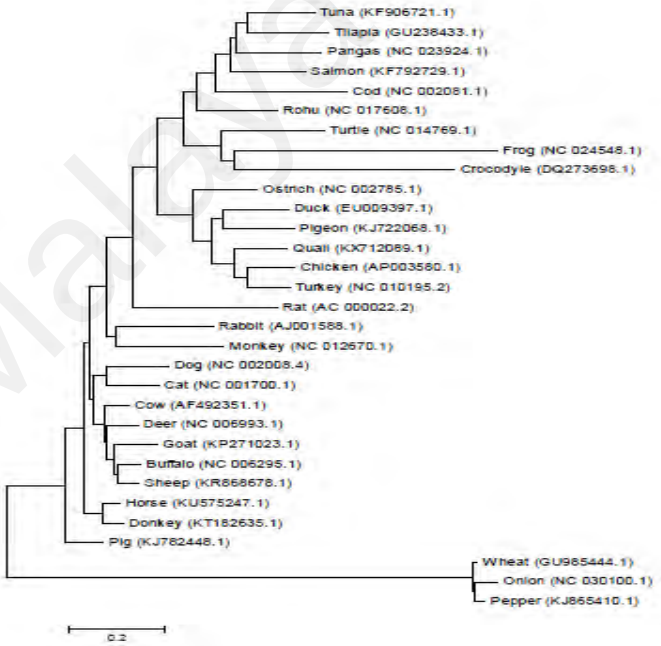


Figure 4.1, continued

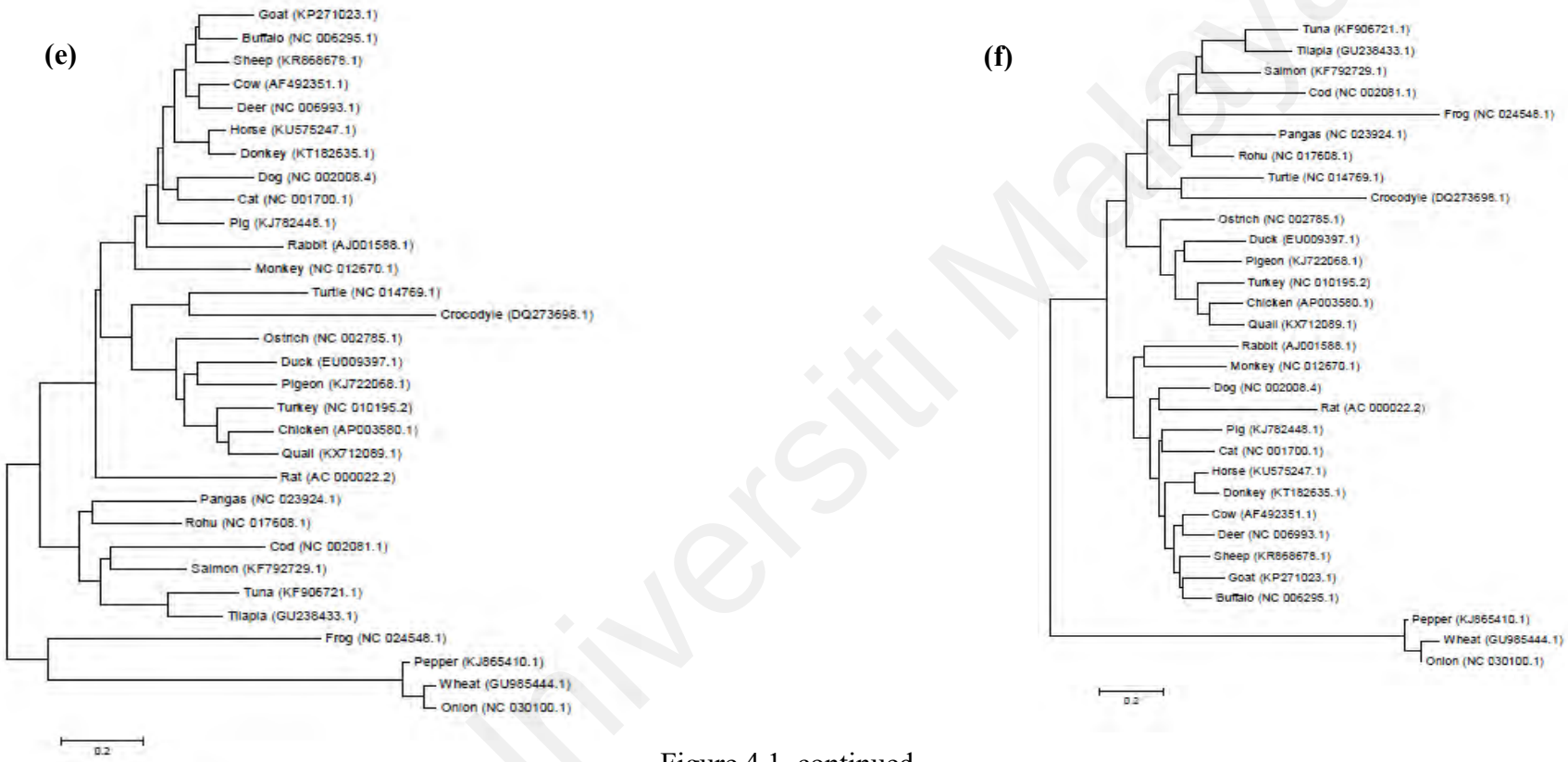


Figure 4.1, continued

(g)

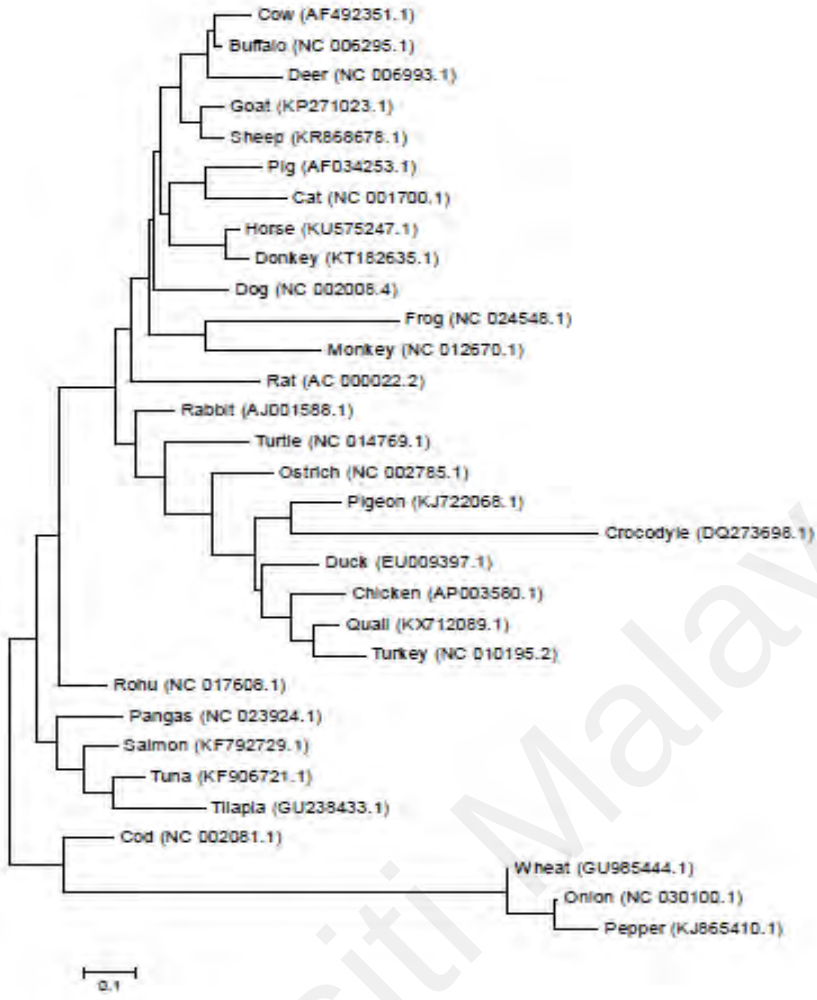


Figure 4.1, continued

4.3 Simplex PCR Assay

4.3.1 Optimization of Simplex PCR Assay

Simplex PCR assay was optimized for each primer pair against the template DNA extracted from each target species. The PCR reactions of seven sets of primers were carried out individually on a gradient thermal cycler with a total of 25 µL reaction volume comprising of appropriate amount of required PCR components (Section 3.5.1). In order to find out the optimum annealing temperature suitable for successful amplifications, the annealing temperatures of all primer sets were carefully checked in the temperature range of 56−61°C in the gradient system. Although amplification was observed for the primer sets at the other temperatures also, however, they were appropriately amplified at 60°C (Figure 4.2). Therefore, 60°C temperature was the optimum annealing temperature for all sets of primers since, all primer pairs in multiplex PCR assays, have to be amplified under a single reaction setting. Next, the universal eukaryotic primer set was added to each simplex system and successful amplification was observed at the same optimizing condition.

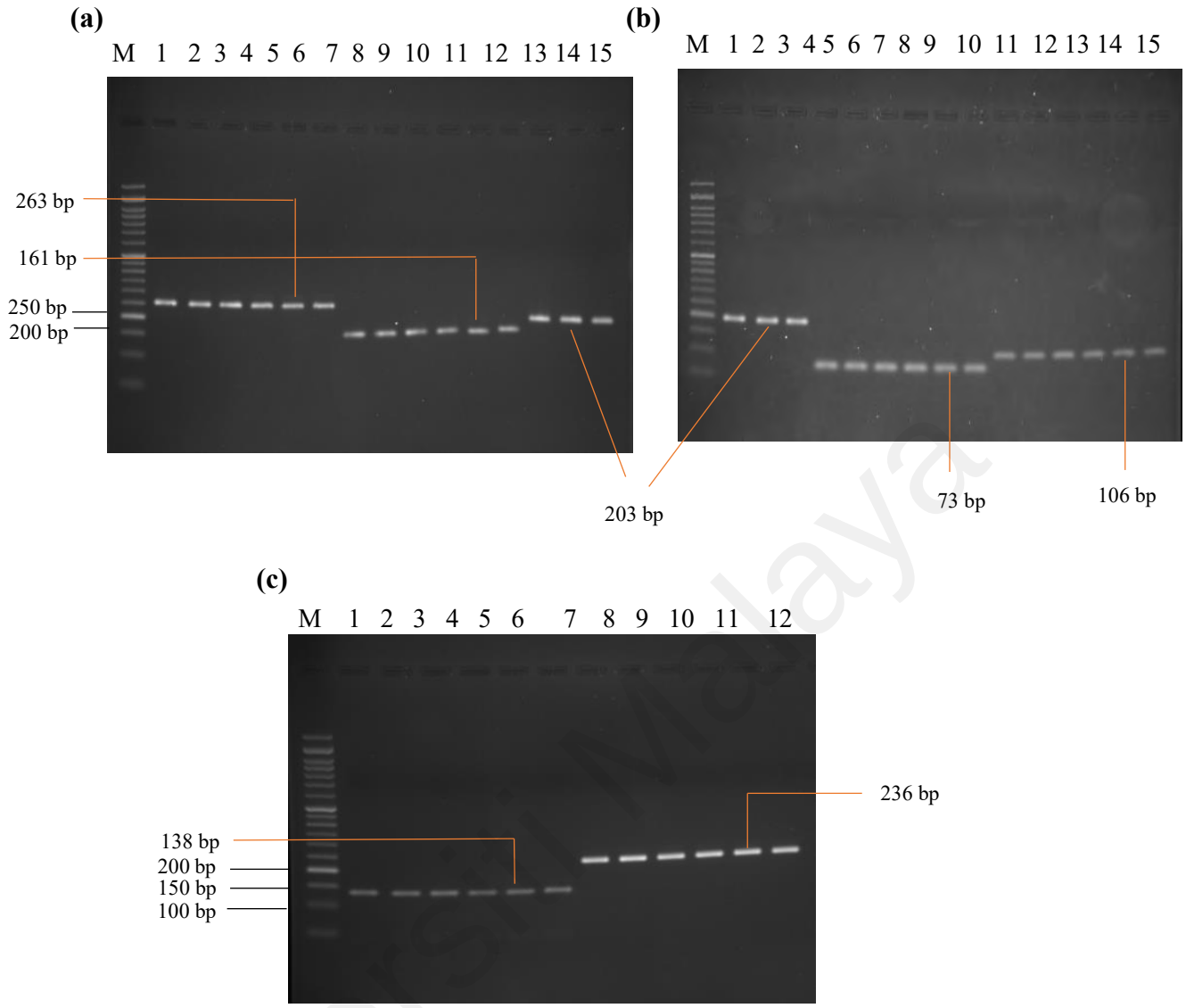


Figure 4.2: Optimization of annealing temperatures of designed sheep ND5, chicken cytb, duck ND5, pig ND5, cow ND5, buffalo ND5 and goat ND5 primer sets. In the gel image (a), lane M is 50 bp ladder DNA, lanes 1-6, 7-12, amplified products for sheep and chicken respectively at 56º, 57º, 58º, 59º, 60º and 61ºC temperatures and lanes 13, 14, 15, PCR products for duck at 56º, 57º and 58ºC. In image (b), lane M is 50 bp DNA ladder, lanes 1-3, PCR products for duck at 59º, 60º and 61ºC and lanes 4-9, 10-15, amplified products for pig and cow respectively at 56º, 57º, 58º, 59º, 60º and 61ºC temperatures. In image (c), lane M is 50 bp DNA ladder, lanes 1-6, 7-12, amplified products for buffalo and goat respectively, at 56º, 57º, 58º, 59º, 60º and 61ºC temperatures.

4.3.2 Specificity of Simplex PCR Assay

To obtain a successful assay, the specificity of the primers plays a vital role. Primers that significantly match the target species and show huge mismatches with non-targets contribute to an increased chance of a precisely specific PCR assay and eliminate non-target amplification possibilities (Murugaiah et al., 2009).

After the simplex PCR was optimized with the universal eukaryotic primer set, the species-specificity of the primers was confirmed by a practical PCR assay through a cross-amplification reaction using DNA templates from one target and 25 non-targets of terrestrial and aquatic animal and plant species (cow, buffalo, goat, sheep, chicken, duck, pig, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic and wheat). The results show that the developed system successfully amplified DNA only from each individual target species while no amplification was observed from any of the nontarget species (Figures 4.3-4.9). Moreover, the universal eukaryotic primers used in this study amplified a 99 bp-length product from all studied species which evidenced the presence of high-quality DNA in all reaction tubes and thus eliminated any chance of false-negative detection. This reflects the high specificity and fidelity of the developed simplex PCR assays. All the assays were carried out in three replicates on three different days, and they gave the same outcomes. A fully automated advanced multicapillary electrophoresis system (QIAxcel, Germany) was used for the separation and visualization of amplified products given their narrow length difference. The system used size marker containing 500, 400, 300, 250, 200, 150, 100, 75, 50 and 25-bp marker DNA and 600 and 15-bp alignment marker for visualization with better resolution. In addition to allowing improved sensitivity and better resolution, even with a minimum of ~5 bp length difference, this device takes shorter analysis time as compared to conventional gel electrophoresis. The clearly separated PCR products corresponding to each target were visualized both as gel images and electropherograms (Figures 4.3-4.9).

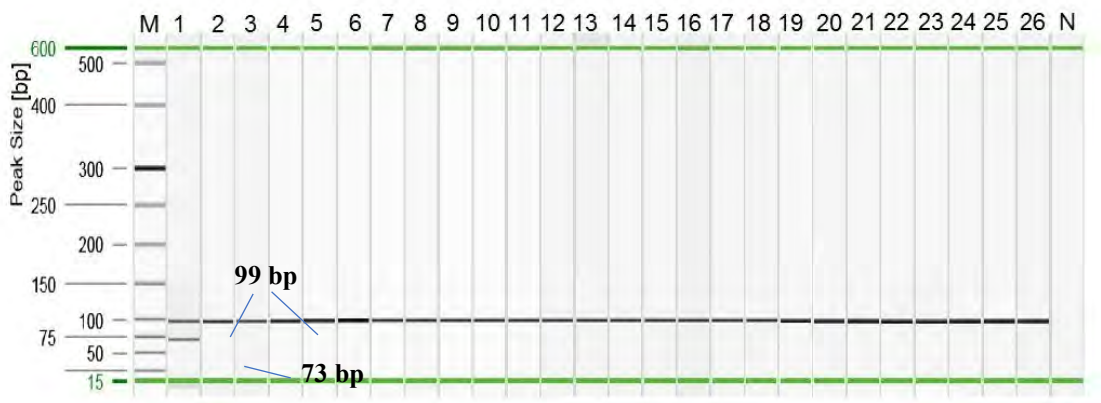


Figure 4.3: The specificity of the simplex PCR of pig ND5 (73 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of pig ND5 (73 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for cow, buffalo, chicken, duck, goat, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

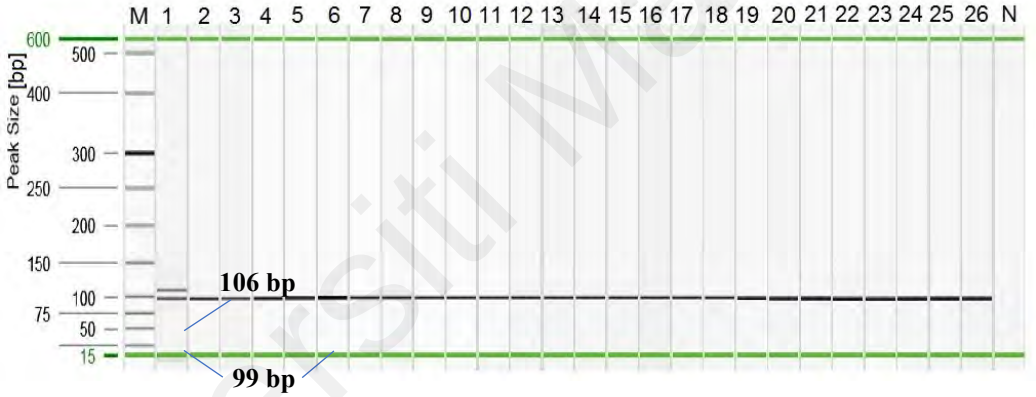


Figure 4.4: The specificity of the simplex PCR of cow ND5 (106 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of cow ND5 (106 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, buffalo, chicken, duck, goat, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

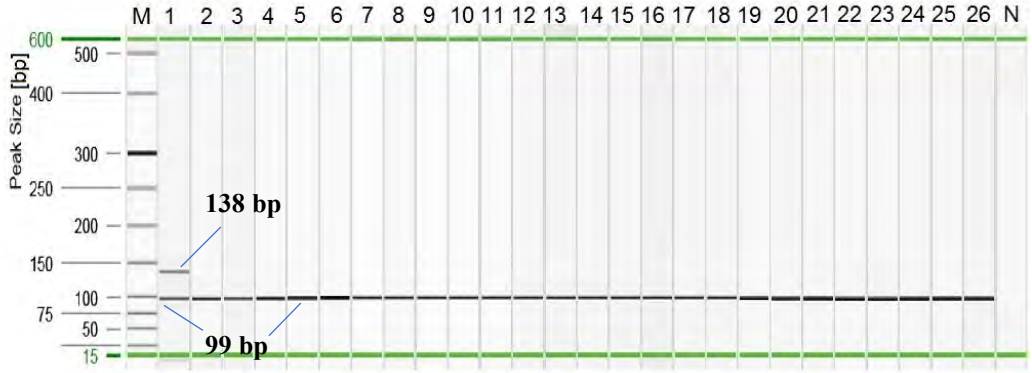


Figure 4.5: The specificity of the simplex PCR of buffalo ND5 (138 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of buffalo ND5 (138 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, cow, chicken, duck, goat, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

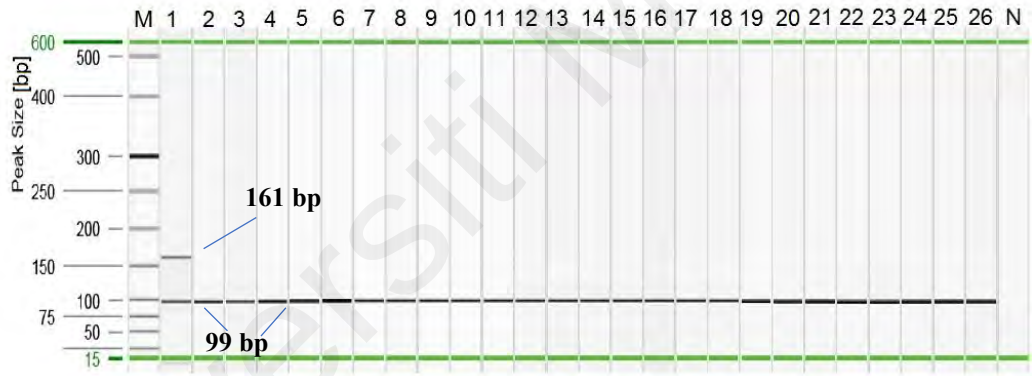


Figure 4.6: The specificity of the simplex PCR of chicken cytb (161 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of chicken cytb (161 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, cow, buffalo, duck, goat, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

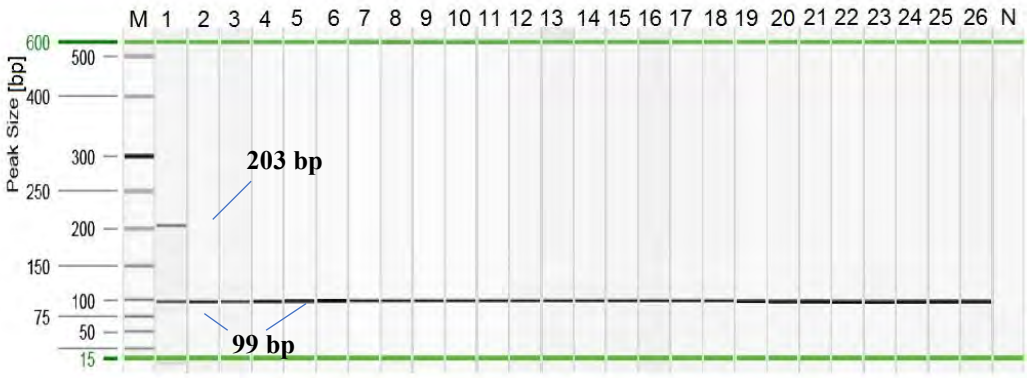


Figure 4.7: The specificity of the simplex PCR of duck ND5 (203 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of duck ND5 (203 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, cow, chicken, goat, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

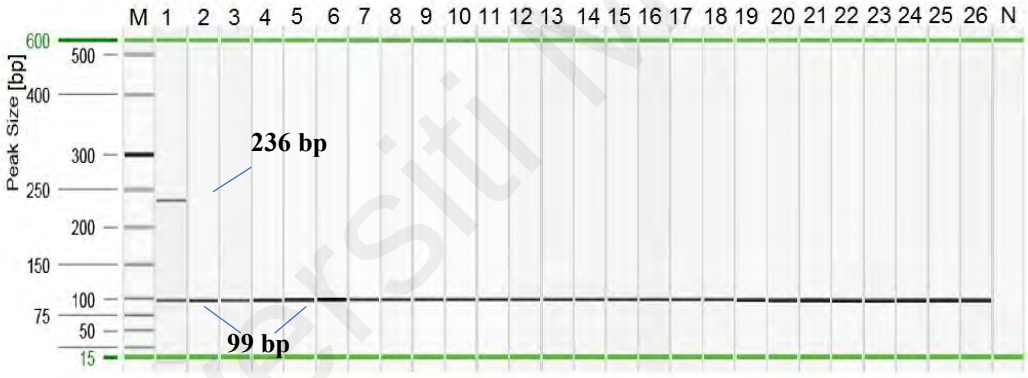


Figure 4.8: The specificity of the simplex PCR of goat ND5 (236 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of goat ND5 (236 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, cow, chicken, duck, sheep, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

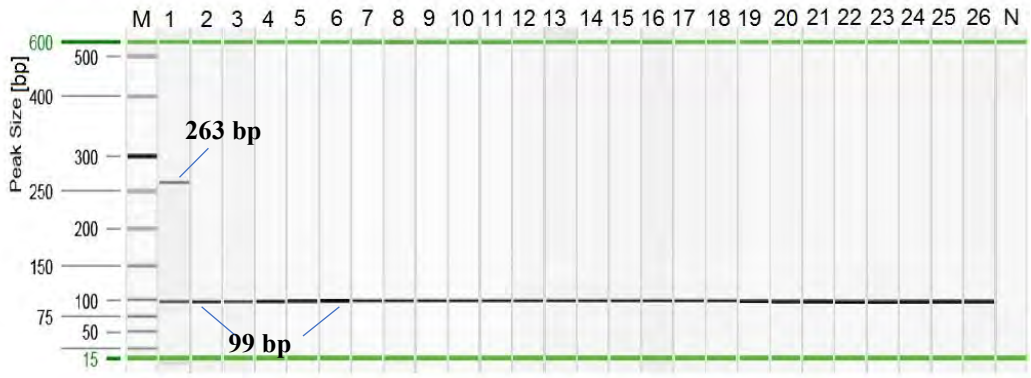


Figure 4.9: The specificity of the simplex PCR of sheep ND5 (263 bp)-specific primer pair with DNA of different species. In the gel image, M, DNA ladder; N, negative template control; *lane 1,* PCR products of sheep ND5 (263 bp) and endogenous control (99 bp); *lanes 2-26,* PCR products of endogenous control (99 bp) for pig, cow, chicken, duck, cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic, and wheat, respectively.

4.4 PCR Product Sequencing

PCR products of cow, buffalo, chicken, duck, goat, sheep and pork were sequenced bi-directionally to ensure the originality of the amplified PCR products, thereby absolutely determining the exact species. The obtained sequences were BLAST with GenBank (www.ncbi. nlm.nih.gov) sequences and checked for matching with any species followed by alignment with specific gene sequences using the MEGA7 alignment tool to find similarities. The sequencing results of different PCR products are summarized in Table 4.16. The sequences of cow, buffalo, chicken, duck, goat, sheep and pig gave the similarity score showing 100%, 98.55%, 100%, 99.38%, 99.01%, 99.5% and 100% homology with *Bos taurus, Bubalus bubalis, Gallus gallus*, *Anas platyrhynchos*, *Capra hircus*, *Ovis aries* and *Sus scrofa* sequences available in GenBank, respectively.

The acceptable limit of sequence similarity for designated species identification is at least 98% (Cawthorn et al., 2013). Therefore, our results prove the reliability and authenticity of the amplified PCR products.

Table 4.16: Sequencing results of PCR products

Target	Target gene	Species	GeneBank Accession ID	Similarity (%)
Cow ND5	ND5	Cow *(Bos taurus)*	V00654.1	100
Buffalo ND5	ND5	Buffalo *(Bubalus bubalis)*	NC_006295.1	98.55
Chicken cytb	Cytb	Chicken *(Gallus gallus)*	NC_001323.1	100
Duck ND5	ND5	Duck *(Anas platyrhynchos)*	EU009397.1	99.38
Goat ND5	ND5	Goat *(Capra hircus)*	KP271023.1	99.01
Sheep ND5	ND5	Sheep *(Ovis aries)*	KR868678.1	99.5
Pig ND5	ND5	Pig *(Sus scrofa)*	AF034253.1	100

4.5 Heptaplex (multiplex) PCR Assay

4.5.1 Optimization of Heptaplex (multiplex) PCR Assay

Following confirmation of the simplex PCR, heptaplex PCR assay was developed stepwise by including additional primer sets for simultaneous detection of all target species in a single assay platform. This system was sequentially developed step by step from singleplex to heptaplex as described in Section 3.6.1 (Figure 4.10). The experimental setup started from individual simplex of pig, cow, buffalo, chicken, duck, goat and sheep (Figure 4.10, lanes 1 − 7) followed by the subsequent stages of duplex for cow and sheep (Figure 4.10, lane 8), triplex for cow, sheep and chicken (Figure 4.10, lane 9), tetraplex for cow, sheep, chicken and pig (Figure 4.10, lane 10), pentaplex for cow, sheep, chicken, pig and buffalo (Figure 4.10, lane 11), hexaplex for cow, sheep, chicken, pig, buffalo and duck (Figure 4.10, lane 12) until the final step of heptaplex for cow, sheep, chicken, pig, buffalo, duck and goat (Figure 4.10, lane 13). All the PCR assays (including the simplex and multiplexes) could successfully amplify the target gene (cytb and ND5) regions producing 73, 106, 138, 161, 203, 236 and 263 bp products from pig, cow, buffalo, chicken, duck, goat and sheep, respectively (Figure 4.10) indicating

complete uniformity with the simplex assay. The electropherogram (Figure 4.10 b) also clearly showed seven peaks corresponding to the seven different bands.

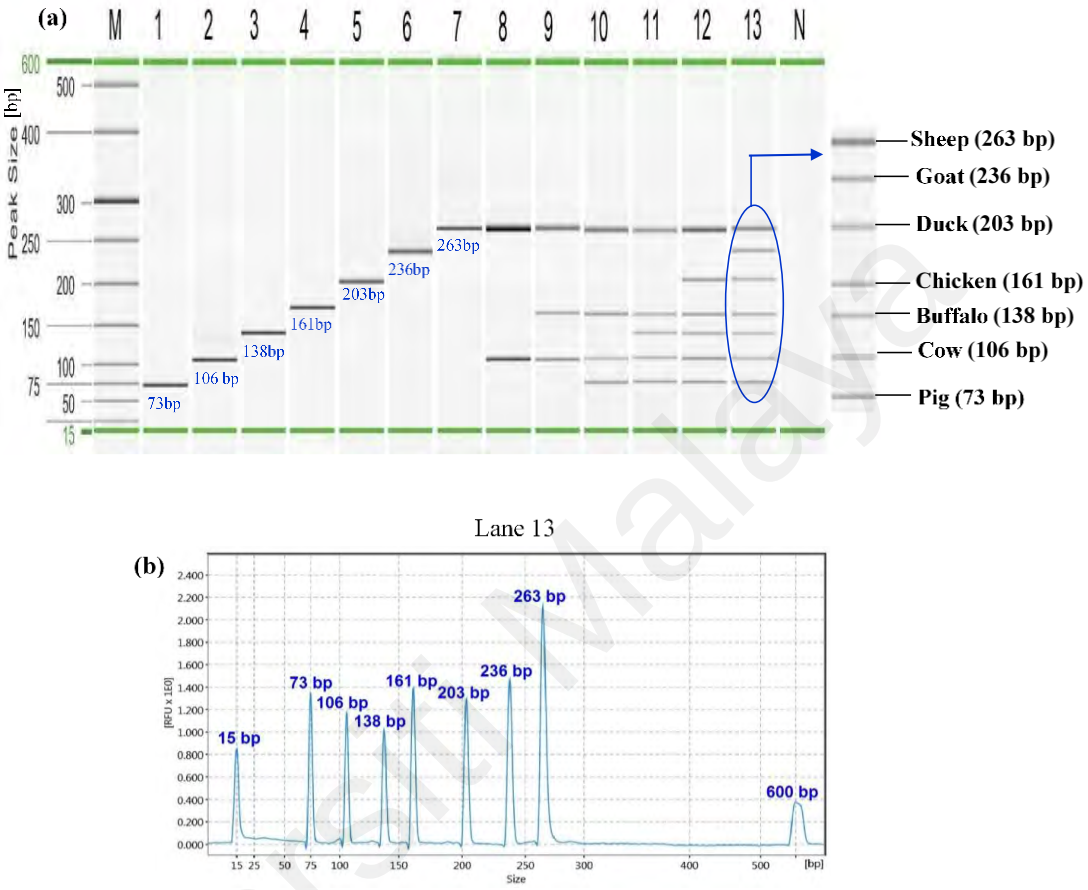


Figure 4.10: Gel image (a) and electropherogram (b) of heptaplex PCR for pig, cow, buffalo, chicken, duck, goat, and sheep species detection. In the gel image (a), *lane* M represents the DNA ladder; *lanes* 1–13 represent the PCR products from pig (*lane* 1); cow (*lane* 2); buffalo (*lane* 3); chicken (*lane* 4); duck (*lane* 5); goat (*lane* 6); sheep (*lane* 7); duplex PCR of cow and sheep (*lane* 8); triplex PCR of cow, sheep and chicken (*lane* 9); tetraplex PCR of cow, sheep, chicken, and pig (*lane* 10); pentaplex PCR of cow, sheep, chicken, pig and buffalo (*lane* 11), hexaplex PCR of cow, sheep, chicken, pig, buffalo and duck (*lane* 12); heptaplex PCR of cow, sheep, chicken, pig, buffalo, duck and goat (*lane* 13); and the negative control (*lane* N). The corresponding electropherograms (b) of *lane* 13 is shown with labels.

4.5.2 Specificity of Heptaplex (multiplex) PCR Assay

The specificity test of the developed heptaplex (mPCR) assay against seven targets (pig, cow, buffalo, chicken, duck, goat, and sheep) and other 19 non-target animal and plant species (Section 3.6.2) revealed that the novel heptaplex PCR system so developed,

amplified all targeted products when DNAs from seven targets were added (Figure: 4.11a, lane 1). Again, when single targets were added individually, it amplified 73, 106, 138, 161, 203, 236, and 263 bp from pork, beef, buffalo, chicken, duck, goat, and sheep, respectively (Figure: 4.11a, lanes 2-8). However, when the assay was run taking DNA from 19 non-target species (Section. 3.6.2), the system yielded no amplified product from any of the non-target species (Figure: 4.11, lanes 9-27), establishing that the heptaplex assay was strictly specific for the differential detection of beef, buffalo, chicken, duck, goat, sheep, and pork.

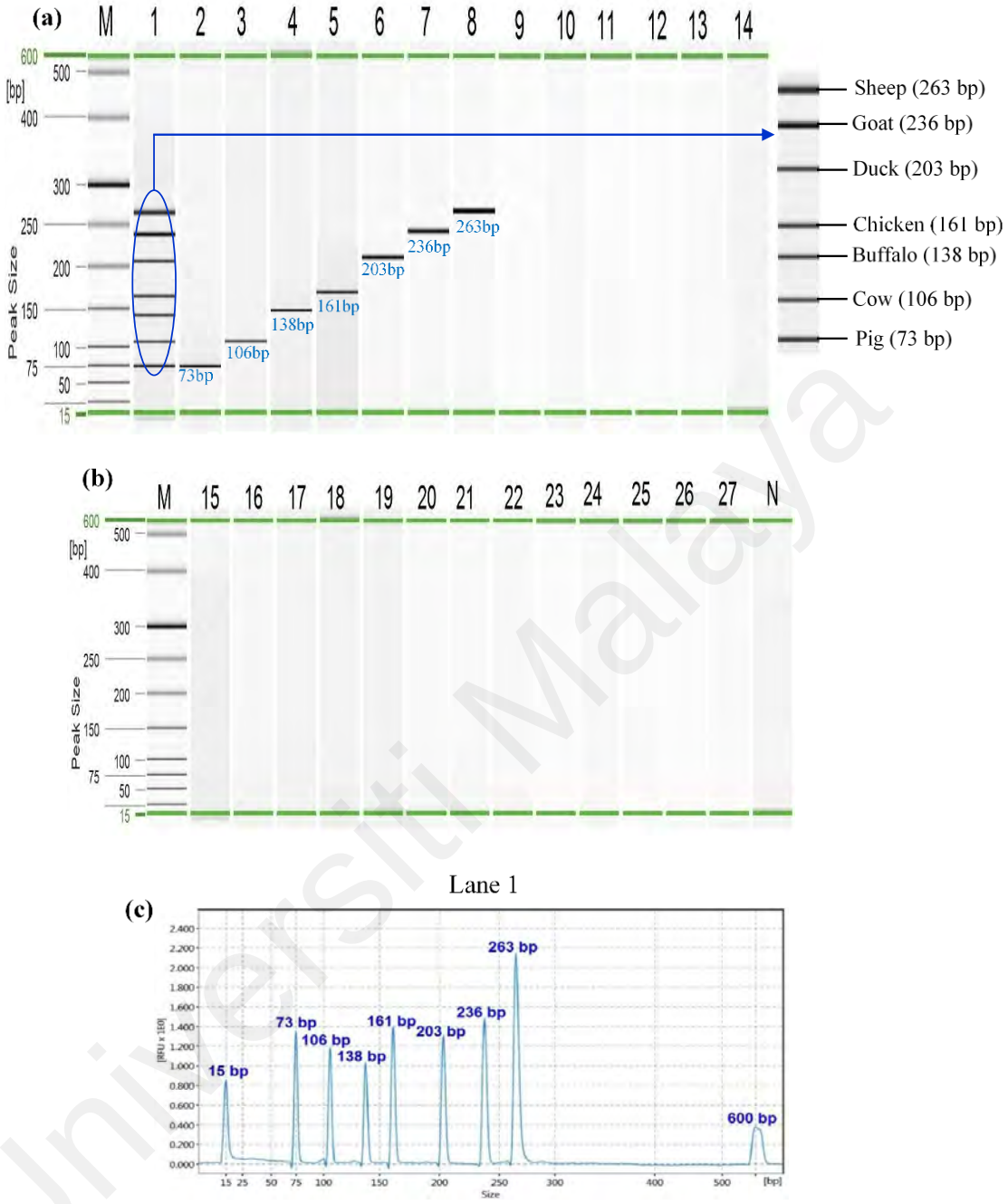


Figure 4.11: The gel images and electropherogram of specificity test of the developed multiplex PCR assay. In the gel images of panel a and panel b, lane M, DNA ladder (50 bp); in panel a, lane 1, PCR products from beef, buffalo, chicken, duck, goat, sheep, and pork; lanes 2-8, PCR products from pork, beef, buffalo, chicken, duck, goat, and sheep, respectively; lanes 9−14, PCR products from dog, cat, rabbit, rat, pigeon, quail, respectively. In panel b, lanes 15−27, PCR products from frog, ostrich, turtle, squirrel, crocodile, tilapia, tuna, sardine, onion, garlic, pepper, ginger, and wheat flour, respectively and lane N, negative template control. The corresponding electropherogram of lane 1 is shown labeled as ‘c’.

4.5.3 Limit of Detection (LOD) of Heptaplex PCR Assay under Raw State

The sensitivity of the heptaplex PCR assay was determined using the diluted DNA mixture (10.0, 1.0, 0.1, 0.01 and 0.005 ng/µL) of all target species (Section 3.6.3). As shown in Figure: 4.12, lanes 1–4, the automated electrophoretic system distinctively showed all the seven bands for PCR products from pig, cow, buffalo, chicken, duck, goat and sheep for DNA concentrations of 10.0−0.01 ng/µL. The electropherogram (Figure 4.12b) also clearly showed seven peaks corresponding to the seven different bands displayed in the gel-view from the 0.01 ng DNA template. However, lane 5 corresponding to 0.005 ng/µL concentration showed bands for products from pig (73 bp), buffalo (138 bp), duck (203 bp), goat (236 bp) and sheep (263 bp). However, no band was observed for cow (106 bp) and chicken (161bp) in lane 5. The electropherogram (Figure 4.12c) also clearly showed five peaks corresponding to the five different bands. Therefore, the LOD for pig, buffalo, duck, goat and sheep was 0.005 ng, whereas that for cow and chicken was 0.01 ng.

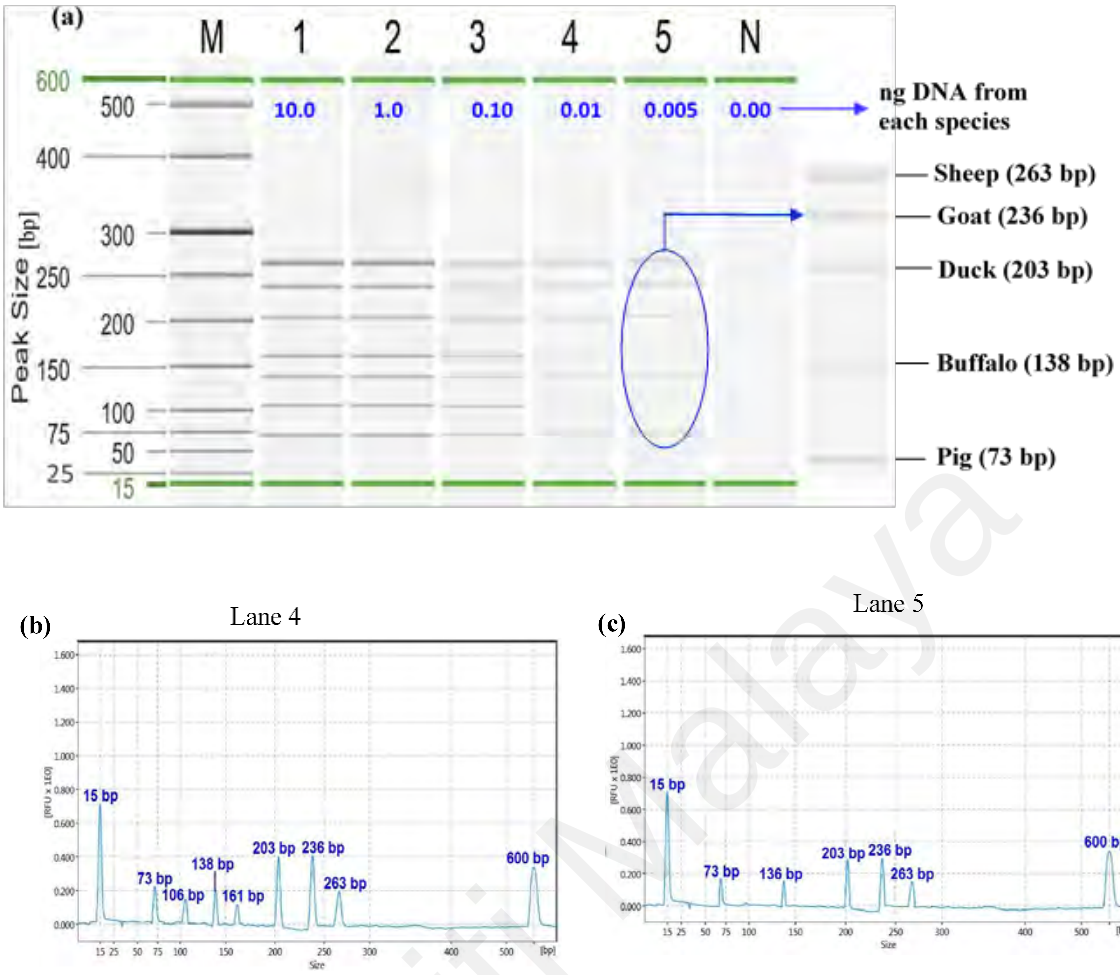


Figure 4.12: Evaluation of multiplex PCR assay sensitivity. In the gel view (a), lane M is the DNA ladder, lanes 1–5 represent the heptaplex PCR products of 10.0, 1.0, 0.1, 0.01, and 0.005 ng of the mixed DNA of pig, cow, buffalo, chicken, duck, goat, and sheep, respectively; and lane N is the negative control (0.00 ng of DNA). The electropherograms (b) and (c) of lanes 4 and 5, respectively, are shown with labels.

4.5.4 Target DNA Stability Test under Heptaplex PCR Assay

Extreme heat or processing treatments usually cause breakdown or degradation of DNA in food products. In order to study the effect of different thermal treatments on target DNA, our developed technique has been evaluated under thermally treated meat samples (Hossain et al., 2017c). Three distinct thermal treatments were applied for the meat of cow, buffalo, goat, sheep, chicken, duck, and pig as described in Section 3.6.4. The assay stability was checked using DNA extracted from the processed samples. The assay could successfully detect all the seven target species from the samples under all heat treatments including the extensive autoclaving at 121°C and 15 psi and microwaving

at 600 and 700 W for 30 min (Figure 4.13). The electropherogram (Figure 4.13b) also clearly showed seven peaks corresponding to the seven different bands.

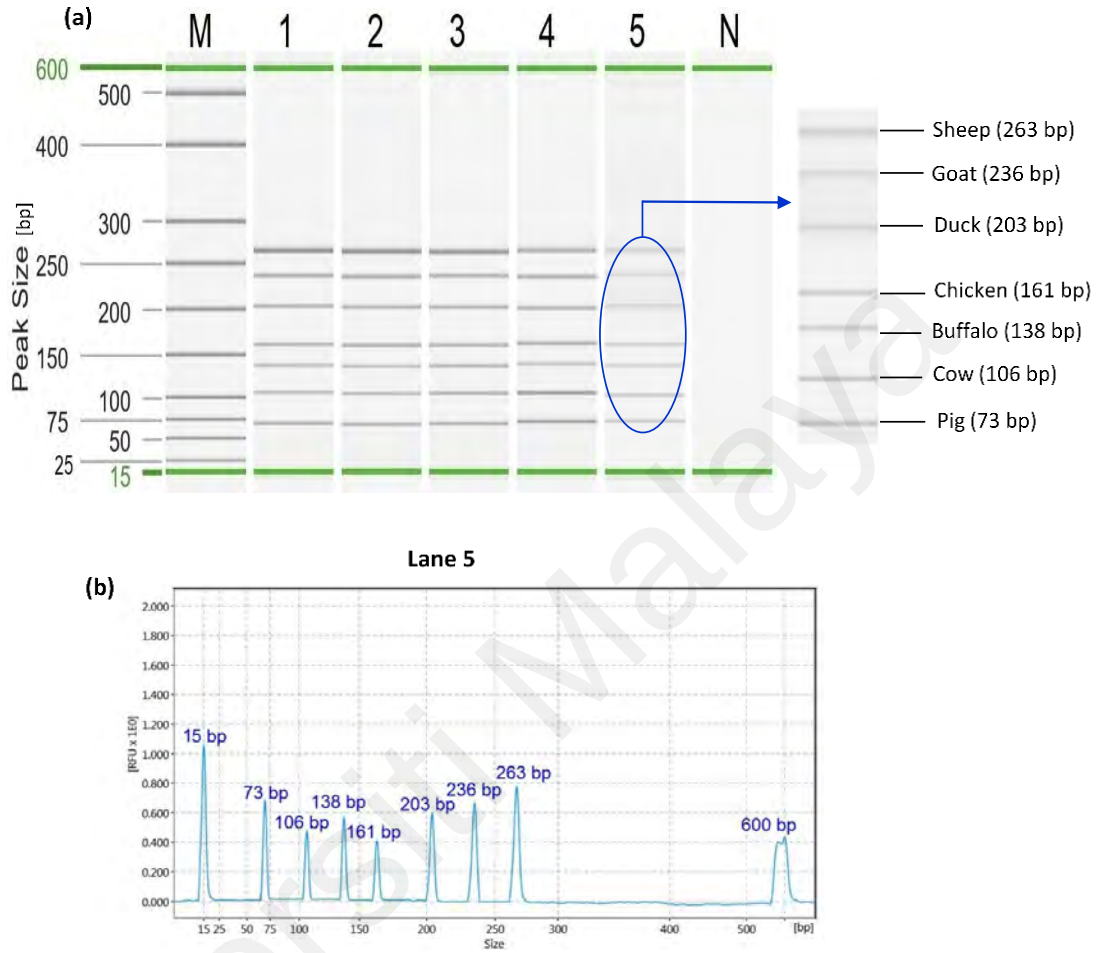


Figure 4.13: Gel image (a) and electropherogram (b) of the stability test of the developed heptaplex PCR assay. In the gel image (a), *lane* M represents the DNA ladder; *lanes* 1–5 represent the PCR products of DNA extracted from pig, cow, buffalo, chicken, duck, goat, and sheep species after heat treatments of boiling at 100 °C for 60 min and 90 min (*lanes* 1-2, respectively), microwaving at 600 and 700 W for 30 min (*lanes* 3–4, respectively), and autoclaving at 121°C under 15 psi for 20 min (lane 5) and *lane* N is the negative control. The corresponding electropherograms (b) of *lane* 5 is shown with labels.

4.5.5 Sensitivity of Heptaplex PCR Assay under Commercial Meat Products (Meatballs, Frankfurters, Burgers)

To simulate the commercial matrices, chicken, beef and pork model meatballs and frankfurters were prepared in the laboratory by deliberate adulteration as described in Section 3.6.5. After preparing deliberately adulterated model meatballs and frankfurters of the three species with six other corresponding target species at 5%, 1%, and 0.5% ratios (Section 3.6.5), the 0.5% adulterated meatballs and frankfurters were subjected to boiling at 100 °C for 90 min and autoclaving at 121°C under 15 psi pressure for 20 min to imitate the cooking effect. The experimental results of meatballs and frankfurters are depicted in Figures 4.14 and 4.15, respectively and the related data are summarized in Table 4.17. Each of the seven targets of the model beef, chicken and pork meatballs and frankfurters adulterated with six other corresponding target species at different ratios (5%, 1%, and 0.5%) was successfully amplified (Figure 4.14; lanes 1−3, 6−8, and 11−13 and Figure 4.15; lanes 1−3, 6-8 and 11−13, respectively). Notably, the 0.5% spiked meatballs and frankfurters treated by boiling and autoclaving also perfectly amplified the seven targets (lanes 4, 9, and 14; lanes 5,10, and 15 respectively of Figures 4.14 and 4.15).

The applicability of the developed heptaplex PCR assay to commercial meat products has been checked by screening 26 meatballs (beef, chicken and pork), 20 curry samples (beef and chicken) and 50 burgers and frankfurters (beef, chicken and pork) of different brands available in Malaysian markets. Analysis of commercial meatballs (Table 4.18) shows that 100% of the tested beef meatballs were mislabelled; two samples did not contain any beef DNA indicating complete species substitution. Out of 10 beef meatballs, 4 samples were found positive for both beef and buffalo, 4 for beef, buffalo and chicken, one for chicken and buffalo, whereas one contained only buffalo DNA. Undeclared buffalo DNA was detected in 90% (9/10) of the beef curry samples. On the other hand, 30% (3/10) of tested chicken meatballs were found to be positive for undeclared species

(beef and/or buffalo). Again, when burgers and frankfurters were analyzed, the incidence of mislabelling was observed in only beef products, which contributed 54 % of the total tested samples. Out of 19 samples labelled as beef, 16 (84 %) were found to contain buffalo DNA, whereas chicken DNA was detected in 2 (10.5 %) samples. Moreover, none of the porcine products tested in this study were found contaminated revealing the integrity of these items (Table 4.18).

Figure 4.14: Gel image of the multiplex PCR products for the detection of cow, buffalo, chicken, duck, goat, sheep and pig species in deliberately adulterated model beef, chicken, and pork meatballs under raw and processed states. In the gel image: *lane* M is the DNA ladder; *lanes* 1–3 represent the multiplex PCR products of beef meatball spiked with 5%, 1%, and 0.5% meat of buffalo, chicken, duck, goat, sheep, and pig, respectively, under raw state; *lanes* 6–8, represent the multiplex PCR products of chicken meatball spiked with 5%, 1%, and 0.5% meat of cow, buffalo, duck, goat, sheep, and pig, respectively, under raw state; *lanes* 11–13 represent the multiplex PCR products of pork meatball spiked with 5%, 1%, and 0.5% meat of cow, buffalo, chicken, duck, goat, and sheep, respectively, under raw state; *lanes* 4, 9, and 14 represent the multiplex PCR products of boiled (at 100 °C for 90 min) 0.5% adulterated beef, chicken, and pork meatballs respectively; *lanes* 5, 10, and 15 represent the multiplex PCR products of autoclaved (at 121 °C under 15 psi pressure for 20 min) 0.5% adulterated beef, chicken, and pork meatballs, respectively; and *lane* N is the negative control.

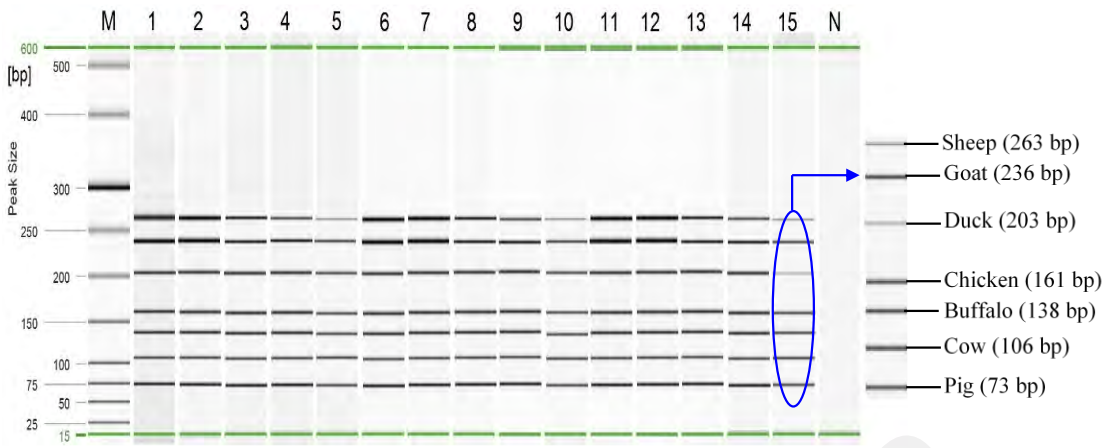


Figure 4.15: Gel image of heptaplex PCR (mPCR) for the detection of beef, buffalo, chicken, duck, goat, sheep, and pork in deliberately adulterated model beef, chicken, and pork frankfurters under raw and processed states. In the gel image, M, Ladder; lanes 1−3, mPCR of beef frankfurter spiked with 5%, 1%, and 0.5% of buffalo, chicken, duck, goat, sheep, and pork, respectively, under raw state; lanes 6−8, mPCR of chicken frankfurter spiked with 5%, 1%, and 0.5% of beef, buffalo, duck, goat, sheep, and pork, respectively, under raw state; lanes 11−13, mPCR of pork frankfurter spiked with 5%, 1%, and 0.5% of beef, buffalo, chicken, duck, goat, and sheep, respectively, under raw state; lanes 4, 9, and 14, mPCR of heat-treated (boiled at 100 °C for 90 min) 0.5% adulterated beef, chicken, and pork frankfurter, respectively; lanes 5, 10, and 15, mPCR of heat-treated (autoclaved at 121°C and 15 psi for 20 min) 0.5% adulterated beef, chicken, and pork frankfurter, respectively; lane N, negative control.

Table 4.17: Screening of model meatball and frankfurter products using developed heptaplex PCR

Meat Products	Adulteration		Number of samples	Type of treatment	Detected species[a]							Detection accuracy
	Species	%			Cow	Buffalo	Chicken	Duck	Goat	Sheep	Pig	
Model meatballs												
Beef meatball	Buffalo, chicken, duck, goat, sheep and pig	5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef meatball	Buffalo, chicken, duck, goat, sheep and pig	1	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef meatball	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef meatball	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef meatball	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken meatball	Beef, buffalo, duck, goat, sheep and pig	5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken meatball	Beef, buffalo, duck, goat, sheep and pig	1	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken meatball	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken meatball	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken meatball	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork meatball	Beef, buffalo, chicken, duck, goat and sheep	5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork meatball	Beef, buffalo, chicken, duck, goat and sheep	1	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork meatball	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork meatball	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork meatball	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100

Table 4.17, continued

Meat Products	Adulteration		Number of samples	Type of treatment	Detected species[a]							Detection accuracy
	Species	%			Cow	Buffalo	Chicken	Duck	Goat	Sheep	Pig	
Model Frankfurters												
Beef Frankfurter	Buffalo, chicken, duck, goat, sheep and pig	5.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef Frankfurter	Buffalo, chicken, duck, goat, sheep and pig	1.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef Frankfurter	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef Frankfurter	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Beef Frankfurter	Buffalo, chicken, duck, goat, sheep and pig	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken Frankfurter	Beef, buffalo, duck, goat, sheep and pig	5.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken Frankfurter	Beef, buffalo, duck, goat, sheep and pig	1.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken Frankfurter	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken Frankfurter	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Chicken Frankfurter	Beef, buffalo, duck, goat, sheep and pig	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork Frankfurter	Beef, buffalo, chicken, duck, goat and sheep	5.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork Frankfurter	Beef, buffalo, chicken, duck, goat and sheep	1.0	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork Frankfurter	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Raw	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork Frankfurter	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Boil	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
Pork Frankfurter	Beef, buffalo, chicken, duck, goat and sheep	0.5	3	Autoclave	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100

[a]Note: The numerator and denominator of each fraction denote the number of positive detection and total number of samples analyzed using the heptaplex PCR assay

Table 4.18: Screening of commercial meatball, frankfurter, burger and curry samples using developed heptaplex PCR

Samples	State	Detected species[a]							Detection accuracy
		Cow	Buffalo	Chicken	Duck	Goat	Sheep	Pig	
Commercial meat products									
Beef meatball	Raw	8/10	10/10	5/10	0/10	0/10	0/10	0/10	100
Chicken meatball	Raw	3/10	2/10	10/10	0/10	0/10	0/10	0/10	100
Pork meatball	Raw	0/6	0/6	0/6	0/6	0/6	0/6	6/6	100
Beef frankfurter	Raw	9/9	9/9	2/9	0/9	0/9	0/9	0/9	100
Chicken frankfurter	Raw	0/10	0/10	10/10	0/10	0/10	0/10	0/10	100
Pork frankfurter & burger	Raw	0/12	0/12	0/12	0/12	0/12	0/12	12/12	100
Beef burger	Raw	9/10	7/10	1/10	0/10	0/10	0/10	0/10	100
Chicken burger	Raw	0/9	0/9	9/9	0/9	0/9	0/9	0/9	100
Beef curry	Raw	1/10	9/10	0/10	0/10	0/10	0/10	0/10	100
Chicken curry	Raw	0/10	0/10	10/10	0/10	0/10	0/10	0/10	100

Note: The numerator and denominator of each fraction denote the number of positive detections and total number of samples analysed using the heptaplex PCR assa

4.6 Authentication of PCR Products by RFLP Analysis

4.6.1 Authentication of Beef, Buffalo, Chicken, Duck, Goat, Sheep and Pork PCR Products of Raw Meat by RFLP Analysis

In this study, the PCR products of pig, cow, buffalo, chicken, duck, goat, and sheep were digested with three restriction endonucleases as mentioned in Section 3.7.1) and distinctive fingerprints were obtained for each of the seven targets (Figure 4.16 and Table 3.8). The PCR products of Pig (73 bp) (Figure 4.16, lane 1), cow (106 bp) (Figure 4.16, lane 3), and Sheep (263 bp) (Figure 4.16, lane 13) were digested with *Fat*I *r*estriction endonuclease which produced two fragments for each target; 52 and 21 bp for pig (lane 2), 87 and 19 bp for cow (lane 4), and 153 and 110 bp for sheep (lane 14). On the other hand, chicken (161 bp) (Figure 4.16, lane 7), duck (203 bp) (Figure 4.16, lane 9), and goat (236 bp) (Figure 4.16, lane 11) products, upon digestion with *Bfa*I*,* resulted in two fragments for each target; 93 and 68 bp for chicken (lane 8), 141 and 62 bp for duck (lane 10), and 130 and 106 bp for goat products (lane 12). Again, *HPY1*88I endonuclease digested Buffalo product (138 bp) (lane 5) generating another two fragments (70 and 68 bp) (Figure 4.16, lane 6). However, given very narrow length difference (only 2 bp), the gel-band for the 70 bp fragment merged with that of 68 bp one and appeared as a thicker band since the technique has the limitation to resolute ≤5 bp difference in fragment length.

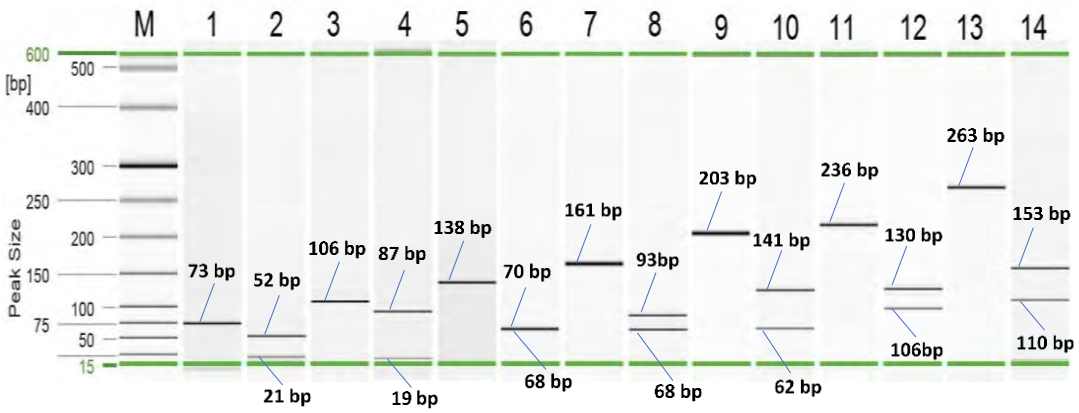


Figure 4.16: RFLP analysis of PCR products before (lanes 1, 3, 5, 7, 9, 11, and 13) and after (lanes 2, 4, 6, 8,10, 12, and 14) restriction digestion. In the gel image, M, Ladder; lanes 1 (undigested) and 2 (digested), PCR products of pork; lanes 3 (undigested) and 4 (digested), PCR products of beef; lanes 5 (undigested) and 6 (digested), PCR products of buffalo; lanes 7 (undigested) and 8 (digested), PCR products of chicken; lanes 9 (undigested) and 10 (digested), PCR products of duck; lanes 11 (undigested) and 12 (digested), PCR products of goat, lanes 13 (undigested) and 14 (digested), PCR products of sheep.

4.6.2 Authentication of PCR Products of Beef, Chicken and Pork Frankfurters by RFLP Analysis

After the heptaplex PCR-RFLP assay was optimized and performed under pure states, it was subsequently validated to screen commercial beef, chicken and pork frankfurters under raw and heat-treated states. Deliberately adulterated model beef, chicken, and pork frankfurters were prepared and subjected to boiling and autoclaving (Section 3.7.2). The PCR products from raw, heat-treated samples were digested and their restriction digestion patterns were analyzed (Figure: 4.17). The digests of all PCR products (Figure: 4.17; lanes 1, 4, 7, 10, 13, 16, and 19 for pork, beef, buffalo, chicken, duck, goat, and sheep, respectively) clearly presented their signature fingerprints (lanes 2 and 3; 5 and 6; 8 and 9; 11 and 12; 14 and 15; 17 and 18; 20 and 21, respectively). Notably, the restriction profiles of various heat-treated (boiled and autoclaved) samples too were very clearly visualized without being affected by thermal treatments.

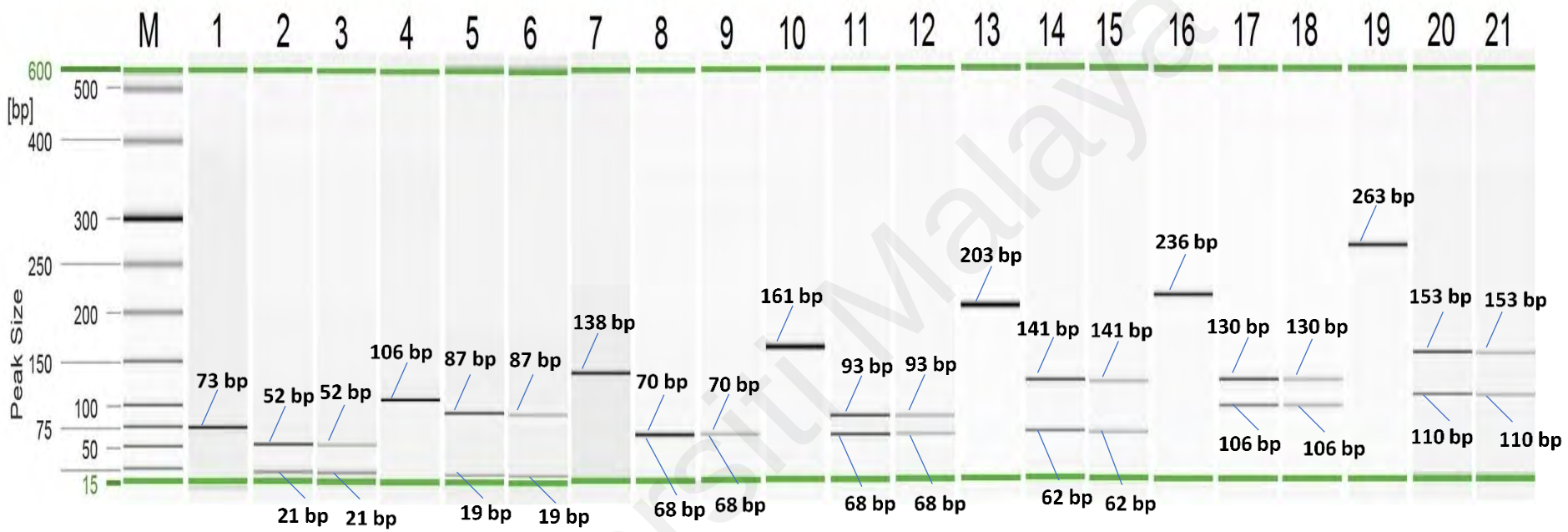


Figure 4.17: PCR-RFLP analysis of PCR products from deliberately adulterated and heat-treated (boiled and autoclaved) frankfurters. In gel image, M, Ladder; lane 1, pork product (from frankfurter) before digestion; lanes 2-3, pork products from frankfurter (boiled at 100 °C for 90 min and autoclaved at 121 °C and 15 psi pressure for 20 min, respectively) after digestion; lane 4, beef product (from frankfurter) before digestion; lanes 5-6, beef products from frankfurter (boiled at 100 °C for 90 min and autoclaved at 121 °C and 15 psi pressure for 20 min, respectively) after digestion; lane 7, buffalo product (from frankfurter) before digestion; lanes 8-9, buffalo products from frankfurter (boiled at 100 °C for 90 min and autoclaved at 121 °C and 15 psi pressure for 20 min, respectively) after digestion; lane 10, chicken product (from frankfurter) before digestion; lanes 11-12, chicken products from frankfurter (boiled at 100 °C for 90 min and autoclaved at 121 °C and 15 psi pressure for 20 min, respectively) after digestion; lane 13,

Figure 4.17, continued: duck product (from frankfurter) before digestion; lanes 14-15, duck products from frankfurter (boiled at 100°C for 90 min and autoclaved at 12°C and 15 psi pressure for 20 min, respectively) after digestion; lane 16, goat product (from frankfurter) before digestion; lane 17-18 goat products from frankfurter (boiled at 100°C for 90 min and autoclaved at 121°C and 15 psi pressure for 20 min, respectively) after digestion; lane 19 sheep product (from frankfurter) before digestion; lanes 20-21, sheep products from frankfurter (boiled at 100°C for 90 min and autoclaved at 121°C and 15 psi pressure for 20 min, respectively) after digestion.

4.7 TaqMan Probe-based Real-time PCR System

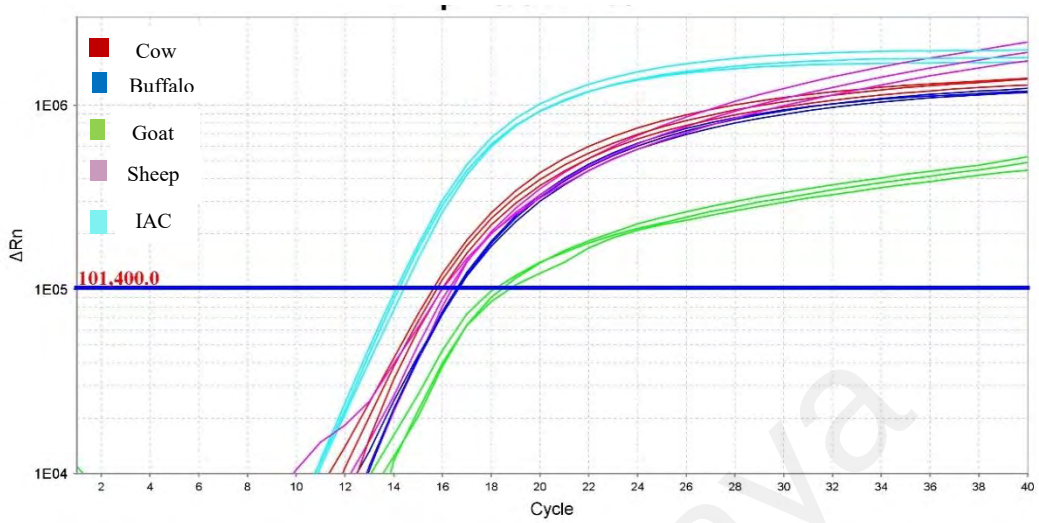
4.7.1 Design of Primers and Probes

The oligonucleotide primers and probes used in real-time PCR assay were designed targeting mitochondrial cytb and ND5 genes. Cytb genes were targeted for cow (*Bos taurus*), buffalo (*Bubalus bubalis*), chicken (*Gallus gallus*) and pig (*Sus scrofa*) species whereas ND5 genes were used for duck (*Anas platyrhychos*), goat (*Capra hircus*) and sheep (*Ovis aries*). In TaqMan-based assays both the specific primers and probes have to bind effectively. Furthermore, the development of mqPCR needs special attention and the melting temperatures (Tm) of all concerned primers and probes must be similar or very close to each other (Cheng et al., 2014). All the primers and probes used in this study were critically evaluated for Tm, GC and sequence mismatching. In this assay, primers with their Tm values within 57.8–61.7°C annealed at 60°C. The probes' Tm values (67.4 - 70.7°C) were 7.2 - 12.0°C higher than those of primers and this facilitated favourable probe binding. The Tm values of probes were maintained at minimum 8ºC higher than that of the primers. The Tm values differences between all primers were kept very narrow (less than 2ºC). Similarly, the Tm values differences between all probes were kept less 2^0 C. Moreover, the GC content of all primers and probes were within the recommended range. The developed primers and probes showed full matching only with the target species while multiple mismatches (3–18 nucleotides in primers and 3-20 nt in probes) were found with other non-targets (Tables 4.19 - 4.25).

4.7.2 Multiplex Real-time PCR Assay

Initially simplex qPCR assays were optimized for each target species using respective primers and probes and next, two different mqPCR systems (mqPCR-1 and mqPCR-2) were optimized by including additional species sequentially. After running PCR, the Ct (threshold cycle) values were generated and the mean Ct values for the mqPCR-1 assay were 15.775, 16.612, 18.490, 16.195 and 14.211 for bovine, buffalo, goat, sheep and IAC respectively. Again, for mqPCR-2, the mean Ct values were obtained 21.034, 19.999, 14.982 and 17.321for chicken, duck, pig and IAC, respectively (Figure 4.18).

(a)



(b)

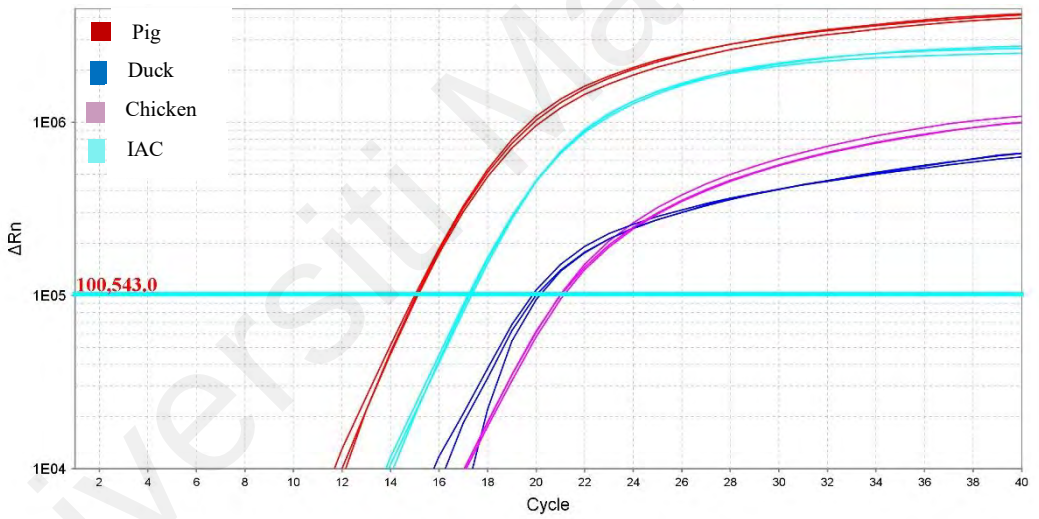


Figure 4.18: Amplification plot plot of mqPCR for cow, buffalo, goat, sheep, chicken, duck, pig and internal amplification control (IAC). Amplification plot for (a) cow, buffalo, goat, sheep and IAC and (b) chicken, duck, pig and IAC.

4.7.3 Specificity of Multiplex Real-time PCR System

The designed primers and probes were ensured of specificity through different testing steps. First, the basic local alignment search tool (BLAST) was used against nonredundant nucleotide sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the target species and the dissimilarity index value with other species. Second, the primers/probes were aligned against target species as well as 30 other nontarget animal and plant species using a ClustalW sequence alignment program (http://www.genome.jp/tools/clustalw/) and the MEGA7 software to determine *in-silico* specificity. Thus, sequence matching was critically evaluated among targets and other related non-target species. Complete sequence matching (100 %) was observed only with the target species. On the other hand, multiple mismatches (3–18 nucleotides in primers and 3-20 nt in probes) were found with other non-target species (Tables 4.19 - 4.25).

The experimental specificity of both mqPCR systems (mqPCR-1 and mqPCR-2) were performed using DNA from seven target species and 23 nontarget species (dog, rabbit, turtle, rat, pigeon, quail, monkey, donkey, ostrich, crocodile, frog, tortoise, cat, salmon, tuna, sardine, rohu, cod, tilapia, wheat, onion, garlic and pepper) on three different days in triplicates. According to the amplification profile it is clear that the 40 cycle PCR assay generates species specific amplification curves as well as background fluorescence for the relevant species eliminating the chances of cross-amplifications (Figure 4.19 a,b). Moreover, the IAC successfully amplified all the eukaryotic targets reflecting good quality of extracted DNA in all the tubes (Figure 4.19 a,b). The mqPCR-1 system gave the amplification signals (Ct values) of 18.16, 19.58, 20.37, 18.31 for bovine, buffalo, goat and sheep and for the mqPCR-2 assay, the Ct values were 24.16, 23.13 and 17.68 for chicken duck and pig respectively. However, none of the nontarget species yielded

any detectable Ct (Tables 4.26 and 4.27). On the other hand, the IAC with the Ct values of 16.41-16.90 and 19.83-20.71 for mqPCR-1 and mqPCR-2, respectively gave significant detectable signals for all the target and nontarget species (Tables 4.26 and 4.27), eliminating the possibility of false positive detection.

Table 4.19: The mismatch comparison of cow Cytb-specific 120 bp site against other 30 non-target species

Species	Forward Primer																				Mis-match	Probe																					Mis-match	Reversed Primer																				Mis-match
Cow (V00654.1)	C	G	G	C	A	C	A	A	A	T	T	T	A	G	T	C	G	A	A	T		T	T	C	T	T	T	C	C	A	T	T	T	T	A	T	C	C	T	T	C	C	A		A	A	T	G	C	C	T	A	T	T	C	T	G	A	G	C	C	C	T	A	G	T	A	G	
Cow (V00654.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Chicken ((NC_001323.1)	T	.	.	A	C	A	C	.	C	C	C	.	.	.	.	A	.	.	G	.	10	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	C	C	.	.	.	C	.	.	C	6	.	.	A	C	.	.	.	.	.	.	.	.	C	T	T	.	.	.	.	.	.	5
Sheep (KR868678.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	3	.	.	T	.	.	.	.	C	.	.	T	.	.	C	.	.	.	.	T	T	.	C	.	.	.	7	.	.	.	.	T	A	.	.	.	.	.	.	A	T	.	.	.	.	.	.	.	4
Duck (EU009397.1)	.	.	.	A	C	A	G	.	C	C	C	.	G	.	.	A	.	.	.	.	9	.	.	.	.	.	.	.	C	A	.	T	.	.	C	.	.	C	C	.	A	.	.	A	.	.	C	9	.	.	C	T	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	G	.	5
Goat (KP271023.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	3	.	.	.	.	.	A	.	.	.	.	.	.	A	T	.	.	.	G	.	.	.	4
Pig (KJ782448.1)	.	.	.	A	.	.	.	G	.	C	C	.	C	.	.	A	.	.	.	.	6	.	.	.	.	.	.	.	C	.	.	T	.	.	C	.	.	.	.	.	.	.	G	.	.	C	5	.	.	.	.	.	.	.	.	.	.	.	.	A	T	A	.	.	.	.	.	.	3
Buffalo (NC 006295.1)	T	.	.	T	.	.	.	G	.	C	.	G	.	.	T	.	.	G	.		7	.	.	.	.	.	.	.	A	.	.	T	.	.	C	.	.	C	.	.	.	.	C	.	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	A	T	T	.	.	.	.	.	.	3
Pigeon (KJ722068.1)	T	.	.	.	C	A	.	.	C	C	C	.	C	.	.	T	.	.	.	.	8	.	.	.	.	.	.	A	.	C	C	.	T	.	.	C	.	.	C	C	.	.	.	C	.	.	C	9	.	.	C	T	.	.	.	.	.	.	.	.	A	T	.	.	.	.	.	C	A	6
Quail (KX712089.1)	.	.	.	.	C	A	.	.	C	C	C	.	.	.	.	A	.	.	.	.	6	.	.	.	.	.	.	.	C	C	.	.	.	.	C	.	.	C	C	.	.	.	C	.	.	.	6	.	.	A	C	.	.	.	.	.	.	.	.	C	T	T	.	.	.	.	.	.	5
Cod (NC 002081.1)	A	.	.	T	G	A	T	G	C	C	.	.	.	.	.	T	C	.	.	.	10	.	.	T	.	.	T	.	.	A	.	.	.	.	.	.	.	C	T	.	A	T	C	.	.	C	9	.	.	A	T	A	.	.	.	.	.	.	.	T	.	.	.	C	.	.	T	.	6
Salmon (KF792729.1)	A	.	.	A	G	G	C	G	C	C	C	.	T	.	.	A	C	.	.	.	12	.	.	T	.	.	.	.	C	.	.	.	.	.	C	.	.	C	C	.	A	T	.	C	.	.	.	8	.	.	.	T	.	T	.	.	.	.	.	.	G	A	.	.	.	G	.	.	.	5
Pangas (NC 023924.1)	A	.	.	A	G	A	C	.	C	C	C	.	.	.	.	A	C	.	.	.	10	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	C	C	.	A	.	.	C	.	.	.	6	.	.	C	T	.	.	.	.	.	.	.	.	A	T	T	.	.	.	.	.	.	5
Tuna (KF906721.1)	T	.	.	A	.	.	T	.	C	C	C	.	C	.	.	T	.	.	.	.	8	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	C	C	.	A	T	.	C	.	.	.	7	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	T	A	.	T	.	5
Tilapia (GU238433.1)	T	.	.	.	.	A	T	T	C	C	.	.	.	.	.	.	C	.	.	.	7	.	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	C	C	.	.	T	.	C	.	.	C	7	.	.	.	T	.	T	.	.	.	.	.	.	C	T	T	T	.	.	A	.	T	.	8
Rohu (NC 017608.1)	A	.	.	A	G	A	C	.	T	A	.	.	.	.	.	.	C	.	.	.	8	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	C	C	.	A	.	.	A	.	.	.	6	.	.	.	T	T	.	.	.	.	.	.	.	A	.	T	T	.	.	.	.	.	.	5
Frog (NC 024548.1)	.	.	.	.	T	.	T	.	.	C	C	.	T	.	.	.	C	.	.	.	6	.	.	T	.	.	T	A	.	A	.	.	T	.	.	C	.	.	C	.	.	T	.	.	C	.	.	.	9	.	.	A	T	.	T	.	T	.	.	T	.	.	.	.	.	T	.	G	A	.	T	.	9
Turtle (NC 014769.1)	T	.	.	.	.	A	T	.	C	C	C	.	T	.	.	A	C	.	.	.	9	.	.	.	.	.	.	A	.	C	.	.	T	.	.	C	.	.	.	C	.	A	.	.	C	.	.	C	8	.	.	A	T	T	.	.	C	C	.	T	.	.	.	T	G	.	T	.	.	.	C	.	.	10
Deer (NC 006993.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	3	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	T	.	.	.	.	.	3
Dog (MH891616.1)	.	.	.	A	.	.	T	G	.	C	.	.	.	.	.	A	.	.	.	.	5	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	T	5	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	T	.	.	.	C	.	5
Rabbit (AJ001588.1)	.	.	.	A	.	.	.	.	C	C	.	.	.	.	.	T	.	.	.	.	4	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	C	.	.	.	T	.	G	.	.	.	5	.	.	G	T	.	.	.	.	.	.	.	.	.	T	T	.	.	C	.	.	C	.	6
Rat (012374.1)	T	.	.	.	.	.	C	.	C	.	C	.	.	.	.	.	.	.	.	.	4	.	.	T	.	.	.	.	A	.	.	.	.	.	C	.	.	C	.	.	.	.	C	.	.	.	5	.	.	A	T	T	.	.	.	.	.	.	.	.	.	A	T	T	.	.	.	.	.	.	7
Monkey (NC 012670.1)	.	.	.	A	.	.	T	.	.	C	C	.	T	.	.	.	C	.	.	.	6	.	.	.	.	.	A	.	C	C	.	G	.	.	C	.	.	.	.	.	.	.	A	.	.	C	7	.	.	.	T	.	.	.	.	.	.	.	.	C	T	.	.	.	A	.	C	A	6
Cat (NC 001700.1)	.	.	.	C	.	.	T	G	.	A	C	.	.	.	.	A	.	.	.	.	6	.	.	.	.	.	T	.	G	C	.	.	.	.	C	.	.	C	.	.	T	.	.	.	.	.	6	.	.	.	.	.	T	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	3
Horse (KU575247.1)	.	.	.	T	.	.	T	.	C	C	C	.	C	.	.	.	.	.	G	.	7	.	.	T	.	.	T	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	A	.	.	C	6	.	.	.	.	.	G	.	.	.	.	.	.	C	T	.	T	.	.	.	G	.	5
Donkey (KT182635.1)	.	.	.	T	.	.	T	.	C	A	C	.	C	.	.	.	.	.	.	.	6	.	.	T	.	.	T	.	.	C	.	.	.	.	C	.	.	.	.	.	T	.	.	A	.	.	C	7	.	.	.	.	.	G	.	G	.	.	.	.	C	T	.	T	.	.	.	.	.	5
Crocodyle (DQ273698.1)	A	.	.	A	G	A	C	T	C	A	A	.	T	.	.	.	A	C	C	.	13	.	.	T	A	C	T	A	.	C	C	.	G	.	.	C	.	.	C	C	.	G	.	.	C	.	.	13	.	.	C	T	T	T	.	.	.	.	.	.	.	T	.	.	.	A	.	C	.	7
Ostrich (NC 002785.1)	.	.	.	A	C	A	.	.	C	C	C	.	.	.	.	A	.	.	G	.	8	.	.	.	.	.	T	.	.	C	C	.	T	.	.	C	C	.	.	C	.	.	.	C	.	.	.	8	.	.	.	T	A	T	.	.	.	.	.	.	T	T	T	.	.	.	.	G	.	7
Turkey (NC 010195.2)	T	.	.	T	C	A	.	.	C	C	C	.	.	.	.	A	.	.	.	.	8	.	.	.	.	.	.	.	.	C	C	.	.	.	.	C	C	.	.	C	.	.	.	C	.	.	C	7	.	.	A	C	.	T	.	.	.	.	.	.	C	T	.	T	.	.	.	.	.	6
Onion (NC 030100.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	G	A	C	T	.		12	.	.	T	.	.	T	A	G	.	C	.	.	.	.	.	C	A	.	T	.	A	.	.	C	.	.	C	11	.	.	G	.	A	A	.	.	.	.	T	.	.	G	T	T	G	.	.	T	T	.	G	.	11
Wheat (GU985444.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	G	A	C	T	.		12	.	.	T	.	.	T	A	G	.	C	.	.	.	.	.	C	A	.	T	.	A	.	.	C	.	.	C	11	.	.	G	.	A	A	.	.	.	.	T	.	.	G	T	T	G	.	.	T	T	.	G	.	11
Pepper (KJ865410.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	G	A	C	T	.		12	.	.	T	.	.	T	A	G	.	C	.	T	.	.	.	C	A	.	T	.	A	.	.	C	.	.	C	12	.	.	G	.	A	A	.	.	.	.	T	.	.	G	T	T	G	.	.	T	T	.	G	.	11

Table 4.20: The mismatch comparison of buffalo Cytb-specific 90 bp site against other 30 non-target

Species	Forward Primer																						Mis-match	Probe																							Mis-match	Reversed Primer																							Mis-match
Buffalo (NC 006295.1)	G	G	G	T	T	C	T	A	G	C	C	C	T	A	G	T	T	C	T	C	T	C	T	C	T	Match	A	A	T	C	C	T	C	A	T	T	C	T	C	A	T	G	C	C	C	C	T	G	C	T	A	C	A	Match	G	G	G	T	T	C	T	A	G	C	C	C	T	A	G	T	T	C	T	C	T	C	T	Match
Buffalo (NC 006295.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Chicken (NC 001323.1)	.	T	.	.	A	.	.	.	.	.	.	.	.	.	C	A	G	C	.	.	.	A	7	C	.	.	.	.	.	T	.	C	.	.	A	.	.	C	.	.	.	T	.	C	.	.	C	.	.	8	.	T	.	.	A	.	.	.	.	.	.	.	.	C	A	G	C	.	.	.	A	7
Sheep (KR868678.1)	.	A	.	.	C	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.	A	6	.	G	.	.	.	.	A	G	.	A	A	.	T	.	.	A	.	.	.	.	.	C	.	.	C	.	.	9	.	A	.	.	C	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.	A	6
Duck (EU009397.1)	.	C	.	.	C	.	.	.	.	.	A	.	.	.	.	C	C	G	C	.	.	.	C	8	.	.	.	.	.	.	A	T	.	C	.	.	G	G	.	C	.	.	.	T	.	C	.	.	C	.	.	9	.	C	.	.	C	.	.	.	.	.	A	.	.	.	.	C	C	G	C	.	.	.	C	8
Goat (KP271023.1)	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	4	.	.	.	.	T	.	A	G	.	A	.	.	T	G	.	A	.	.	.	T	.	C	.	.	C	.	.	10	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	4
Pig (KJ782448.1)	.	A	.	.	G	T	.	.	.	.	.	.	.	.	.	A	A	C	.	.	.	C	7	.	.	.	.	.	.	A	.	.	.	T	.	A	.	.	.	.	.	.	A	.	A	.	.	.	.	.	5	.	A	.	.	G	T	.	.	.	.	.	.	.	.	.	A	A	C	.	.	.	C	7
Cow (AF492351.1)	.	A	.	.	A	.	.	.	.	.	.	.	.	.	C	C	T	.	.	.	.	.	5	.	.	.	T	.	.	T	G	C	.	.	.	A	.	.	C	.	.	.	.	.	A	.	.	.	.	.	6	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	C	C	T	.	.	.	.	.	5
Pigeon (KJ722068.1)	.	A	.	.	A	.	.	.	.	.	T	.	.	.	C	C	G	C	.	.	.	A	8	.	.	.	T	.	.	A	T	.	C	.	.	.	.	C	C	.	.	.	.	.	A	.	.	.	.	.	8	.	A	.	.	A	.	.	.	.	.	.	T	.	.	.	C	C	G	C	.	.	.	A	8
Quail (KX712089.1)	.	C	.	.	A	.	.	T	.	.	.	.	T	.	C	A	G	C	.	.	.	A	9	T	.	.	.	.	.	.	C	.	.	.	.	A	.	.	C	.	.	.	T	.	C	.	.	C	.	.	7	.	C	.	.	A	.	.	T	.	.	.	.	T	.	C	A	G	C	.	.	.	A	9
Cod (NC 002081.1)	.	C	.	.	A	.	.	T	.	.	A	.	.	C	C	.	A	T	.	.	.	G	9	.	G	.	.	.	.	.	.	G	G	.	T	G	.	A	.	.	.	T	.	T	.	.	C	.	.	9	.	C	.	.	A	.	.	T	.	.	A	.	.	C	C	.	A	T	.	.	.	G	9
Salmon (KF792729.1)	.	A	.	.	A	.	.	C	.	.	.	.	.	C	T	.	A	T	.	.	.	G	8	G	G	.	.	.	T	.	.	A	G	.	.	G	.	C	.	.	.	A	.	C	.	.	C	.	.	10	.	A	.	.	A	.	.	C	.	.	.	.	.	C	T	.	A	T	.	.	.	G	8
Pangas (NC 023924.1)	.	A	.	.	A	.	.	.	.	.	.	.	.	G	C	.	A	T	.	.	.	.	6	.	G	.	A	T	.	A	.	.	A	G	.	T	G	.	C	.	.	.	.	.	A	T	.	.	.	.	11	.	A	.	.	A	.	.	.	.	.	.	.	.	G	C	.	A	T	.	.	.	.	6
Tuna (KF906721.1)	.	A	.	.	A	.	.	.	.	.	.	.	.	C	C	.	A	G	C	.	.	.	C	8	C	G	.	A	.	.	T	.	.	A	G	.	A	G	.	G	C	.	.	.	T	.	C	.	.	.	.	11	.	A	.	.	A	.	.	.	.	.	.	.	.	C	C	.	A	G	C	.	.	.	C	8
Tilapia (GU238433.1)	.	A	.	.	C	.	.	C	.	.	.	.	.	C	T	.	A	T	.	.	.	A	8	T	G	.	.	.	G	.	.	A	G	.	T	G	.	A	.	.	.	A	.	T	.	.	C	.	.	11	.	A	.	.	C	.	.	C	.	.	.	.	.	C	T	.	A	T	.	.	.	.	A	8
Rohu (NC 017608.1)	.	A	.	.	C	.	.	T	.	.	A	T	.	.	C	.	A	T	.	.	.	A	9	.	G	.	A	T	.	A	.	.	A	G	.	A	G	.	.	.	.	.	.	C	.	.	.	.	.	9	.	A	.	.	C	.	.	T	.	.	A	T	.	.	C	.	A	T	.	.	.	.	A	9
Frog (NC 024548.1)	.	A	.	.	C	.	.	.	.	.	.	.	T	C	.	C	.	.	A	.	.	A	7	G	G	.	T	.	.	.	T	.	C	.	.	G	.	.	A	.	.	.	.	.	C	A	C	T	.	.	11	.	A	.	.	C	.	.	.	.	.	.	.	T	C	.	C	.	.	A	.	.	A	7
Turtle (NC 014769.1)	.	T	.	.	A	.	.	T	.	.	.	.	C	C	.	A	T	C	.	.	.	C	9	T	G	.	A	.	.	A	T	.	.	A	.	A	G	.	A	.	.	A	A	C	C	.	.	G	.	.	14	.	T	.	.	A	.	.	T	.	.	.	.	.	C	C	.	A	T	C	.	.	.	C	9
Deer (NC 006993.1)	.	A	.	.	C	T	.	.	.	.	.	.	.	.	.	C	T	C	A	.	.	C	8	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	T	.	.	5	.	A	.	.	C	T	.	.	.	.	.	.	.	.	.	C	T	C	A	.	.	C	9
Dog (MH891616.1)	.	T	.	.	A	.	.	C	.	.	.	.	.	.	.	A	T	.	.	.	.	C	6	.	.	.	.	T	.	G	G	C	A	T	.	.	.	.	T	.	.	A	.	.	C	.	.	C	.	.	10	.	T	.	.	A	.	.	C	.	.	.	.	.	.	.	.	A	T	.	.	.	.	C	6
Rabbit (AJ001588.1)	.	A	.	.	C	.	.	.	.	.	T	.	.	.	.	C	.	.	A	.	.	C	6	T	G	.	T	.	.	A	G	C	C	T	.	.	.	.	C	.	.	A	T	.	C	.	.	T	.	.	10	.	A	.	.	C	.	.	.	.	.	T	.	.	.	.	C	.	.	A	.	.	C	6
Rat (012374.1)	.	A	.	.	A	G	.	.	.	.	.	T	.	.	.	.	C	.	.	A	.	.	A	7	.	.	.	.	T	.	A	G	C	C	T	.	T	C	.	A	.	.	A	T	.	C	T	.	.	.	13	.	A	.	.	A	G	.	.	.	.	.	T	.	.	.	.	C	.	.	A	.	.	A	7
Monkey (NC 012670.1)	.	C	.	.	A	.	.	.	.	.	A	T	.	.	T	.	C	.	.	A	.	.	A	8	C	.	.	.	T	.	A	G	C	A	G	C	.	.	.	C	.	.	.	A	.	A	.	.	T	.	.	12	.	C	.	.	A	.	.	.	.	.	A	T	.	.	T	.	C	.	.	A	.	.	A	8
Cat (NC 001700.1)	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	4	.	G	.	A	.	.	A	G	C	A	A	.	.	.	.	T	.	.	A	A	.	C	.	.	C	.	.	12	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	4
Horse (KU575247.1)	.	C	.	.	A	T	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	C	6	G	.	.	.	.	.	A	G	C	A	.	.	.	.	.	C	.	.	.	A	C	C	.	.	C	.	.	10	.	C	.	.	A	T	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	C	6
Donkey (KT182635.1)	.	T	.	.	A	T	.	.	.	.	.	.	T	A	.	C	.	.	T	.	.	C	8	.	.	.	.	.	.	A	G	C	A	.	.	.	.	.	C	.	.	.	A	C	C	.	.	.	.	.	8	.	T	.	.	A	T	.	.	.	.	.	.	.	T	A	.	C	.	.	T	.	.	C	8
Crocodyle (DQ273698.1)	.	A	.	.	C	A	.	T	.	.	.	A	.	.	T	.	.	G	C	A	.	.	C	10	.	G	.	A	T	.	A	C	.	A	.	.	.	C	.	A	.	.	T	G	C	A	.	.	.	.	12	.	A	.	.	C	A	.	T	.	.	.	A	.	.	T	.	.	G	C	A	.	.	C	10
Ostrich (NC 002785.1)	.	T	.	.	A	.	.	T	.	.	.	T	.	.	.	C	C	G	C	.	.	.	C	9	.	.	.	.	.	.	A	T	.	C	.	.	A	.	.	C	.	.	A	.	.	C	.	.	G	.	.	8	.	T	.	.	A	.	.	T	.	.	.	T	.	.	.	C	C	G	C	.	.	.	C	9
Turkey (NC 010195.2)	.	T	.	.	C	.	.	.	.	.	.	T	.	.	.	C	A	G	C	A	.	.	A	9	C	.	.	T	.	.	T	C	.	C	.	.	T	.	.	C	.	.	.	T	C	.	.	T	.	.	10	.	T	.	.	C	.	.	.	.	.	.	T	.	.	.	C	A	G	C	A	.	.	A	9
Onion (NC 030100.1)	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13	.	T	C	T	.	.	G	T	.	G	G	C	T	T	.	A	.	.	.	T	.	T	T	.	T	A	.	16	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13
Wheat (GU985444.1)	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13	.	T	C	T	.	.	.	T	.	G	G	C	T	T	.	A	.	.	T	T	.	T	T	.	T	A	.	16	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13
Pepper (KJ865410.1)	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13	.	T	G	T	.	.	G	T	.	G	G	C	T	T	.	A	.	.	.	T	.	T	T	.	T	A	.	16	.	T	.	.	A	G	C	C	.	.	A	A	.	.	.	C	A	.	C	A	G	T	.	13

Table 4.21: The mismatch comparison of goat ND5-specific 236 bp site against other 30 non-target

Species	Forward Primer																									Mis-	Probe																									Mis-	Reversed Primer																									Mis-
Goat (KP271023.1)	T	G	T	T	T	T	C	T	T	C	T	C	T	T	G	C	A	C	T	A	A	C	C	C	A	C	match	C	C	G	C	A	C	C	C	A	T	C	A	T	A	A	T	A	A	C	C	A	A	C	C	T	C	match	C	C	T	T	A	A	A	T	T	A	T	C	G	C	T	C	A	G	C	T	T	C	A	A	G	match
Goat (KP271023.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (KJ782448.1)	C	A	.	.	C	G	.	C	.	.	A	.	.	C	A	.	.	T	.	.	.	.	.	.	.	.	9	.	.	A	T	C	.	.	A	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	T	C	A	9	.	G	.	A	G	.	.	C	.	C	.	.	T	.	.	T	.	.	.	.	.	T	.	.	A	9
Cow (AF492351.1)	.	A	.	.	C	.	.	C	.	.	A	.	.	C	T	.	.	.	.	.	G	T	T	.	.	.	9	.	T	A	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	A	.	G	.	T	.	T	9	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	A	4
Buffalo (NC 006295.1)	.	A	.	.	C	C	.	.	.	.	A	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	6	.	A	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	5	T	.	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	A	5
Duck (EU009397.1)	.	C	A	.	.	A	A	C	.	.	C	.	.	C	A	.	.	.	.	C	C	T	.	.	.	.	11	T	.	C	T	C	.	T	A	.	C	T	C	C	.	.	.	C	C	.	T	C	C	.	.	A	C	T	18	.	T	.	C	.	.	.	A	.	C	C	.	C	.	.	.	A	.	.	C	.	A	.	.	A	10
Chicken (AP003580.1)	.	C	C	.	.	A	A	C	A	.	C	.	.	C	A	.	.	A	C	.	T	T	A	.	.	.	14	.	.	C	T	C	.	T	T	.	C	.	C	C	T	.	.	T	C	.	T	T	C	.	.	C	.	15	.	T	.	C	.	.	G	A	.	C	C	.	C	A	.	.	T	C	.	C	.	A	.	.	A	13
Sheep (KR868678.1)	.	A	.	.	.	.	.	C	.	.	C	.	.	C	A	.	.	.	.	.	G	T	.	.	.	.	7	.	.	A	T	.	.	.	.	.	.	G	C	.	G	C	.	.	T	.	.	.	T	T	.	T	10	.	.	.	C	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	4
Dog (NC 002008.4)	.	A	.	.	.	.	.	.	.	.	A	T	G	C	A	T	.	A	.	C	.	.	A	G	.	.	11	.	T	T	T	G	.	.	.	.	.	.	.	T	.	.	T	.	.	T	C	T	A	C	T	12	T	A	.	A	.	.	.	C	.	.	.	.	C	A	.	G	.	.	T	.	.	.	.	.	A	9
Pigeon (KJ722068.1)	.	C	C	.	.	A	A	C	A	.	C	T	C	C	A	T	C	.	.	C	C	T	A	.	.	.	17	T	T	A	T	T	.	T	T	.	.	A	C	C	C	.	.	C	.	.	T	G	C	.	A	C	T	18	.	T	.	C	.	.	.	A	.	C	C	.	A	A	.	.	T	.	.	.	C	.	T	.	.	A	11
Quail (KX712089.1)	.	C	C	.	A	A	A	.	A	.	C	.	.	C	A	.	.	.	C	.	T	T	A	.	.	.	13	T	.	C	T	C	.	T	T	.	C	.	C	C	C	.	.	T	C	.	T	T	C	.	.	C	.	16	.	T	.	C	.	.	.	A	.	C	C	.	A	.	.	A	.	C	.	C	.	.	.	.	A	10
Cod (NC 002081.1)	.	A	.	A	.	A	.	.	.	.	A	A	G	C	C	T	T	T	.	.	T	T	A	.	T	.	15	T	T	T	A	C	.	.	A	C	.	T	T	.	.	G	.	.	C	T	T	T	.	.	A	.	.	15	.	.	.	A	G	.	T	A	.	T	A	.	C	T	.	A	.	.	.	.	.	T	.	.	.	10
Salmon (KF792729.1)	.	C	C	.	A	A	G	C	.	.	A	A	C	C	C	T	T	T	.	.	.	T	A	.	T	.	17	T	.	T	A	T	.	.	T	C	.	T	.	.	T	.	C	T	C	.	T	T	.	A	.	C	.	15	.	T	.	C	G	.	C	A	.	T	A	A	C	.	.	.	.	.	.	.	T	.	.	A	11
Pangas (NC 023924.1)	.	T	A	.	G	A	.	.	A	.	.	A	C	C	C	T	G	.	.	C	C	T	A	.	.	.	15	T	.	T	G	.	.	.	C	.	T	C	.	.	.	.	.	.	T	.	.	A	.	C	.		.	T	.	C	G	.	C	A	.	C	A	A	T	A	.	.	.	.	C	.	.	.	A	12
Tuna (KF906721.1)	.	A	A	.	A	A	.	A	A	.	A	A	G	C	C	T	.	A	.	C	.	T	.	.	T	.	16	.	A	T	A	C	.	.	T	G	.	G	T	.	C	.	C	.	C	.	T	C	T	.	.	C	.	15	.	T	.	.	G	.	T	A	.	C	A	A	T	A	.	T	.	.	C	.	G	.	.	A	13
Tilapia (GU238433.1)	.	A	A	.	A	G	.	.	A	.	C	A	G	C	C	T	.	A	.	C	.	T	.	.	T	.	16	.	A	T	T	T	.	.	.	G	.	.	C	.	T	.	C	C	C	T	T	C	T	.	.	C	.	15	.	T	.	.	G	.	T	G	.	.	A	A	T	A	.	.	.	.	C	.	.	.	.	A	10
Rohu (NC 017608.1)	.	-	A	C	A	C	T	C	A	T	C	A	C	.	T	-	-	.	.	C	C	T	A	.	T	.	17	T	A	T	A	C	.	.	A	C	.	A	.	.	C	.	C	C	.	.	T	.	.	A	.	C	.	14	A	T	.	.	G	.	T	A	.	C	A	A	T	A	.	T	.	.	.	.	.	.	.	A	12
Frog (NC 024548.1)	.	.	.	.	A	.	A	.	C	.	C	.	.	C	C	.	C	.	.	T	T	G	.	C	T	.	12	.	.	.	.	.	A	T	.	C	.	.	.	.	.	.	T	C	.	G	.	G	.	.	C	T	.	9	.	A	.	A	T	.	T	C	.	C	C	A	T	T	.	T	G	.	T	C	.	.	C	.	A	16
Turtle (NC 014769.1)	C	A	A	.	A	A	A	C	.	.	C	A	C	C	C	T	T	.	.	C	T	T	A	.	.	.	18	.	A	C	T	.	.	.	T	T	.	A	.	.	.	.	.	.	T	A	T	.	T	A	.	C	.	12	A	T	.	.	.	.	C	A	.	T	A	A	C	A	.	A	.	.	.	.	.	T	.	.	A	12
Deer (NC 006993.1)	.	A	.	.	C	.	.	C	.	.	C	T	.	C	A	.	G	.	.	.	G	T	T	.	.	.	11	.	T	A	T	C	.	.	.	.	.	.	.	.	.	C	T	.	.	A	.	G	.	T	C	T	11	T	A	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	5
Rabbit (AJ001588.1)	.	A	.	.	C	.	.	C	A	.	C	T	C	.	.	T	.	G	C	T	G	T	A	T	.	.	15	T	.	C	T	C	.	.	T	.	.	T	G	.	G	G	C	C	T	.	T	T	T	.	A	C	T	18	.	T	.	A	.	.	.	.	.	A	.	A	A	C	.	.	.	.	.	T	.	.	A	8
Monkey (NC 012670.1)	.	.	C	A	C	A	.	C	C	.	C	A	.	C	A	T	.	A	.	.	.	.	A	.	.	.	13	.	.	C	T	T	.	.	A	.	.	T	T	.	T	G	C	C	.	.	.	C	T	.	A	.	.	13	A	T	.	A	G	.	C	C	.	.	A	.	A	.	.	.	A	.	.	.	.	T	.	.	A	11
Cat (NC 001700.1)	.	A	.	.	.	A	.	C	C	.	A	.	.	C	A	T	.	.	.	.	.	.	T	G	.	.	10	T	.	C	T	G	.	.	.	.	.	.	.	.	T	.	.	T	.	T	.	T	.	.	A	C	.	9	T	.	.	C	.	.	G	C	.	.	.	.	A	.	.	A	.	.	.	.	.	T	.	.	A	8
Horse (KU575247.1)	.	.	.	.	C	.	.	C	.	.	C	.	.	C	A	T	.	.	.	G	T	T	T	.	.	.	10	.	.	C	T	C	.	.	A	.	.	.	.	.	T	.	.	T	.	A	.	T	.	T	.	.	9	.	.	.	C	.	.	.	C	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	A	5
Rat (AC 000022.2)	.	A	A	.	A	A	.	A	A	T	C	.	.	A	A	T	C	.	.	C	.	T	A	.	T	.	16	.	A	A	.	.	.	.	A	.	.	.	.	C	-	.	.	T	T	T	.	.	.	T	A	A	.	10	T	A	.	.	.	.	C	.	T	A	.	T	A	.	A	.	.	T	.	.	.	.	.	A	10
Ostrich (NC 002785.1)	.	T	.	.	C	A	A	C	A	.	C	T	C	C	C	T	T	T	.	.	C	T	.	.	.	.	16	T	T	C	T	T	.	T	T	.	C	A	C	C	.	.	.	C	C	.	T	G	C	.	.	C	T	18	T	.	.	C	.	.	.	A	.	C	C	.	A	.	.	.	.	.	.	.	.	.	A	6
Donkey (KT182635.1)	.	A	.	.	C	.	.	C	.	.	C	.	.	C	A	T	G	.	.	.	G	.	T	T	.	.	11	.	T	C	T	.	.	.	A	.	.	.	.	.	T	C	.	T	.	A	.	T	.	.	.	.	9	.	.	.	C	.	.	.	C	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	A	5
Crocodyle (DQ273698.1)	C	A	.	.	A	G	.	C	.	T	A	A	.	.	A	T	T	A	.	.	C	T	G	T	T	.	17	.	A	A	T	C	T	T	G	.	C	A	C	.	C	C	C	.	.	T	.	.	.	A	C	.	16	A	A	C	C	.	C	.	A	.	T	C	G	C	G	.	T	.	.	.	.	.	.	C	A	14
Turkey (NC 010195.2)	.	A	C	.	A	A	A	C	A	.	C	T	.	C	A	.	T	.	.	.	C	T	A	.	.	.	15	T	T	C	T	T	.	T	.	.	C	.	C	C	.	.	.	T	T	T	T	.	T	.	A	C	.	16	.	T	.	C	.	.	.	A	.	T	C	.	A	A	.	.	T	C	T	C	.	A	.	.	.	12
Wheat (GU985444.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	T	G	T	.	12	T	.	.	G	T	A	G	T	T	C	.	G	.	.	G	C	C	T	T	T	T	T	T	T	.	G	G	20	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	.	.	T	T	T	15
Onion (NC 030100.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	T	G	T	.	12	T	.	.	G	T	A	G	T	T	C	.	G	.	.	G	C	C	T	T	T	T	T	T	T	.	G	G	20	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	C	T	G	T	.	16
Pepper (KJ865410.1)	.	.	.	.	.	.	.	.	A	T	G	T	A	.	C	T	.	.	.	T	.	T	.	G	T	.	11	T	.	.	G	T	A	G	T	T	.	T	G	.	.	G	C	.	T	T	G	T	T	T	T	.	G	G	19	G	T	.	.	G	.	T	G	C	T	.	.	T	T	G	G	G	.	.	.	.	T	T	.	14

Table 4.22: The mismatch comparison of sheep ND5-specific 263 bp site against other 30 non-target

Species	Forward Primer																					Mis-match	Probe																					Mis-match	Reversed Primer																					Mis-match
Sheep (KR868678.1)	T	T	C	C	T	C	C	C	T	C	A	C	A	C	T	T	A	G	T	C	A	C	C		C	T	A	A	C	C	A	T	A	C	C	C	C	A	T	C	G	C	A	G	C	A	A	T	C	A	A		G	A	C	T	T	C	T	T	C	T	C	A	A	T	A	A	T	A	T	T	C	G	T	T	C	C	A	G	
Sheep (KR868678.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (KJ782448.1)	C	G	.	.	.	A	.	.	.	.	.	T	.	.	A	C	.	.	.	A	7	.	.	.	.	.	.	.	C	.	.	A	.	.	T	A	T	.	A	T	.	T	C	.	.	.	9	.	.	.	.	A	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	T	A	.	.	.	.	T	.	6
Goat (KP271023.1)	.	.	.	T	.	.	T	.	.	T	G	.	.	.	.	A	C	.	.	.	6	T	.	.	.	.	.	G	C	.	.	.	.	.	.	A	T	.	A	T	.	.	C	.	.	.	8	.	.	.	.	A	T	.	.	T	.	.	G	.	.	.	.	.	.	.	T	.	.	C	.	.	.	6
Cow (AF492351.1)	C	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	T	.	.	T	5	.	.	.	.	.	T	.	.	.	.	.	.	T	A	T	.	A	T	.	.	.	A	.	G	8	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	C	.	.	.	.	5
Buffalo (NC 006295.1)	C	C	.	T	.	.	A	.	.	T	.	T	.	.	.	.	A	C	.	.	.	.	8	T	.	.	.	.	A	T	.	.	.	.	.	.	.	A	T	.	A	T	.	.	C	A	.	G	10	.	.	T	.	A	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	5
Duck (EU009397.1)	C	.	.	.	C	T	.	A	C	A	C	T	C	.	.	C	A	C	A	C	T	A	16	.	.	C	C	T	A	.	C	T	.	.	A	.	.	A	T	C	C	T	C	C	C	T	C	C	18	.	.	.	C	A	A	.	A	T	.	.	.	.	.	.	C	.	.	.	C	.	C	.	.	C	A	10
Chicken (AP003580.1)	C	A	.	.	C	T	.	A	C	A	.	.	.	T	.	.	A	C	A	C	T	A	14	.	.	C	C	T	T	.	C	C	.	.	T	.	.	T	A	T	C	C	T	T	C	C	.	C	C	18	.	.	.	A	.	A	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	C	.	.	C	A	8
Dog (NC 002008.4)	.	.	.	T	.	.	A	T	G	.	.	T	.	A	.	C	A	C	A	G	.	.	11	.	.	T	.	.	T	T	.	G	.	.	.	.	.	A	T	T	A	T	.	T	C	.	T	C	13	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	A	.	.	T	.	5
Pigeon (KJ722068.1)	C	A	.	.	.	.	.	A	.	.	C	T	C	.	.	.	A	C	A	T	T	T	12	A	.	T	C	T	T	.	.	.	.	.	.	.	.	.	C	T	.	C	T	G	C	C	T	C	.	14	.	.	T	C	A	A	.	A	.	.	.	C	C	.	.	.	.	.	.	T	C	.	C	.	.	C	.	11
Quail (KX712089.1)	.	A	.	.	C	T	.	A	C	A	C	.	.	T	.	.	A	C	A	C	T	T	14	.	.	C	C	T	T	.	C	C	.	.	.	.	T	A	T	C	C	T	T	C	C	.	C	C	17	.	.	.	.	.	A	.	A	T	.	.	C	.	.	.	.	.	.	.	T	T	.	C	.	.	.	A	8
Cod (NC 002081.1)	.	.	.	A	A	G	.	.	.	T	T	T	.	T	.	.	A	.	A	T	T	T	12	.	.	T	C	T	T	T	A	C	.	.	A	C	.	T	T	T	.	.	T	.	.	C	T	T	.	16	.	.	.	C	A	.	.	A	T	.	.	T	.	.	T	.	.	T	.	.	T	A	.	.	.	.	T	A	11
Salmon (KF792729.1)	C	.	.	A	A	.	.	.	.	T	T	T	.	A	.	.	A	.	.	T	T	.	10	.	.	C	C	T	.	T	A	T	.	.	T	C	.	T	A	T	T	A	.	T	.	C	T	T	.	17	.	.	.	C	A	.	.	A	.	.	.	C	.	.	C	.	.	T	.	.	T	A	C	C	.	.	.	A	11
Pangas (NC 023924.1)	.	A	.	T	A	.	.	.	.	G	C	T	C	.	.	.	A	C	.	C	T	A	12	.	.	.	.	T	.	T	G	.	.	.	C	.	T	C	T	.	A	T	.	.	C	T	.	.	11	.	.	.	C	A	.	.	A	.	.	.	.	C	.	.	.	.	C	.	.	T	A	C	C	.	.	.	A	10
Tuna (KF906721.1)	A	A	.	A	A	G	.	.	.	A	.	T	C	A	.	C	A	.	.	T	T	.	13	.	.	.	G	.	A	T	A	C	.	.	T	G	.	G	T	T	C	A	.	.	.	C	T	C	T	16	.	.	.	C	A	.	.	A	T	.	.	.	.	C	.	.	T	.	.	.	A	C	C	.	.	.	A	10
Tilapia (GU238433.1)	.	A	.	.	A	G	.	.	.	A	.	T	C	A	.	C	A	.	T	T	T	T	13	.	.	C	G	.	A	T	.	T	.	.	G	.	.	C	T	T	A	.	C	T	C	T	C	T	16	.	.	T	A	.	T	.	A	.	.	.	.	.	T	.	.	C	.	.	.	A	C	C	.	.	C	.	10
Rohu (NC 017608.1)	C	.	.	A	.	.	A	.	.	T	C	T	C	.	.	.	A	.	.	T	T	.	10	.	.	.	G	T	A	T	A	C	.	.	A	C	.	A	A	T	C	A	.	C	.	C	T	.	.	16	.	.	.	C	A	.	.	A	.	.	.	T	C	.	.	.	.	C	.	.	.	.	C	.	.	.	A	8
Frog (NC 024548.1)	.	C	.	.	C	T	.	.	C	.	C	T	T	T	T	G	C	C	.	T	G	.	G	14	.	.	G	G	.	.	G	C	.	A	T	.	C	.	.	A	T	.	A	T	T	.	C	G	.	G	15	.	.	.	C	.	A	.	A	.	A	.	T	G	.	T	T	.	G	.	.	T	C	.	C	.	.	C	A	14
Turtle (NC 014769.1)	C	.	.	.	A	.	.	.	.	T	C	T	C	T	.	.	A	C	A	C	T	A	13	.	.	.	.	.	A	C	.	.	.	.	T	T	.	A	A	T	.	A	T	.	C	C	T	.	T	13	.	.	T	C	A	G	.	A	.	.	.	C	.	.	T	.	.	.	.	.	T	A	.	A	.	.	C	A	12
Deer (NC 006993.1)	C	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	T	.	.	.	4	.	.	.	.	.	T	.	.	C	.	.	.	.	.	A	T	.	A	.	T	.	C	A	.	G	9	.	.	T	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	5
Rabbit (AJ001588.1)	C	.	.	.	A	.	.	T	C	T	G	T	.	G	C	T	.	.	A	T	.	A	13	T	.	.	G	T	.	C	.	C	.	.	T	.	.	T	.	T	G	.	.	C	T	C	T	T	T	15	.	.	T	.	A	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	C	C	.	.	.	A	8
Monkey (NC 012670.1)	C	A	.	.	C	.	.	A	.	.	.	T	.	A	.	.	A	C	A	.	.	.	9	.	.	.	.	.	.	C	.	T	.	.	A	.	.	T	T	T	T	.	.	C	.	C	.	C	T	11	.	.	T	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	A	6
Cat (NC 001700.1)	.	A	.	.	C	.	A	.	.	.	.	T	.	.	.	.	A	C	T	G	.	A	9	.	.	.	C	T	.	C	.	G	.	.	.	.	.	A	T	T	A	T	.	T	C	T	.	.	12	.	.	T	.	A	T	.	.	.	.	.	.	C	C	.	.	C	.	.	T	A	.	C	.	.	T	.	10
Horse (KU575247.1)	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	T	.	A	5	.	.	.	.	.	.	C	.	C	.	.	A	.	.	A	T	.	T	T	.	T	C	A	.	T	11	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	4
Rat (AC 000022.2)	A	A	.	A	A	T	.	.	.	A	.	T	C	.	.	C	A	.	A	.	.	T	T	11	.	.	T	.	.	A	.	C	.	.	.	A	.	.	.	A	.	-	A	T	T	T	.	.	.	9	.	.	.	.	A	T	.	.	.	.	.	.	.	C	C	.	.	.	.	C	.	A	T	.	.	.	7
Ostrich (NC 002785.1)	C	A	.	.	.	.	.	.	.	T	T	T	.	.	.	C	A	C	A	C	T	A	12	.	.	T	C	T	T	.	C	.	.	.	A	.	.	.	C	T	C	C	T	G	C	C	T	C	C	17	.	.	.	C	A	A	.	A	T	.	.	.	.	.	.	C	.	.	.	.	C	.	C	.	.	T	.	9
Donkey (KT182635.1)	C	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	C	T	T	.	A	7	T	.	.	.	.	T	C	.	.	.	.	A	.	.	.	A	T	.	T	.	.	T	C	A	.	T	11	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	C	.	.	T	.	.	A	.	.	.	.	5
Crocodyle (DQ273698.1)	C	.	T	A	A	T	T	A	.	T	.	T	.	.	.	G	T	.	.	C	T	G	14	.	.	.	G	.	A	.	.	C	T	T	G	.	C	A	C	T	C	C	.	.	.	.	T	.	.	13	.	.	.	A	.	T	.	A	.	.	.	C	.	C	C	T	.	C	.	.	.	T	.	A	.	.	C	A	12
Turkey (NC 010195.2)	C	A	.	.	.	T	.	A	C	T	C	T	.	.	.	.	A	C	.	.	T	A	10	.	.	T	C	T	.	.	C	C	.	.	A	.	.	T	A	T	T	T	T	.	T	C	.	C	T	16	.	.	.	C	.	A	.	A	.	.	.	C	.	.	.	.	.	.	.	.	C	.	C	.	.	C	A	8
Wheat (GU985444.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	T	G	T	.	13	T	.	G	C	T	.	G	G	T	A	G	T	T	C	.	.	T	.	.	.	C	G	G	T	T	T	19	.	.	T	A	G	.	C	.	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	17
Onion (NC 030100.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	T	G	T	.	13	.	.	G	C	T	.	G	G	T	A	G	T	T	C	.	.	T	.	.	.	C	G	G	T	T	T	18	.	.	T	A	G	.	C	C	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	18
Pepper (KJ865410.1)	.	.	.	.	A	T	G	T	A	T	C	T	.	.	.	T	A	.	.	G	T	A	13	.	.	G	C	T	.	G	G	T	A	G	T	T	.	T	.	T	.	.	.	G	G	G	T	T	17	.	.	T	A	G	.	C	C	G	A	.	C	G	.	.	G	.	G	A	.	G	T	.	A	A	T	T	.	18

Table 4.23: The mismatch comparison of chicken Cytb-specific 161 bp site against other 30 non-target

Species	Forward Primer																									Mis-match	Probe																						Mis-match	Reversed Primer																						Mis-match
Chicken (NC 001323.1)	C	T	T	T	G	C	A	A	T	C	G	C	A	G	G	T	A	T	T	A	C	T	A	T			C	C	C	C	T	A	G	G	C	A	T	C	T	C	A	T	C	C	G	A	C	T	C	T			A	C	T	C	T	C	A	T	A	C	T	C	A	C	C	C	C	A	T	T	C	C	
Chicken (NC 001323.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Sheep (KR868678.1)	A	.	.	C	A	T	C	.	.	.	.	.	.	.	C	C	C	.	C	G	.	C	.	.	11		.	.	.	A	C	.	.	.	A	.	.	T	C	.	.	.	.	G	.	.	.	A	.	A	8		.	T	C	.	.	A	C	.	.	A	.	.	C	T	.	A	T	C	C	.	.	A	12
Duck (EU009397.1)	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	C	C	.	6		.	.	.	.	.	.	.	.	T	C	.	T	G	T	.	.	.	A	.	.	.	.	G	.	7		.	T	C	.	.	.	.	.	G	.	.	T	.	.	.	.	.	C	C	.	.	A	7
Goat (KP271023.1)	A	.	.	C	A	T	C	.	.	.	A	.	.	.	C	C	C	.	C	G	.	C	.	.	12		.	.	.	A	C	.	.	.	A	.	.	T	C	.	.	.	.	A	.	.	.	A	.	A	8		.	T	G	.	.	A	C	.	.	A	.	T	C	T	T	G	T	T	C	.	A	A	15
Pig (KJ782448.1)	.	.	.	C	.	T	C	.	.	T	A	.	C	.	C	C	C	.	C	G	.	A	G	C	14		.	.	.	A	C	C	.	.	A	.	.	T	C	.	.	.	.	A	.	.	.	A	T	A	10		T	T	A	T	.	T	.	.	.	A	.	A	C	T	A	A	T	C	C	.	A	.	15
Cow (AF492351.1)	A	.	.	.	A	T	C	.	.	.	A	T	.	.	C	A	.	.	.	G	.	C	.	.	10		.	.	A	A	C	.	.	.	A	.	.	T	.	.	C	.	.	A	.	.	.	G	T	A	10		C	T	C	T	.	A	C	.	.	A	.	T	C	T	A	G	.	T	C	.	A	A	16
Buffalo (NC 006295.1)	A	.	.	C	A	T	T	.	.	.	.	.	.	.	C	A	C	.	.	G	.	A	.	.	10		.	.	A	A	C	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	A	7		C	T	A	.	.	A	T	.	.	A	.	.	C	T	A	G	.	C	C	.	A	A	14
Pigeon (KJ722068.1)	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	C	C	.	C	.	.	C	.	.	6		.	.	A	.	.	.	.	.	.	.	.	A	.	C	.	.	.	A	.	.	.	G	C		6		.	T	G	A	.	A	.	.	.	.	.	C	T	.	.	.	C	C	.	A	A	10
Quail (KX712089.1)	A	.	.	C	T	T	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	6		.	.	A	T	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	4		.	.	A	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	3
Cod (NC 002081.1)	.	.	.	.	.	T	T	G	.	T	.	.	T	.	C	.	T	.	.	.	.	A	.	.	8		.	.	.	A	C	.	.	.	A	.	.	.	A	A	T	.	.	A	A	.	T	G	.	A	11		G	.	.	G	.	G	.	.	G	.	.	T	C	T	G	G	G	C	.	.	A	A	13
Salmon (KF792729.1)	A	.	.	C	.	T	T	.	.	T	.	.	.	.	C	.	G	C	C	.	.	A	G	.	11		.	.	A	G	C	.	.	.	.	.	.	.	A	A	C	.	.	.	.	.	T	G	.	C	9		G	T	A	G	C	.	.	.	.	.	.	A	C	T	T	G	G	C	C	.	A	A	15
Pangas (NC 023924.1)	A	.	.	C	.	T	.	G	.	T	A	T	C	.	C	A	G	C	.	.	.	A	C	.	14		.	.	A	A	.	T	.	.	T	T	.	A	A	A	C	.	.	.	.	.	G	.	A	11		.	T	A	A	.	.	C	.	C	.	.	T	.	.	A	G	.	C	C	.	.	G	11
Tuna (KF906721.1)	A	.	.	C	.	T	C	.	.	.	.	.	.	.	C	C	.	.	G	.	.	A	.	.	8		.	.	A	A	.	C	.	.	A	T	.	A	A	A	C	.	.	A	A	.	T	G	.	A	14		G	T	G	A	.	.	C	.	G	.	.	A	G	T	G	G	.	.	C	.	T	G	14
Tilapia (GU238433.1)	.	.	.	C	A	T	C	.	.	T	.	.	.	.	C	.	G	C	A	.	.	A	.	.	10		.	.	.	A	C	.	.	.	.	C	.	A	A	A	C	.	.	A	.	.	.	G	.	C	10		G	.	A	A	.	T	C	.	T	T	.	A	.	T	T	G	.	C	C	.	T	A	15
Rohu (NC 017608.1)	A	.	.	.	A	T	C	.	.	T	.	.	C	.	C	C	G	C	A	.	.	C	C	.	13		.	.	A	A	.	T	.	.	A	C	.	A	A	A	C	.	.	A	.	.	.	G	.	A	12		G	T	A	A	.	T	.	.	.	.	.	A	C	T	A	G	.	C	C	.	.	A	13
Frog (NC 024548.1)	A	.	.	.	A	T	T	.	.	T	A	.	C	.	C	.	G	.	.	.	G	.	C	.	11		.	.	.	A	C	.	.	.	A	C	.	T	A	A	C	.	.	.	A	.	.	C	T	A	12		G	T	.	A	.	.	C	.	.	.	.	.	G	G	G	G	.	.	C	.	.	G	10
Turtle (NC 014769.1)	.	.	.	C	.	.	T	.	.	.	.	T	.	.	.	C	.	A	G	.	A	G	C		9		.	.	A	A	C	.	.	.	A	T	.	A	A	A	C	.	.	A	A	.	.	G	.	.	12		.	T	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	C	C	.	A	T	8
Deer (NC 006993.1)	A	.	.	.	A	T	C	.	.	.	.	.	.	.	C	A	C	.	.	G	.	.	.	.	8		.	.	A	A	C	.	.	.	A	.	.	.	C	.	.	.	.	G	.	.	.	G	.	A	8		T	T	A	.	.	T	C	.	.	G	.	A	C	T	.	T	T	C	C	.	A	A	15
Dog (MH891616.1)	T	.	.	C	A	T	C	.	.	.	.	.	.	.	C	.	C	.	A	G	.	A	.	.	10		.	.	T	T	C	.	.	.	A	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	A	7		C	T	.	A	C	T	C	.	.	.	.	.	C	T	A	A	T	C	C	.	A	A	15
Rabbit (AJ001588.1)	A	.	.	.	A	T	C	.	.	T	.	.	.	A	C	.	T	.	A	G	T	C	T	.	13		.	.	.	A	C	.	.	.	A	.	.	T	C	.	T	.	.	A	A	.	.	.	.	A	9		C	T	.	G	.	A	C	C	.	T	.	.	T	T	.	T	.	C	.	C	.	T	13
Rat (012374.1)	A	.	.	C	A	T	C	.	.	.	.	.	C	.	C	C	C	.	.	G	.	A	.	.	11		.	.	.	A	C	.	.	.	A	C	.	A	A	A	A	C	.	.	T	.	.	.	G	.	A	11		T	T	C	A	.	G	T	.	.	.	.	.	C	T	A	T	T	T	C	.	A	A	15
Monkey (NC 012670.1)	.	.	.	C	A	T	C	.	.	.	A	T	C	.	C	C	C	.	C	G	.	A	G	.	14		.	.	T	T	G	T	.	.	A	.	.	T	.	.	C	.	.	.	.	.	.	.	.	G	8		.	T	C	.	.	.	C	.	T	.	.	.	T	T	.	A	T	C	C	.	A	G	12
Cat (NC 001700.1)	A	.	.	C	A	T	T	.	.	.	T	.	.	.	C	C	T	.	A	G	.	A	G	G	14		.	.	.	T	C	.	.	.	A	.	.	T	A	.	.	.	.	.	.	T	.	.	A	7		C	T	A	G	.	A	C	.	.	G	.	T	T	T	A	A	.	.	C	.	.	A	14
Horse (KU575247.1)	.	.	.	C	A	T	C	.	.	.	A	.	.	.	C	C	C	.	G	G	T	A	G	.	13		.	.	.	T	C	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	T	A	T	G	8		C	T	C	.	.	.	C	.	G	A	.	.	T	T	G	.	T	C	C	.	A	.	13
Donkey (KT182635.1)	.	.	.	.	A	T	C	.	.	.	A	.	G	.	C	C	C	.	G	G	T	A	.	.	12		.	.	.	T	C	.	.	.	A	.	.	.	C	.	.	.	.	T	.	.	.	A	T	A	8		C	T	.	.	.	.	C	.	.	G	.	.	C	T	A	.	T	C	C	.	A	.	11
Crocodyle (DQ273698.1)	A	.	.	.	A	T	C	C	.	.	A	T	.	.	C	C	.	C	C	.	T	C	C	.	14		.	.	A	.	.	.	.	.	A	T	.	A	A	T	C	.	.	T	A	.	.	G	.	C	11		G	.	C	.	.	G	G	C	C	A	C	.	G	T	.	.	T	.	.	.	A	A	13
Ostrich (NC 002785.1)	A	.	.	.	.	T	.	.	.	.	.	T	.	.	C	.	.	C	.	.	C	C	.		7		.	.	.	.	.	C	.	.	A	.	.	.	A	T	C	.	.	T	C	.	.	.	G	C	9		.	.	G	.	.	A	.	.	.	T	.	.	.	T	.	.	.	C	C	.	A	.	7
Turkey (NC 010195.2)	.	.	.	C	A	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.		5		.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	G	.	.	5		.	.	A	A	.	.	.	.	.	.	A	.	.	.	.	.	C	.	.	A	.	5
Onion (NC 030100.1)	.	C	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	.	G	.	C	.	13		.	.	A	T	.	G	.	.	T	G	.	A	C	A	T	.	.	A	.	.	G	A	T	G	14		G	T	A	G	C	T	C	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.	15
Wheat (GU985444.1)	.	C	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	.	G	.	C	.	13		.	.	A	T	.	G	.	.	T	G	.	A	C	A	T	.	.	T	.	.	G	A	T	G	14		G	T	A	G	C	T	C	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.	15
Pepper (KJ865410.1)	.	.	.	.	A	T	T	T	.	A	.	T	.	.	.	C	G	C	C	.	G	.	C	.	12		.	.	A	T	.	G	.	.	T	G	.	A	C	A	T	.	.	A	.	.	G	A	T	G	14		G	T	A	G	C	T	T	.	G	.	.	A	T	.	T	T	T	T	.	.	T	.	15

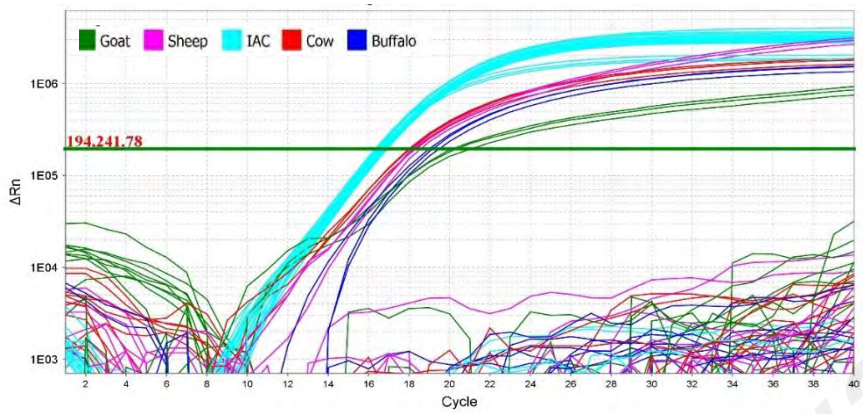
Table 4.24: The mismatch comparison of duck ND5-specific 203 bp site against other 30 non-target

Species	Forward Primer																									Mis-	Probe																									Mis-	Reversed Primer																									Mis-
Duck (EU009397.1)	C	A	C	C	G	T	A	A	A	A	G	C	T	G	C	A	T	T	T	C	T	A	A	match	C	C	G	G	A	C	T	A	G	A	A	T	C	C	A	T	T	A	C	C	T	G	C	C	A	C	match	C	C	A	T	T	C	T	A	C	A	A	T	T	C	G	C	C	A	T	G	T	match
Duck (EU009397.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig (KJ782448.1)	.	.	.	.	.	.	.	T	C	C	T	A	C	.	.	C	.	.	C	A	C	T	C	12	.	A	.	.	C	.	A	.	.	.	A	T	A	.	.	C	.	T	.	.	C	A	A	.	.	11	.	A	.	.	.	A	.	.	G	.	.	.	.	.	T	.	.	.	.	A	.	5
Goat (KP271023.1)	G	G	T	.	.	.	T	T	C	.	T	G	.	.	.	C	.	.	.	A	.	C	.	11	.	A	.	.	.	.	A	.	.	.	A	T	A	G	.	C	.	T	T	.	C	A	A	.	.	12	.	.	.	.	.	A	.	.	G	.	.	.	.	.	T	.	A	.	.	.	.	4
Cow (AF492351.1)	A	G	.	T	A	.	C	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	13	.	A	.	.	C	.	A	.	.	.	C	T	A	.	.	.	.	T	T	.	C	A	A	.	.	11	.	T	.	.	.	A	.	.	G	.	.	.	.	.	T	.	A	.	.	A	.	6
Buffalo (NC 006295.1)	A	G	.	.	A	.	T	T	C	.	T	G	C	.	.	C	.	.	C	A	.	T	.	13	.	A	.	.	.	.	A	.	.	.	A	T	A	.	.	C	.	T	T	.	C	A	A	.	T	12	.	T	.	.	.	A	.	.	G	.	.	.	.	.	T	.	A	.	.	A	.	6
Chicken (AP003580.1)	A	.	.	.	A	.	T	.	.	.	A	.	C	.	.	C	.	.	C	.	.	.	.	7	.	A	.	.	.	G	C	.	.	.	A	G	.	.	.	G	.	.	A	C	.	.	.	.	8	.	.	.	.	C	T	.	.	G	.	G	.	.	.	.	A	.	C	A	.	7
Sheep (KR868678.1)	A	.	.	.	A	.	T	T	C	.	T	G	C	.	.	T	.	.	C	A	.	C	.	12	.	A	.	.	.	.	A	.	.	.	A	T	A	.	.	.	.	T	.	.	C	A	A	.	.	9	.	T	.	.	.	A	.	.	G	.	.	.	.	.	T	.	A	.	.	A	.	6
Dog (NC 002008.4)	.	G	.	T	A	C	T	T	C	T	T	A	C	.	.	G	.	.	C	A	.	.	.	14	.	A	.	.	.	.	A	G	.	.	A	.	A	.	.	C	.	T	T	.	C	A	A	.	.	11	.	T	.	.	C	A	.	.	G	.	.	.	.	.	T	.	T	.	.	A	.	7
Pigeon (KJ722068.1)	.	.	.	T	.	.	C	.	.	.	A	T	C	.	.	.	.	.	.	.	.	.	.	5	T	A	.	.	C	.	C	.	.	.	.	A	G	.	.	.	C	.	T	.	.	C	.	.	.	9	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	A	.	.	C	A	.	5
Quail (KX712089.1)	A	.	.	.	.	.	T	.	.	.	A	.	C	.	.	C	.	.	C	A	.	.	.	7	.	A	.	.	C	A	C	.	.	.	C	A	G	.	.	.	C	.	T	T	A	C	.	T	.	.	13	.	A	.	.	C	.	.	.	G	.	.	.	.	.	T	.	.	A	.	C	A	.	7
Cod (NC 002081.1)	A	G	.	A	.	.	.	.	.	G	T	.	.	.	.	T	.	.	C	T	.	.	.	8	.	A	.	.	T	A	.	.	.	G	G	.	T	G	.	A	.	.	T	.	.	C	T	T	C	G	14	.	T	.	.	.	T	.	.	G	.	.	.	.	.	T	.	.	T	.	C	A	.	7
Salmon (KF792729.1)	T	G	.	T	A	.	C	.	.	.	A	T	A	.	.	T	.	.	C	T	.	.	G	12	A	A	.	.	G	A	C	C	.	.	.	A	.	A	.	.	.	G	T	T	A	C	T	A	.	T	16	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	A	T	C	C	.	6
Pangas (NC 023924.1)	.	G	.	T	.	.	.	.	G	C	A	.	.	.	.	.	.	.	.	.	T	.	T	.	7	A	G	.	.	.	A	C	.	.	.	.	A	A	T	G	.	C	.	T	.	A	C	.	A	C	T	15	.	A	.	.	.	.	.	G	G	.	.	.	.	T	.	.	A	T	C	A	.	8
Tuna (KF906721.1)	.	G	.	A	.	.	T	.	.	.	C	T	A	.	.	.	.	.	.	.	T	.	T	G	9	A	G	.	.	G	G	C	C	.	.	G	.	.	.	.	.	T	T	A	C	.	A	.	.	12	.	A	.	.	C	.	.	C	G	.	.	.	.	.	.	.	A	T	C	A	.	8
Tilapia (GU238433.1)	A	G	.	A	.	.	.	.	.	.	T	G	A	.	.	C	.	.	C	T	.	T	.	10	A	A	.	.	C	G	C	.	.	.	A	.	A	.	.	.	.	T	.	A	C	.	A	G	T	13	.	.	.	.	C	.	.	.	G	.	G	.	.	T	.	.	.	T	C	C	.	7
Rohu (NC 017608.1)	.	G	.	.	.	.	C	.	G	C	T	.	C	.	.	.	.	.	C	T	.	T	.	9	T	A	A	A	.	A	C	.	.	.	G	G	.	.	.	.	T	.	A	C	A	A	.	T	14	.	A	.	.	C	T	.	.	G	.	.	.	.	.	T	.	.	A	C	.	A	.	8
Frog (NC 024548.1)	A	G	.	T	.	.	.	.	.	.	A	.	C	T	.	.	.	.	.	T	.	C	C	9	A	.	A	.	T	.	A	T	.	.	C	G	.	T	T	C	C	C	T	.	G	C	.	T	C	.	17	G	T	.	.	C	T	.	.	G	.	.	.	.	.	T	.	.	T	C	C	.	9
Turtle (NC 014769.1)	A	.	.	T	.	.	.	.	.	.	A	.	A	.	.	.	.	.	C	T	.	C	.	7	T	A	.	A	C	A	.	T	.	.	.	.	.	A	.	.	C	.	T	T	A	C	.	A	.	.	13	.	.	.	.	C	.	.	.	G	.	.	.	.	T	A	.	.	T	A	T	.	7
Deer (NC 006993.1)	A	.	.	T	A	.	C	T	C	.	T	A	.	.	.	T	.	.	C	A	.	C	.	12	.	T	.	.	C	.	A	.	.	.	A	T	A	.	.	.	.	T	.	.	C	A	A	.	.	10	.	.	.	.	.	A	.	.	G	.	.	.	.	.	T	.	A	.	.	.	.	4
Rabbit (AJ001588.1)	T	T	.	A	.	.	T	C	T	T	A	.	.	.	.	.	.	C	A	C	T	.	.	12	.	T	A	.	T	.	A	.	.	.	A	T	A	.	.	A	G	T	A	.	C	.	A	.	.	13	.	T	.	.	C	A	.	.	G	.	.	.	.	.	T	T	.	.	.	.	A	.	7
Monkey (NC 012670.1)	A	.	.	.	.	.	.	.	T	.	T	G	.	.	.	T	.	.	.	A	.	T	.	7	T	A	A	A	C	.	A	.	.	.	A	.	A	.	C	C	.	T	T	.	.	A	A	G	T	16	.	.	.	.	.	A	.	.	G	.	.	.	.	.	T	.	G	C	.	A	.	6
Cat (NC 001700.1)	.	.	.	A	A	.	C	T	C	T	T	A	C	.	.	C	.	.	C	A	.	C	.	13	.	A	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	T	.	.	C	A	A	.	.	8	.	.	.	.	C	A	.	.	G	.	.	.	.	.	T	.	A	.	.	.	.	5
Horse (KU575247.1)	.	.	.	T	A	.	C	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	11	.	T	.	.	.	.	A	.	.	.	A	.	A	.	.	.	.	T	.	.	C	A	A	.	.	8	.	T	.	.	.	A	.	G	G	.	.	.	.	.	T	.	.	C	.	A	.	7
Rat (AC 000022.2)	A	T	.	A	A	.	T	.	.	.	T	T	C	T	.	.	.	C	.	.	C	C	12	A	.	A	A	C	A	C	.	.	.	.	.	A	.	.	.	A	.	T	T	A	C	T	A	.	.	14	.	A	.	.	.	A	.	.	.	.	.	.	.	.	T	.	T	T	C	A	.	7
Ostrich (NC 002785.1)	.	.	.	T	.	.	C	.	.	.	A	T	A	.	.	C	.	.	C	T	.	T	.	9	.	A	.	.	.	A	C	.	.	.	G	.	A	.	.	.	.	T	T	.	C	.	.	.	.	8	.	T	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	4
Donkey (KT182635.1)	.	.	T	.	A	.	T	T	C	.	T	A	.	.	.	C	.	.	C	A	.	T	.	11	.	T	.	.	.	.	A	.	.	.	A	.	A	.	.	C	.	T	.	.	C	A	A	.	.	9	.	T	.	.	C	A	.	.	G	.	.	.	.	.	T	.	.	C	.	A	.	7
Crocodyle (DQ273698.1)	G	G	.	A	.	C	T	.	.	.	C	T	G	.	.	C	.	.	C	.	.	T	.	11	A	T	.	A	C	T	.	.	A	C	C	A	T	.	.	C	.	.	.	A	.	A	.	G	.	A	15	.	T	.	.	C	A	.	.	G	.	G	.	.	T	A	.	.	G	.	A	C	10
Turkey (NC 010195.2)	A	.	.	.	.	.	T	.	.	.	A	.	.	.	.	C	.	.	C	.	.	.	C	6	.	.	.	.	C	.	.	.	.	.	.	A	G	T	.	.	.	T	T	A	C	.	T	.	.	9	.	A	.	.	C	.	.	.	G	.	G	.	.	.	.	.	.	.	C	A	.	6
Wheat (GU985444.1)	G	T	G	.	.	.	T	T	C	.	T	T	.	T	.	T	.	C	G	A	.	C	T	15	G	G	.	A	G	.	.	.	T	G	C	T	T	G	C	.	.	T	.	.	C	A	G	.	A	16	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	.	.	.	T	.	12
Onion (NC 030100.1)	G	T	G	.	.	.	T	T	C	.	T	T	.	T	.	T	.	C	A	A	.	C	T	15	A	G	.	A	G	.	.	.	T	G	C	T	T	G	C	.	.	T	.	.	C	A	G	.	A	16	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	A	.	.	T	.	13
Pepper (KJ865410.1)	G	T	G	.	.	.	T	T	C	.	T	T	C	T	.	T	.	C	G	A	.	C	T	16	G	G	.	A	G	.	.	.	T	G	C	T	T	G	C	.	.	T	.	.	A	A	G	.	A	16	T	G	G	.	C	.	A	T	.	T	T	.	A	T	T	.	.	.	.	T	.	12

Table 4.25: The mismatch comparison of pig Cytb-specific 146 bp site against other 30 non-target

Species	Forward Primer	Mis-match	Probe	Mis-match	Reversed Primer	Mis-match
Pig (AF034253.1)	TATCCCTTATATCGGAACAGACCTC		CCTGCCATTCATCATTACCGCCCC		CCGTACATCTCCTAATTCCTCTGC	
Pig (AF034253.1)	.....	0	.....	0	.....	0
Chicken (NC 001323.1)	A..T..C..C..T...CACACC...A	11	...C..C..TGCA..CG.A.GTA	12	T.A.C...C...ACC.....A.	8
Sheep (KR868678.1)	A..T..A.....T..C...A....A	7	TT.C.....CG.A.....	6	TA..T..C..A..C.....C.	7
Duck (EU009397.1)	CC....A..C.....CAGACC...G	10	A..A..C..TT.A..CG.A.GAA	12	TA..C..CT.AAACC.....A.	10
Goat (KP271023.1)	A.....A.....T..C...A....A	6	...C.....C..A.....	3	TA..C..C..G..C.....C.	7
Cow (AF492351.1)	A.....A..C.....C...A.TT.A	8	...T.....T.....C.TA..AA	7	TA..C..C..A.....C.	6
Buffalo (NC 006295.1)	A.....A..C..T..T...AGT..G	9	...C.....T..CG.A..A.	6	TA..C..C..AT....T..C.	8
Pigeon (KJ722068.1)	.G....C..C..T..CCA.AC....	9	...C..C..T..A..CG.A.G..	8	T.A.C..C...ACC.....A.	7
Quail (KX712089.1)	.G.A..C..C.....CCA.AC...A	10	...C.....T.A..CG.A.GAA	9	T.A.C..C...AC.....A.	7
Cod (NC 002081.1)	.G.T.....G.A..TGAT.C.T.A	11	AT.C..C..TG.TG..G.T..TT	12	TACC.C..C..A..T..T..C.	9
Salmon (KF792729.1)	.G....C..CG.A...GGC.C...T	10	AT.C.....G.T...G.A..TG	9	TACC.C.....T.....TTA.	8
Pangas (NC 023924.1)	CG....C.....A...GACAC...A	10	A..C.....G.AG...T...AG	7	TAC.....GC...G..T..A.	8
Tuna (KF906721.1)	AG....A...G.T.....TAC....	8	AT.C.....G....CG.A...A	8	TTC.T..C..T..T.....T.	8
Tilapia (GU238433.1)	A.....C..T..C.ATTC.T.A	10	.T.C..C.....G.A..TG	7	TA..C..CT.AA.T..T..T.	10
Rohu (NC 017608.1)	CG.A..A..C..A...GACATAT.A	14	A..A.....T.....G.....G	5	TTA.T..C.....C.	6
Frog (NC 024548.1)	CGC...C..C.....CT.TA....T	10	T..C.....T..T.....TG	6	TAA.T..C.....TTA.	8
Turtle (NC 014769.1)	C.C...C..C..T..C.ATAC...T	11	A..C..C...GCT..CG.T.GT.	11	.....T.A..C.....C.	4
Deer (NC 006993.1)	A..T..A.....T..C...A....A	7	T..T.....T.....CG.A..A.	7	TA.....CT.A..C.....T.	7
Dog (MH891616.1)	C.....T...T.A	4	...C..T.....CG.A..T.	6	TA.....C.....T..A.	5
Rabbit (AJ001588.1)	A.....A.....AC.T.A	6	.T.....T.....G.AAA.TT	7	TAA.T..C.....C..T..A.	8
Rat (012374.1)	C..T..C..C..T..C..CACT..A	11	...C.....CG.....	3	TT.....C.....C.	4
Monkey (NC 012670.1)	A.....G..C.....TA....T	6	...A..C.....C.T.....	4	T.....CT.A.....A.	5
Cat (NC 001700.1)	A..T..A..C.....G..T..A..A	8	T..T.....T..CT.A....T	7	GA.....C...T.....T.	4
Horse (KU575247.1)	A..T..C..C.....T..TAC....	8	...A..C.....C..A.....	4	T.....T.A.....T..T.	5
Donkey (KT182635.1)	A.....C..C.....T..TACA...	8	T..A..C..T.....C..G.....	6	T...C.....A.....C.	4
Crocodyle (DQ273698.1)	C.....C..CG.A...GACTCAAT	13	G..C.....T...C.C.TA...A	8	T.AC...C...A.C.....C.	7
Ostrich (NC 002785.1)	C.....C..C.....CA.AC...A	8	...C.....TG.A..CG.T.G.A	9	TG.....AC.....	4
Turkey (NC 010195.2)	A.....C..C..T..TCA.AC...A	10	...C..C.....A..CG.A.GAA	9	T.A.C..C...A.....T.....	6
Onion (NC 030100.1)	C..A...GTAG.A...GATAC.A.A	14	A..C..CC.T..TT.AGTA.G.G	13	TTC.T.....GGCCGCAT...	12
Wheat (GU985444.1)	C..A..AGTAG.A...GATAC.A.A	15	A..C..CC.T..TT.AGTA.G.G	13	TTC.T.....GGCCGCAT...	12
Pepper (KJ865410.1)	C..A...GTAG.A...GATAC.A.A	14	A..C..C..T..TT.AGTA.G.G	12	TTC.T.....GGCCGCAT...	12

(a)



(b)

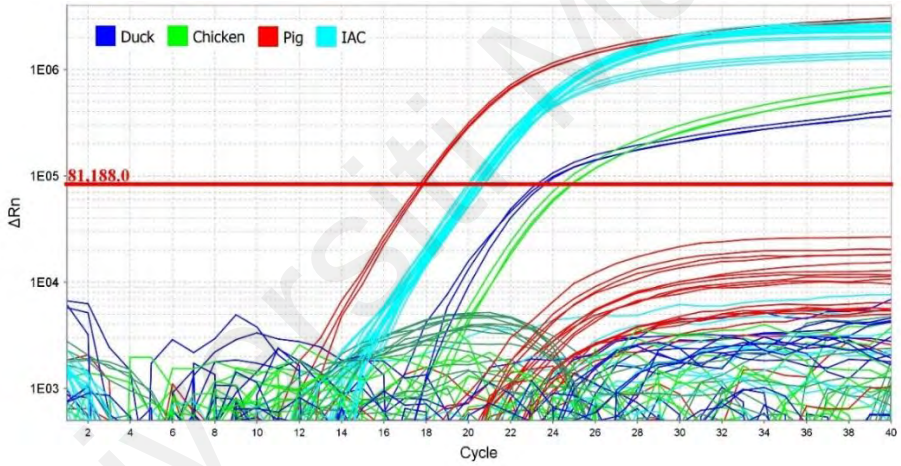


Figure 4.19: Multiplex qPCR amplification plot (a) for cow (red), buffalo (blue), goat (deep green) and sheep (pink), (b) for chicken (green), duck (blue) and pig (red) along with the endogenous control for eukaryotes (sky blue) against 23 other species (below the threshold cycle).

Table 4.26: Specificity /cross-reactivity tests of mqPCR-1 and endogenous system

Animal species tested	Multiplex real-time PCR system			Internal amplification control PCR system		
	Increase of fluorescence signal	Mean Ct value	RSD (%)	Increase of fluorescence signal	Mean Ct value	RSD (%)
Cow	+	18.16	0.92	+	16.42	0.62
Buffalo	+	19.58	0.22	+	16.78	0.78
Goat	+	20.37	0.79	+	16.82	0.43
Sheep	+	18.31	0.20	+	16.55	0.73
Chicken	-	-		+	16.41	0.27
Duck	-	-		+	16.43	0.46
Pig	-	-		+	16.78	0.51
Dog	-	-		+	16.89	0.74
Rabbit	-	-		+	16.79	0.54
Turtle	-	-		+	16.33	0.43
Rat	-	-		+	16.67	0.36
Pigeon	-	-		+	16.81	0.63
Quail	-	-		+	16.9	1.03
Monkey	-	-		+	16.69	0.60
Donkey	-	-		+	16.41	0.55
Ostrich	-	-		+	16.88	0.48
Crocodile	-	-		+	16.44	0.32
Frog	-	-		+	16.73	0.49
Tortoise	-	-		+	16.9	0.57
Cat	-	-		+	16.53	0.52
Salmon	-	-		+	16.44	0.68
Tuna	-	-		+	16.54	0.97
Sardine	-	-		+	16.89	0.56
Rohu	-	-		+	16.66	0.51
Cod	-	-		+	16.49	0.26
Tilapia	-	-		+	16.77	0.51
Wheat	-	-		+	16.48	0.44
Onion	-	-		+	16.79	0.53
Garlic	-	-		+	16.41	0.27
Pepper	-	-		+	16.88	0.67

Note: '+' indicates positive PCR result (Ct value <40) and '-' indicates no increase of the fluorescence signal within 40 cycles.

Table 4.27: Specificity /cross-reactivity tests of mqPCR-2 and endogenous system

Animal species tested	Multiplex real-time PCR system			Internal amplification control PCR system		
	Increase of fluorescence signal	Mean Ct value	RSD (%)	Increase of fluorescence signal	Mean Ct value	RSD (%)
Chicken	+	24.16	0.31	+	19.88	0.66
Duck	+	23.13	0.35	+	19.94	0.70
Pig	+	17.68	0.67	+	20.32	0.65
Cow	-	-		+	20.65	0.62
Buffalo	-	-		+	20.32	0.59
Goat	-	-		+	20.29	0.77
Sheep	-	-		+	20.12	0.53
Dog	-	-		+	20.44	0.60
Rabbit	-	-		+	19.89	0.68
Turtle	-	-		+	20.02	0.82
Rat	-	-		+	19.98	6.06
Pigeon	-	-		+	20.43	0.93
Quail	-	-		+	19.88	1.11
Monkey	-	-		+	20.46	1.20
Donkey	-	-		+	20.66	0.72
Ostrich	-	-		+	19.92	0.93
Crocodile	-	-		+	20.22	0.84
Frog	-	-		+	20.68	0.54
Tortoise	-	-		+	19.94	0.86
Cat	-	-		+	20.71	0.48
Salmon	-	-		+	19.88	0.98
Tuna	-	-		+	20.58	0.67
Sardine	-	-		+	20.35	0.60
Rohu	-	-		+	20.69	0.54
Cod	-	-		+	19.87	1.04
Tilapia	-	-		+	20.7	0.49
Wheat	-	-		+	20.48	0.54
Onion	-	-		+	19.83	0.76
Garlic	-	-		+	20.62	0.70
Pepper	-	-		+	20.51	0.44

Note: '+' indicates positive PCR result (Ct value <40) and '-' indicates no increase of the fluorescence signal within 40 cycles.

4.7.4 Limit of Detection (LOD)

To determine the LOD of both mqPCR systems, serially diluted (10-fold) mixed genomic DNA of the target species, starting from 30 ng to 0.003 ng concentration, was prepared. Then 2 µL of each diluted mixed DNA solution was added to 20 µL of multiplex reaction mixture. As a result, the reaction mixture contained 60, 6, 0.6, 0.06, 0.006, ng of DNA for each species. After running PCR using all diluted DNA mixtures, amplification plots clearly reflected detectable Ct from all concentrations of DNA mixtures. Thus, the

assay was sensitive to detect and quantify down to 0.006 ng of target DNA (Figure 4.20). Table 4.28 presents the Ct values and corresponding relative standard deviation (RSD) for all dilutions of DNA. In this assay, RSD were found to be less than 1.0 (0.07-0.7) for all diluted DNA.

4.7.5 Quantification of Targets and Determination of Multiplex qPCR Efficiency

To determine the quantity of the target species DNA, individual standard curves were constructed for each of the seven target species. Both mqPCR assays (mqPCR-1 and mqPCR-2) were carried out using each serially diluted DNA mixture (starting from 30 ng to 0.003 ng concentration) obtained from the two admixtures prepared by mixing of equal amount of meat from each target species. After running experiments, the individual standard curves were generated for cow, buffalo, goat, sheep and IAC (mqPCR-1) and for chicken, duck, pig and IAC (mqPCR-2) by plotting Ct values against logarithmic concentrations of DNA (Figure 4.20 a-i). Good linear regression was observed for all the standard curves as evidenced by the high regression coefficient (R^2) of 0.9993, 0.9987, 0.9987, 0.9974 and 0.9992 for cow, buffalo, goat, sheep and IAC (mqPCR-1) and 0.9991, 0.9966, 0.9981 and 0.9888 for chicken, duck pig and IAC (mqPCR-2), respectively. The corresponding slopes were found -3.447, -3.501, -3.49, -3.525 and -3.391 (for mqPCR-1) and, - 3.411, - 3.542, - 3.39 and - 3.106 (for mqPCR-2) for the aforementioned species and IAC, respectively. Similarly, the PCR efficiencies (E) as calculated following the formula mentioned in methodology, were 95.03%, 93.03%, 93.43%, 92.17% and 97.20% (mqPCR-1) and 96.41, 91.57%, 97.24% and 109.84 (mqPCR-2) respectively.

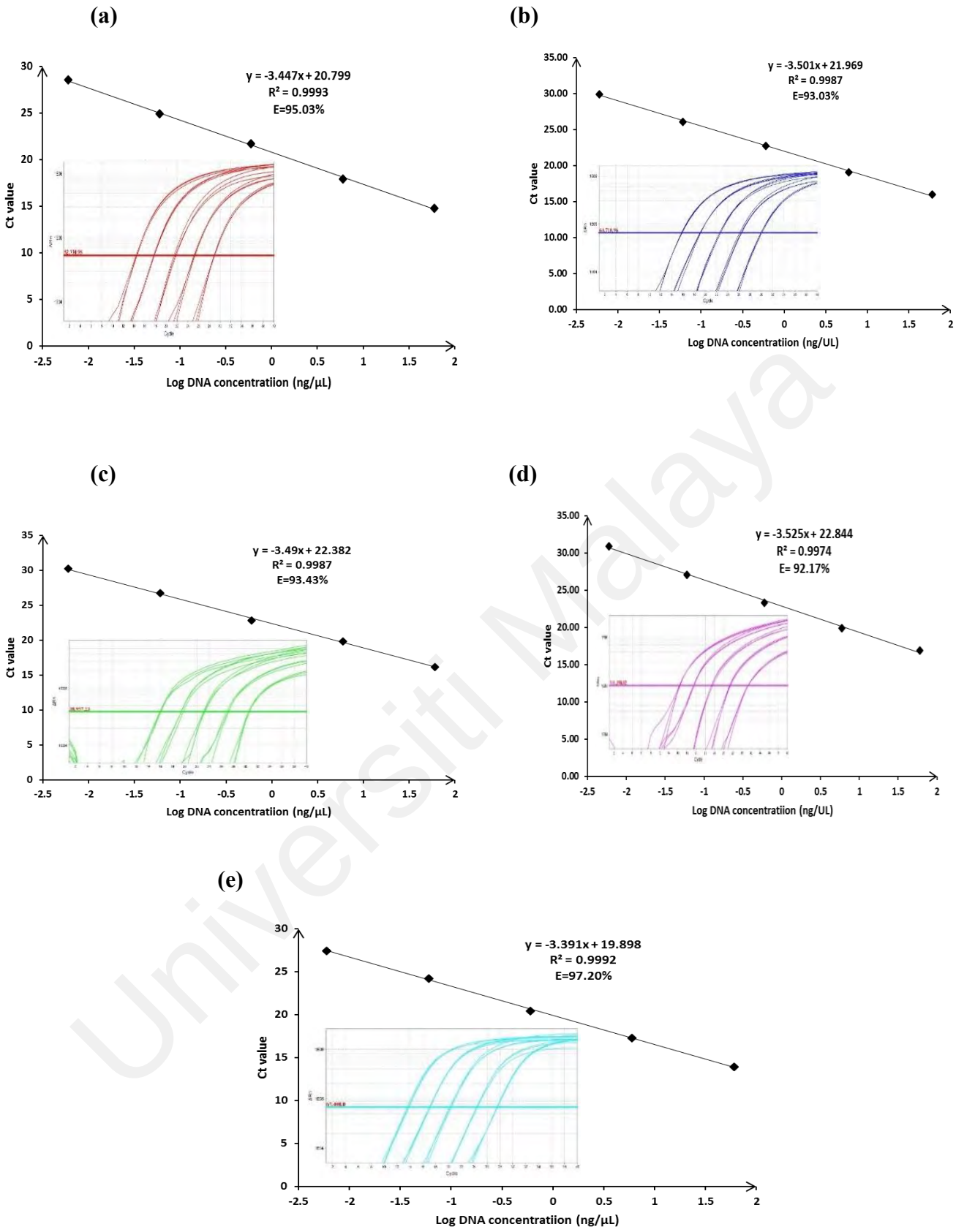


Figure 4.20: Amplification plots and standard curves (a−i) of multiplex qPCR products obtained from 10-fold serially diluted mixed DNA of seven target species. Amplification plots and standard curves for beef (a), buffalo (b), goat (c), sheep (d) and internal amplification control (IAC) (e) (mqPCR-1) and for chicken (f), duck (g), pork (h) and IAC (i) (mqPCR-2) in qPCR systems.

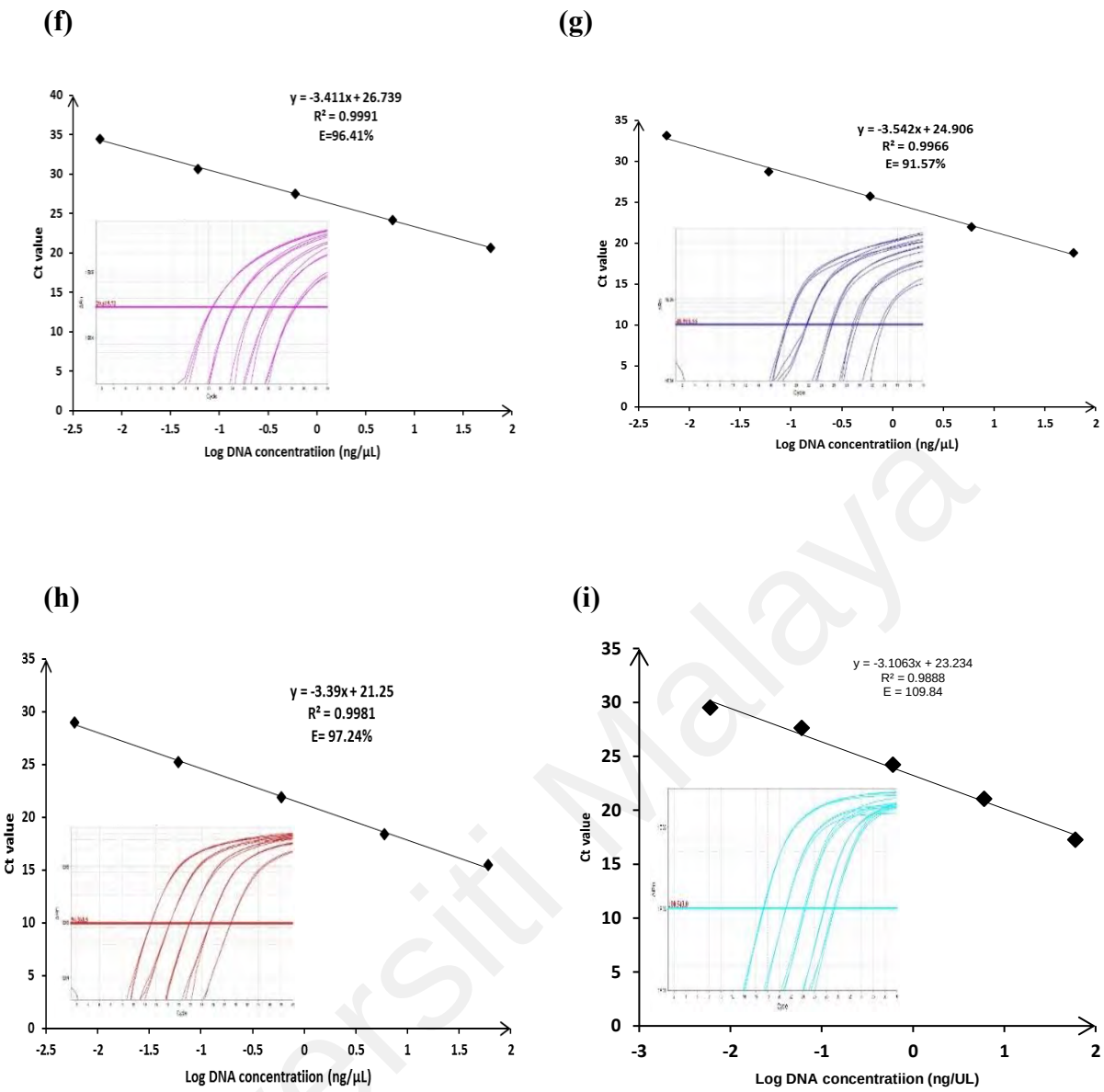


Figure 4.20, continued.

Table 4.28: Ct values of each target species and IAC obtained from the amplification plot with 10-fold serially diluted DNA of each target species for the determination of LOD and generation of standard curves.

DNA concentration (ng) →		60	6	0.6	0.06	0.006
Cow	**Ct value**	14.774	18.019	21.617	24.801	28.536
		14.808	17.828	21.773	25.007	28.498
		14.754	17.903	21.693	24.907	28.524
	Mean Ct value	14.78	17.92	21.69	24.91	28.52
	SD	0.027	0.096	0.078	0.103	0.019
	RSD (%)	0.18	0.54	0.36	0.41	0.07
Buffalo	**Ct value**	15.906	19.028	22.632	26.158	29.907
		16.005	19.001	22.794	26.186	30.05
		15.983	19.101	22.707	25.857	29.901
	Mean Ct value	15.96	19.04	22.71	26.07	29.95
	SD	0.052	0.052	0.081	0.182	0.084
	RSD (%)	0.33	0.27	0.36	0.70	0.28
Goat	**Ct value**	16.235	19.809	22.746	26.624	30.218
		16.204	19.905	22.889	26.743	30.287
		16.132	19.835	22.829	26.766	30.124
	Mean Ct value	16.19	19.85	22.82	26.71	30.21
	SD	0.053	0.050	0.072	0.076	0.082
	RSD (%)	0.33	0.25	0.31	0.29	0.27
Sheep	**Ct value**	16.941	19.923	23.399	27.178	30.856
		16.897	19.884	23.345	27.101	30.992
		16.884	19.859	23.156	27.023	30.938
	Mean Ct value	16.91	19.89	23.30	27.10	30.93
	SD	0.030	0.032	0.128	0.078	0.068
	RSD (%)	0.18	0.16	0.55	0.29	0.22
IAC (mqPCR-1)	**Ct value**	13.863	17.275	20.425	24.248	27.424
		13.812	17.301	20.363	24.201	27.378
		14.074	17.334	20.413	24.179	27.451
	Mean Ct value	13.92	17.30	20.40	24.21	27.42
	SD	0.139	0.030	0.033	0.035	0.037
	RSD (%)	1.00	0.17	0.16	0.15	0.13
Chicken	**Ct value**	20.621	24.144	18.305	30.72	34.431
		20.646	24.188	18.406	30.669	34.518
		20.721	24.202	18.312	30.574	34.501
	Mean Ct value	20.66	24.18	27.51	30.65	34.48
	SD	0.052	0.030	0.056	0.074	0.046
	RSD (%)	0.25	0.13	0.21	0.24	0.13
Duck	**Ct value**	18.824	21.967	25.705	28.81	33.017
		18.767	22.073	25.795	28.754	33.137
		18.839	21.905	25.831	28.718	33.239
	Mean Ct value	18.81	21.98	25.78	28.76	33.13
	SD	0.038	0.085	0.065	0.046	0.111
	RSD (%)	0.20	0.39	0.25	0.16	0.34
Pig	**Ct value**	15.511	18.393	21.875	25.193	28.908
		15.475	18.377	21.905	25.207	29.063
		15.488	18.365	21.911	25.252	29.093
	Mean Ct value	15.49	18.38	21.90	25.22	29.02
	SD	0.018	0.014	0.019	0.031	0.099
	RSD (%)	0.12	0.08	0.09	0.12	0.34
IAC (mqPCR-2)	**Ct value**	17.18	21.025	24.242	27.578	29.493
		17.33	21.014	24.236	27.678	29.528
		17.244	21.103	24.133	27.592	29.474
	Mean Ct value	17.251	21.047	24.204	27.616	29.498
	SD	0.075	0.049	0.061	0.054	0.027
	RSD (%)	0.44	0.23	0.25	0.20	0.09

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

4.7.6 Sensitivity and Validity of Multiplex qPCR Assay under Model Meat Products

Both the developed mqPCR assays were tested for sensitivity to determine the level of beef, buffalo, goat, sheep, chicken, duck and pork in deliberately adulterated model meat products. After running mqPCR using the extracted DNA from adulterated meat products (beef, goat, chicken and pork meatballs and frankfurters), all the seven species could be successfully detected down to 1% adulteration by the two mqPCR systems. The Ct values corresponding to minimum detectable quantity (1%) were 15.29 - 24.47 (Table 4.29) in mqPCR-1 (For cow, buffalo, goat and sheep) and 15.67 – 28.27 (Table 4.30) in mqPCR-2 (for chicken, duck and pig). However, IAC maintained constant Ct value ranges of 13.26-13.62 in qPCR-1 and 18.43 – 18.68 in qPCR-2 for all percentages of adulteration. The inter day relative standard deviations (RSDs) were calculated from the mean Ct values of model meat products spiked at different levels (10, 5 and 1%) and were obtained between 0.03 and 1.25% (for both mqPCR-1 and mqPCR-2). RSDs were found less than 1.0% for 80 samples out of 84 samples. However, only a few samples (4) yielded RSDs ≥ 1.0% (Tables 4.29 and 4.30). Thus, the developed mqPCR systems were highly sensitive, specific and authentic and can unambiguously determine the targets even from 1% adulterated samples.

The validity of mqPCR systems were checked through the analysis of processed meat products (frankfurters and meatballs). The analysis results of the target species for different spiking ratios are presented in Table 4.31 (for cow, buffalo, goat, sheep in mqPCR-1) and in Table 4.32 (for chicken, duck and pig in mqPCR-2). The target recoveries from the spiking levels (10, 5 and 1%) were found between 88.43 and 112.20% along with systematic errors between − 11.57 and + 12.20% and RSDs 0.47−13.54% in qPCR-1. The maximum recovery of 112.20% was observed in 5% sheep adulterated goat meatball and a minimum of 88.43% was in beef meatball spiked with 10% sheep.

Maximum RSD value was found for goat meatball containing 5% goat adulteration and minimum RSD value was found for 5% sheep adulterated goat meatball. On the other hand, for mqPCR-2, the target recoveries were achieved 87.33 – 113.72% along with systematic errors -12.67 – 13.72 and RSDs 1.29 – 14.0. The maximum and minimum recoveries were observed from chicken frankfurter adulterated with 5% chicken and 1% pig respectively. The maximum and minimum RSDs were found for 5% pig adulterated chicken meatball and 1% duck adulterated chicken frankfurter respectively.

Table 4.29: Mean CT values and inter day RSD of different model meat products for mqPCR-1

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Beef frankfurter	10	Beef	15.781	15.732	15.822	0.045	0.28
		Buffalo	19.841	19.734	19.869	0.071	0.36
		Goat	20.242	20.199	20.191	0.027	0.14
		Sheep	20.792	20.581	20.611	0.114	0.55
	5	Beef	15.426	15.604	15.622	0.108	0.69
		Buffalo	20.821	20.938	21.001	0.091	0.43
		Goat	21.391	21.422	21.238	0.099	0.46
		Sheep	21.833	21.711	21.734	0.065	0.30
	1	Beef	15.390	15.263	15.351	0.065	0.42
		Buffalo	23.401	23.311	23.284	0.061	0.26
		Goat	23.821	23.635	23.611	0.115	0.49
		Sheep	24.357	24.471	24.410	0.057	0.23
Goat frankfurter	10	Beef	18.864	18.781	18.603	0.133	0.72
		Buffalo	19.758	19.815	19.732	0.042	0.22
		Goat	17.441	17.393	17.193	0.132	0.77
		Sheep	20.799	20.623	20.654	0.094	0.45
	5	Beef	19.792	19.678	19.612	0.091	0.46
		Buffalo	21.015	20.751	20.791	0.142	0.68
		Goat	17.138	17.035	16.929	0.105	0.62
		Sheep	21.811	21.898	21.686	0.107	0.49
	1	Beef	22.206	22.312	22.347	0.073	0.33
		Buffalo	23.569	23.529	23.235	0.182	0.78
		Goat	16.690	16.731	16.787	0.049	0.29
		Sheep	24.398	24.129	24.108	0.162	0.67

Table 4.29, continued

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Beef meatball	10	Beef	15.887	15.721	15.759	0.087	0.55
		Buffalo	19.792	19.930	19.962	0.090	0.45
		Goat	20.382	20.398	20.238	0.088	0.44
		Sheep	20.795	20.843	21.108	0.169	0.80
	5	Beef	15.599	15.459	15.419	0.095	0.61
		Buffalo	20.997	20.921	20.993	0.043	0.20
		Goat	21.412	21.368	21.298	0.057	0.27
		Sheep	21.661	21.891	21.821	0.118	0.54
	1	Beef	15.265	15.411	15.221	0.099	0.65
		Buffalo	23.492	23.513	23.256	0.143	0.61
		Goat	23.610	23.692	23.812	0.102	0.43
		Sheep	24.421	24.361	24.116	0.162	0.67
Goat meatball	10	Beef	18.794	18.644	18.604	0.100	0.54
		Buffalo	19.738	19.776	19.911	0.091	0.46
		Goat	17.401	17.462	17.237	0.116	0.68
		Sheep	20.656	20.601	20.576	0.041	0.20
	5	Beef	19.662	19.699	19.628	0.036	0.18
		Buffalo	20.759	20.812	20.795	0.027	0.13
		Goat	16.910	17.288	17.210	0.200	1.16
		Sheep	21.610	21.607	21.597	0.007	0.03
	1	Beef	22.050	22.337	22.242	0.146	0.66
		Buffalo	23.204	23.284	23.156	0.065	0.28
		Goat	16.728	16.748	16.678	0.036	0.22
		Sheep	24.295	24.476	24.445	0.097	0.40

Table 4.30: Mean CT values and inter day RSD of different model meat products for mqPCR-2

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Chicken frankfurter	10	Chicken	21.514	21.502	21.706	0.114	0.53
		Duck	22.821	22.921	22.872	0.050	0.22
		Pig	19.291	19.282	19.111	0.101	0.53
	5	Chicken	21.272	21.301	21.151	0.080	0.37
		Duck	23.994	23.901	23.734	0.132	0.55
		Pig	20.345	20.301	20.241	0.052	0.26
	1	Chicken	21.221	21.175	21.551	0.205	0.96
		Duck	26.486	26.462	26.491	0.016	0.06
		Pig	22.731	22.762	22.893	0.086	0.38
Pork frankfurter	10	Chicken	24.590	24.793	24.822	0.126	0.51
		Duck	22.824	22.692	22.643	0.094	0.41
		Pig	16.061	16.185	16.231	0.088	0.54
	5	Chicken	25.825	25.891	25.899	0.041	0.16
		Duck	23.721	23.689	23.649	0.036	0.15
		Pig	16.132	16.101	15.871	0.143	0.89
	1	Chicken	28.211	28.271	28.202	0.038	0.13
		Duck	26.431	26.471	26.422	0.026	0.10
		Pig	15.897	15.801	15.921	0.063	0.40

Table 4.30, continued

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Chicken meatball	10	Chicken	21.712	21.634	21.771	0.069	0.32
		Duck	22.824	22.661	22.634	0.103	0.45
		Pig	19.103	19.114	19.191	0.048	0.25
	5	Chicken	21.592	21.481	21.411	0.091	0.42
		Duck	24.031	23.861	23.881	0.093	0.39
		Pig	20.311	20.124	19.901	0.205	1.02
	1	Chicken	21.463	21.411	21.367	0.048	0.22
		Duck	26.432	26.372	26.245	0.095	0.36
		Pig	22.643	22.680	22.712	0.035	0.15
Pork meatball	10	Chicken	24.571	24.612	24.691	0.061	0.25
		Duck	22.689	22.651	22.701	0.026	0.12
		Pig	16.021	16.423	16.189	0.202	1.25
	5	Chicken	25.606	25.532	25.541	0.040	0.16
		Duck	23.791	23.822	23.970	0.096	0.40
		Pig	15.887	15.847	15.734	0.079	0.50
	1	Chicken	28.219	28.119	28.067	0.077	0.27
		Duck	26.456	26.386	26.256	0.101	0.38
		Pig	15.678	15.811	16.067	0.198	1.25

Table 4.31: Reproducibility and Recovery of the Target Species in Model Meat Products for mqPCR-1

Products	Spike level (%)	Species	Content of target determined (%)				SD	RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean				
Beef frankfurter	10	Beef	71.40	73.77	69.45	71.54	2.163	3.02	102.20	2.20
		Buffalo	10.10	10.87	9.95	10.31	0.494	4.79	103.07	3.07
		Goat	10.26	10.55	10.61	10.47	0.187	1.79	104.73	4.73
		Sheep	9.55	10.96	10.75	10.42	0.761	7.30	104.20	4.20
	5	Beef	90.50	80.35	79.40	83.42	6.153	7.38	98.14	-1.86
		Buffalo	5.30	4.90	4.70	4.97	0.306	6.15	99.33	-0.67
		Goat	4.23	4.71	5.32	4.75	0.546	11.49	95.07	-4.93
		Sheep	4.84	5.24	5.16	5.08	0.212	4.17	101.60	1.60
	1	Beef	92.70	100.90	95.15	96.25	4.209	4.37	99.23	-0.77
		Buffalo	0.97	1.03	1.05	1.02	0.042	4.10	101.67	1.67
		Goat	0.96	1.09	1.11	1.05	0.081	7.73	105.33	5.33
		Sheep	0.93	0.86	0.89	0.89	0.035	3.93	89.33	-10.67
Goat frankfurter	10	Beef	9.10	9.62	10.84	9.85	0.893	9.06	98.53	-1.47
		Buffalo	10.70	10.30	10.88	10.63	0.297	2.79	106.27	6.27
		Goat	65.10	67.21	76.69	69.67	6.173	8.86	99.52	-0.48
		Sheep	9.50	10.66	10.45	10.20	0.618	6.06	102.03	2.03
	5	Beef	4.89	5.28	5.52	5.23	0.318	6.08	104.60	4.60
		Buffalo	4.68	5.57	5.42	5.22	0.476	9.12	104.47	4.47
		Goat	79.50	85.12	91.28	85.30	5.892	6.91	100.35	0.35
		Sheep	4.91	4.63	5.32	4.95	0.347	7.01	99.07	-0.93
	1	Beef	0.98	0.91	0.89	0.93	0.047	5.10	92.67	-7.33
		Buffalo	0.87	0.89	1.08	0.95	0.116	12.24	94.67	-5.33
		Goat	106.80	104.00	100.20	103.67	3.313	3.20	106.87	6.87
		Sheep	0.90	1.08	1.09	1.02	0.107	10.45	102.33	2.33

Table 4.31, continued

Products	Spike level (%)	Species	Content of target determined (%)				SD	RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean				
Beef meatball	10	Beef	66.52	74.32	72.45	71.10	4.072	5.73	101.57	1.57
		Buffalo	10.46	9.55	9.35	9.79	0.592	6.05	97.87	-2.13
		Goat	9.35	9.25	10.29	9.63	0.574	5.96	96.30	-3.70
		Sheep	9.53	9.23	7.77	8.84	0.942	10.65	88.43	-11.57
	5	Beef	80.63	88.53	90.90	86.69	5.377	6.20	101.98	1.98
		Buffalo	4.74	4.98	4.75	4.82	0.136	2.81	96.47	-3.53
		Goat	4.74	4.88	5.11	4.91	0.187	3.80	98.20	-1.80
		Sheep	5.41	4.66	4.87	4.98	0.387	7.77	99.60	-0.40
	1	Beef	100.78	91.42	103.79	98.66	6.451	6.54	101.71	1.71
		Buffalo	0.92	0.90	1.07	0.96	0.093	9.65	96.33	-3.67
		Goat	1.11	1.05	0.97	1.04	0.07	6.73	104.33	4.33
		Sheep	0.89	0.92	1.09	0.97	0.108	11.16	96.67	-3.33
Goat meatball	10	Beef	9.54	10.54	10.80	10.29	0.665	6.46	102.93	2.93
		Buffalo	10.84	10.57	9.67	10.36	0.613	5.91	103.60	3.60
		Goat	66.86	64.22	74.50	68.53	5.339	7.79	97.90	-2.10
		Sheep	10.44	10.82	11.00	10.75	0.286	2.66	107.53	7.53
	5	Beef	5.34	5.21	5.46	5.34	0.125	2.34	106.73	6.73
		Buffalo	5.54	5.35	5.41	5.43	0.097	1.79	108.67	8.67
		Goat	92.43	72.03	75.83	80.10	10.85	13.54	94.23	-5.77
		Sheep	5.59	5.60	5.64	5.61	0.026	0.47	112.20	12.20
	1	Beef	1.08	0.89	0.95	0.97	0.097	9.98	97.33	-2.67
		Buffalo	1.11	1.05	1.15	1.10	0.05	4.56	110.33	10.33
		Goat	104.23	102.86	107.70	104.93	2.495	2.38	108.18	8.18
		Sheep	0.97	0.86	0.87	0.90	0.061	6.76	90.00	-10.00

Table 4.32: Reproducibility and recovery of the target species in model meat products for mqPCR2

Products	Spike level (%)	Species	Content of target determined (%)				SD	RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean				
Chicken frankfurter	10	Chicken	85.06	85.7	74.72	81.83	6.16	7.53	102.28	2.28
		Duck	9.69	9.08	9.38	9.38	0.31	3.25	93.83	-6.17
		Pig	9.45	9.51	10.68	9.88	0.69	7.02	98.80	-1.20
	5	Chicken	100.16	98.21	108.68	102.35	5.57	5.44	113.72	13.72
		Duck	4.52	4.8	5.35	4.89	0.42	8.64	97.80	-2.20
		Pig	4.62	4.76	4.96	4.78	0.17	3.57	95.60	-4.40
	1	Chicken	103.66	106.93	82.96	97.85	13.00	13.28	99.85	-0.15
		Duck	0.89	0.91	0.89	0.90	0.01	1.29	89.67	-10.33
		Pig	0.91	0.89	0.82	0.87	0.05	5.41	87.33	-12.67
Pork frankfurter	10	Chicken	10.66	9.3	9.12	9.69	0.84	8.69	96.93	-3.07
		Duck	9.67	10.54	10.88	10.36	0.62	6.02	103.63	3.63
		Pig	84.84	77.98	75.59	79.47	4.80	6.04	99.34	-0.66
	5	Chicken	4.63	4.43	4.4	4.49	0.13	2.79	89.73	-10.27
		Duck	5.4	5.51	5.66	5.52	0.13	2.36	110.47	10.47
		Pig	80.85	82.57	96.53	86.65	8.60	9.92	96.28	-3.72
	1	Chicken	0.92	0.89	0.93	0.91	0.02	2.28	91.33	-8.67
		Duck	0.93	0.9	0.93	0.92	0.02	1.88	92.00	-8.00
		Pig	94.82	101.23	93.31	96.45	4.21	4.36	98.42	-1.58

Table 4.32, continued

Products	Spike level (%)	Species	Content of target determined (%)				SD	RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean				
Chicken meatball	10	Chicken	74.4	78.44	71.51	74.78	3.48	4.65	93.48	-6.52
		Duck	9.67	10.76	10.95	10.46	0.69	6.60	104.60	4.60
		Pig	10.74	10.66	10.12	10.51	0.34	3.21	105.07	5.07
	5	Chicken	80.7	86.98	91.19	86.29	5.28	6.12	95.88	-4.12
		Duck	4.41	4.93	4.86	4.73	0.28	5.96	94.67	-5.33
		Pig	4.73	5.37	6.25	5.45	0.76	14.00	109.00	9.00
	1	Chicken	88.04	91.19	93.93	91.05	2.95	3.24	92.91	-7.09
		Duck	0.93	0.96	1.05	0.98	0.06	6.37	98.00	-2.00
		Pig	0.97	0.95	0.92	0.95	0.03	2.66	94.67	-5.33
Pork meatball	10	Chicken	10.8	10.5	9.96	10.42	0.43	4.09	104.20	4.20
		Duck	10.56	10.83	10.48	10.62	0.18	1.73	106.23	6.23
		Pig	87.18	66.35	77.8	77.11	10.43	13.53	96.39	-3.61
	5	Chicken	5.37	5.64	5.61	5.54	0.15	2.67	110.80	10.80
		Duck	5.16	5.05	4.59	4.93	0.30	6.13	98.67	-1.33
		Pig	95.48	98.11	105.94	99.84	5.44	5.45	110.94	10.94
	1	Chicken	0.92	0.98	1.02	0.97	0.05	5.17	97.33	-2.67
		Duck	0.91	0.95	1.04	0.97	0.07	6.89	96.67	-3.33
		Pig	110.07	100.54	84.49	98.37	12.93	13.14	100.37	0.37

A paired-samples t-test was conducted to compare the reference (actual) values (M1) and the experimental values (M2) of the mqPCR systems. There was no significant difference between the actual and reference values for beef (M1= 44.66, M2= 45.02), buffalo (M1= 5.33, M2= 5.46), goat (M1= 44.66, M2= 45.34), sheep (M1= 5.33, M2= 5.42), chicken (M1=47.33, M2= 47.18), duck (M1=5.33, M2=5.39) and pig (M1=47.33, M2=47.02) with the corresponding p values for beef (p=0.25), buffalo (p=0.11), goat (p=0.51), sheep (p=0.58), chicken (p=0.91), duck (p=0.61) and pig (p=0.80), respectively at 10, 5 and 1% adulterations (Table 4.33). The analysis results show that the experimental quantity (mean value) obtained from both the mqPCR assays for adulterated model frankfurter and meatball of four target species (cow, goat, chicken and pig) were fairly close to the actual (mean) values that were used during their laboratory preparation. This reflects a good precision and accuracy of the developed mqPCR systems for the determination of 1-10% adulteration of beef, buffalo, goat, sheep, chicken, duck and pork in meat products.

Table 4.33: Results of group difference between the experimental and reference values of the target species for different model meat products

Species in meat products	Mean Difference (actual - recovered value)	t value (t)	Degree of Freedom (df)	Sig. (2-tailed) value (p)	95% Confidence Interval of the Difference	
					Lower	Upper
Beef	-0.36	-1.23	11.00	0.25	-0.99	0.28
Buffalo	-0.13	-1.73	11.00	0.11	-0.29	0.03
Goat	-0.68	-0.68	11.00	0.51	-2.84	1.5
Sheep	-0.09	-0.57	11.00	0.58	-0.41	0.24
Chicken	0.15	0.11	11.00	0.91	-2.85	3.16
Duck	-0.06	-0.52	11.00	0.61	-0.29	0.18
Pig	0.31	0.26	11.00	0.80	-2.33	2.94

4.7.7 Analysis of Commercial Meat Products

A total of 49 popular meat products including 17 burgers (beef, chicken and lamb), 17 frankfurters (beef, chicken and pork), 4 cocktails (pork), 4 sausages (pork) and 7 cooked whole muscle meat (lamb) were purchased from different Malaysian outlets, and these were analyzed using the developed multiplex qPCR assay. The results presented in Table 4.34 reveals considerable species substitution rate with the highest incidences in beef products. The screening results showed that 85%, 100%, 100%, and 100% of beef burgers, frankfurters, meatballs and cocktails contained buffalo adulteration of which 9% contained no beef at all indicating species substitution in addition to mislabelling. On the other hand, pork products (frankfurter, sausage and cocktail) contained chicken DNA in 50% of samples. Undeclared DNA was detected in 23% of chicken burgers and frankfurters. Moreover, lamb burgers and cooked whole lamb meat contained either beef or buffalo in 15% of tested samples.

Table 4.34: Screening results of model and commercial meat products performed by developed multiplex qPCR assays

Sample	Adulteration		Detected species[a]							Detection accuracy
	Species	%	Cow	Buffalo	Chicken	Duck	Goat	Sheep	Pig	
Model frankfurters										
Beef	Buffalo, goat and sheep	10	3/3	3/3	-	-	3/3	3/3	-	100
Beef	Buffalo, goat and sheep	5	3/3	3/3	-	-	3/3	3/3	-	100
Beef	Buffalo, goat and sheep	1	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	10	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	5	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	1	3/3	3/3	-	-	3/3	3/3	-	100
Chicken	Duck and pig	10	-	-	3/3	3/3	-	-	3/3	100
Chicken	Duck and pig	5	-	-	3/3	3/3	-	-	3/3	100
Chicken	Duck and pig	1	-	-	3/3	3/3	-	-	3/3	100
Pork	Chicken and duck	10	-	-	3/3	3/3	-	-	3/3	100
Pork	Chicken and duck	5	-	-	3/3	3/3	-	-	3/3	100
Pork	Chicken and duck	1	-	-	3/3	3/3	-	-	3/3	100
Model meatballs										
Beef	Buffalo, goat and sheep	10	3/3	3/3	-	-	3/3	3/3	-	100
Beef	Buffalo, goat and sheep	5	3/3	3/3	-	-	3/3	3/3	-	100
Beef	Buffalo, goat and sheep	1	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	10	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	5	3/3	3/3	-	-	3/3	3/3	-	100
Goat	Cow. Buffalo and sheep	1	3/3	3/3	-	-	3/3	3/3	-	100
Chicken	Duck and pig	10	-	-	3/3	3/3	-	-	3/3	100
Chicken	Duck and pig	5	-	-	3/3	3/3	-	-	3/3	100
Chicken	Duck and pig	1	-	-	3/3	3/3	-	-	3/3	100

Table 4.34, continued

Sample	Adulteration		Detected species[a]							Detection accuracy
	Species	%	Cow	Buffalo	Chicken	Duck	Goat	Sheep	Pig	
Pork	Chicken and duck	10	-	-	3/3	3/3	-	-	3/3	100
Pork	Chicken and duck	5	-	-	3/3	3/3	-	-	3/3	100
Pork	Chicken and duck	1	-	-	3/3	3/3	-	-	3/3	100
Commercial meat products										
Beef burger	-	-	6/7	6/7	0/7	0/7	0/7	0/7	0/7	100
Beef frankfurter	-	-	4/4	4/4	0/4	0/4	0/4	0/4	0/4	100
Chicken burger	-	-	0/4	0/4	4/4	0/4	0/4	0/4	0/4	100
Chicken frankfurter	-	-	1/9	3/9	9/9	0/9	0/9	0/9	0/9	100
Pork frankfurter	-	-	0/4	0/4	2/4	0/4	0/4	0/4	4/4	100
Pork cocktail	-	-	0/4	0/4	3/4	0/4	0/4	0/4	4/4	100
Pork sausage	-	-	0/4	0/4	1/4	0/4	0/4	0/4	4/4	100
Lamb burger	-	-	1/6	0/6	0/6	0/6	0/6	6/6	0/6	100
Lamb cooked whole muscle meat	-	-	0/7	1/7	0/7	0/7	0/7	7/7	0/7	100

[a]Note: The numerator and denominator of each fraction denote the number of positive detection and total number of samples analyzed using the multiplex qPCR assays.

CHAPTER 5: DISCUSSION

5.1 DNA Extraction

To obtain reproducible, reliable and quality PCR results, the quantity and quality of template DNA play a vital role. Therefore, both DNA extraction and DNA quantification are crucial steps in PCR assays. The salient features that ideal DNA extraction methods should possess include high yield of DNA, removal of impurities and inhibitors, and high–throughput processing. Certain factors that determine the yield of extracted genomic DNA include quantity and quality of starting materials and the nature of samples whether raw, processed, thermally or chemically treated. Extraction kit and extraction protocol are also important. It is notable that commercial DNA extraction kits offer greater DNA yields compared to conventional (liquid-liquid extraction) techniques since the latter involves aqueous and organic phases resulting in wastage of DNA. Furthermore, commercial kits are less hazardous in to handling and there are minimum chances of DNA damage during the extraction procedure (Al Amin, 2015).

In this study, three different kinds of commercially available DNA extraction kits have been used to extract total genomic DNA from three different sample types such as raw meat, meat products (meatballs, burgers, frankfurters, sausages etc.) and plant tissues since each commercial extraction kit is developed targeting specific sample type depending on the presence of proteins, ingredients, additives, color etc. The Genomic DNA Mini Kit was developed to purify total DNA (both mitochondrial DNA and genomic DNA) from different animal tissues. Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) was used to extract total DNA from raw meat. The supplied micropestle was used to facilitate the disintegration of homogenized tissue and thus to enhance cell lysis. Lysis buffer helped to break the cell wall/cell membrane and release

DNA and proteinase K, to degrade protein thereby eliminating protein contamination. Absolute ethanol helped to remove protein part and wash buffer was used to remove potential contaminants. Finally low salt containing elution buffer (TE buffer) helped to stabilize stored DNA PH. Total DNA was extracted from meat products using DNeasy mericon Food Kit (QIAGEN GmgH, Hilden, Germany) where plant DNA was extracted by the DNeasy Plant Mini Kit (QIAGEN GmgH, Hilden, Germany).

The yield of total extracted DNA was determined by estimating its absorbance at 260 nm and the purity of DNA was checked on the basis of the ratio of absorbances at 260 nm and 280 nm (Nejad et al., 2014). At 260 nm, the absorbance value reflects the maximum absorbance of Nucleic acids while absorbance at 280 nm indicates the absorbance maxima of proteins. However, the ratio of A260/A280 provides information about DNA purity with respect to protein contamination (Oliveira et al., 2014). I found higher DNA concentration from raw samples as compared to thermally treated samples. The highest yield of DNA was obtained from raw animal tissue (150−408 ng/µL) whereas the lowest, from autoclave (121°C and 15 psi pressure) treated samples (32−82 ng/µL) (Table 4.1) since, extensive heat and pressure are simultaneously applied in autoclaving that caused highest degree of denaturation and degradation of the DNA. Microwaved (at 700W) samples gave the second lowest yield of DNA (58-98 ng/µl). Maybe, severe thermal treatment caused DNA degradation (Ali et al., 2016). However, the DNA yield from boiled samples were found to be a bit higher (90-160 ng/µl for raw meat and 72-110 ng/µl for meat products) than that from autoclaved and microwaved samples (Table 4.1) and this outcome might be the result of less degradation and denaturation of DNA under the milder thermal effect of boiling. It is notable that, the purity and the quantity of DNA was comparatively higher from all pure meat tissues (raw, boiled and autoclaved) than that from the meat products (raw, boiled and autoclaved); this might be due to the fact that commercial meat products contain additional quantities of fat and some added

ingredients including spices, salt, vegetables, flours, color and other food additives. For all the samples, the extracted DNA showed the absorbance ratio of A260/A280 between 1.8 and 2.0. Thus, the overall DNA extraction results reflect good quality of extracted DNA, and it was quite suitable for successful PCR amplification (Nejad et al., 2014).

5.2 Development of Biomarkers

Successful PCR amplification crucially depends on the design of suitable biomarkers and thus, successful detection of species is possible by appropriate design and development of acceptable primers. Inaccurately designed primers may result in inadequate product, or it may lead to formation of primer-dimer and/or non-specific products. Primer design, especially for mPCR assays, is extremely critical since they demand more stringent specificity. In this study, I developed seven sets of species-specific primers targeting the interspecies hyper variable and intraspecies conserved regions of mitochondrial cytb and ND5 genes of cow, buffalo, goat, sheep, chicken, duck and pig (Table 3.1). Mitochondrial cytb gene was targeted to design primer set of chicken while mitochondrial ND5 gene was targeted to design primer pairs of cow, buffalo, duck, goat, sheep and pork (Table 3.1). DNA biomarkers using mitochondrial DNA (mtDNA) are much preferred over nuclear DNA (nDNA) for authentication purposes since they have proven higher efficiency given their ubiquitous presence in multiple copies in all cells, especial protection by mitochondrial membrane and unique features for maternal inheritance (Rashid et al., 2015). The reason behind selection of cytb and ND5 genes as targets is that they met required criteria such as suitable target length, high level of intra-species conserved regions within the species and interspecies polymorphism, in addition to sequence database availability for most animals and plants (Mohamad et al., 2013; Razzak et al., 2015). During primer design, primer size should be considered. Due to too long primers, template DNA binding efficiency may be decreased at normal annealing

temperature because of possibility of secondary structure formation; again, too short primers may reduce specificity resulting in non-specific amplification (Abd-Elsalam, 2003). An ideal primer should be of 18-28 nucleotides length (Dieffenbach et al., 1993). All the developed primers in this study were kept within the recommended nucleotide length (between 21 and 28 bp size).

In this study, all the targets were kept within the length of 263 bp since earlier reports (Ali et al., 2015a) suggested higher stability of small-length targets over the longer ones. Longer targets are more susceptible to degradation under food processing and thus the assay certainly loses its efficiency and applicability in processed meat analysis (Rashid et al., 2015). Thus, the short length of our targets ranging from 73 to 263 bp probably contributed to successful outcome. The designed seven sets of primers for each of cow, buffalo, goat, sheep, chicken, duck and pig amplified short length PCR products within the range of 73−263 bp (cow ND5: 106 bp, buffalo ND5: 138 bp, goat ND5: 236 bp, sheep ND5: 263 bp, chicken cytb: 161 bp, duck ND5: 203 bp and pig ND5: 73 bp). Biomarker targets within this range made this assay highly efficient and stable under extreme food processing treatments and allowed to detect all the targets unambiguously even in degraded samples.

The efficiency of an mPCR assay crucially depends on primer specificity and melting temperature (Tm). In designing specific primers, oligonucleotide mismatch should be cautiously calculated since a critical mismatch, even by a single base pair, at the primer binding site might interfere PCR efficiency by reducing the amplification or making it unsuccessful (Wu et al., 2009). Considering these factors, our selected primers were critically evaluated about base mismatches in primer annealing sites. Sequence matching was cautiously evaluated within species (intraspecies) as well as with other non-related species by aligning the primers against three individuals of the same species and 7–11

species of the same genus as well as 30 different non-targets. The developed seven sets of primers possess 100 % matching only with the individuals of the target species and almost full sequence matching (maximum 1 base mismatch in few cases) was found with different species of the same genus. On the other hand, multiple mismatches of 3–18 nucleotides (13.63−76.19 %) were found with other non-target species (Tables 4.2−4.8). This eliminated the possibility of any cross-amplification reaction even with closely related species during PCR assay. Melting temperature (Tm) has an important role in primer annealing. Usually, primers having Tm within 55-65°C perform best in most PCR assays. Moreover, in multiplex PCR, a small difference in Tm between the primer sets might affect assay efficiency since, herein, all targets amplification occurs simultaneously in a single reaction tube and so all the concerned primers should have Tm of close proximity. Although, a variation of 3−5°C in Tm of the primers is acceptable, ≤ 2 °C Tm variation gives better results. If Tm of a primer is extremely lower than the annealing temperature (Ta) of PCR, annealing and extension might be failed, again, significantly higher Tm may cause non-hybridization and extension at an incorrect location along the DNA. Identical Tm of ~ 60°C (58.6−61.7°C) of the seven primers used in this study ensured that all primers could anneal only with the target template thereby eliminating the chances of annealing with any non-target species (Table 3.1) (Razzak et al., 2015). To get good PCR products, a satisfactory GC content is important since GC (%) largely determines Tm and annealing temperature (Ta) (Rychlik et al., 1990). Ideally, GC content should be 40-60% and the 3′-end containing 3 or more number of G or C may negatively affect primer specificity. Long polyG or polyC stretches in primers should be avoided to eliminate the chance of non-specific annealing (Ali et al., 2014a). Having fulfilled all the required criteria, the GC content and Tm of all primers were kept in the recommended values. The pairwise distance was also calculated for each target using the neighbour-joining technique (Tables 4.9−4.15). Cow showed the minimum distance (0.150) with

buffalo and the maximum distance (1.403) with pepper. Similarly, buffalo, goat, sheep, chicken, duck and pig had the lowest distances with cow (0.187), sheep (0.214), buffalo (0.206), quail (0.175), pigeon (0.287) and cat (0.257), respectively and the highest distances with onion (2.214), pepper (1.419), wheat (1.647), onion (0.892), onion (1.699) and pepper (1.597), respectively. In addition, the phylogenetic tree (Figure 4.1, a−g) constructed based on genome sequences by the neighbour-joining method also showed a significant level of distance among the species. Thus, the bioinformatics analysis demonstrated enough genetic distances among the species studied, thereby eliminating the possibility of any cross-target detection (Hossain et al., 2016). Finally, theoretical findings were verified by running PCR experiments with the extracted DNA from three individual species of both targets and nontargets and successful amplification was observed only from target species with no cross amplification from the nontargets.

5.3 PCR Assay Optimization

Optimization of the PCR reaction is the key step in getting successful PCR products. In this study, I initially optimized simplex PCR assay followed by stepwise optimization of duplex, triplex, tetraplex, pentaplex, hexaplex and finally heptaplex. Individual components involved in the reaction were sequentially optimized. Reaction volume was the first thing to consider; the higher the reaction volume, the higher the assay cost, but very low volume might not be sufficient for perfect amplification of concerned primers, especially for multiplex PCR system. Therefore, I optimized the reaction volume at 25 µL, which incurred low cost but was fairly sufficient for a multiplex reaction. Buffer concentration also has important role in PCR reaction. The cations of buffer help in neutralizing the negative charges of the phosphate groups of DNAs, thereby decreasing the electro repulsive forces between the DNA stands. Consequently, primers come into contact with DNA strands and thus, the annealing between them becomes facilitated. I

carried out PCR reactions in a final 25 µL volume containing 5 µL of 5× GoTaq Flexi Buffer adhering to manufacturers' instructions. The role of magnesium chloride is critical in PCR amplification. Mg^{2+} acts as a cofactor of the enzyme polymerase. By forming soluble complexes with dNTPs, it helps in preparing an identifiable substrate for Polymerase. Indeed, Mg^{2+} has a significant role on DNA polymerase activity as well as on the efficiency and specificity of PCR. It may also affect the annealing of primers and the formation of primer dimers. However, an excess of Mg^{2+} may be responsible for nonspecific amplification because of nonspecific annealing of primers, whereas insufficient Mg^{2+} may decrease the yield of amplified products. Thus, polymerase enzyme activity becomes optimum when free magnesium is more easily available compared to that bound with dNTP and template DNA (Markoulatos et al., 2002). In this study, I carried out several PCR experiments with varying concentrations of $MgCl_2$ to make the final optimized concentration of 2.5 mM for simplex assay, 3.0 mM for duplex and triplex reactions, 3.5 mM for tetraplex and pentaplex and 4.0 mM for hexaplex and heptaplex assays. Again, dNTPs (dATP, dCTP, dGTP and dTTP) were used in two different concentrations (0.20 mM for simplex to tetraplex and 0.25 mM for pentaplex to heptaplex) for the optimization of simplex to heptaplex reactions. The concentration of dNTPs also determines the fidelity and efficiency of a PCR amplification; the excess amount of dNTPs may inhibit amplification because of increase error rate of polymerase (Kramer & Coen, 2001). Free Mg^{2+} concentration depends on the quantity of dNTPs as Mg^{2+} binds with dNTPs and thus, DNA polymerase activity reduces due to the imbalance in the amount of four dNTPs (Kunz & Kohalmi, 1991). Annealing temperature (T_a) is another important parameter to determine experimentally for successful amplification. It has been experimentally revealed that the highest annealing temperature is favorable for better amplification since it increases specificity by reducing chances of non-specific primer binding (Wu et al., 2009). For multiplex PCR assays, melting temperature (T_m)

should be identical for all primers since all primers are to amplify in a single assay tube under same reaction conditions. Although there were different T_m values (58.6 - 61.7°C) of our developed seven sets of primers, all the primer sets were able to amplify successfully at the fixed temperature (60°C) that was quite favorable for developing mPCR assay (Figure 4.2 a–c). The GC content of the primers were within the range of 35.7–50% (Table 3.1). The cycling condition applied in the PCR experiments was as follows: the initial denaturation was at 95°C for 3 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 40 s, elongation at 72°C for 40 s and the final elongation at 72◦C for 5 min.

After optimization of simplex PCRs, multiplex PCR assays were sequentially optimised through duplex (cow and sheep), triplex (cow, sheep and chicken), tetraplex (cow, sheep, chicken and pig), pentaplex (cow, sheep, chicken, pig and buffalo), hexaplex (cow, sheep, chicken, pig, buffalo and duck) and, finally, heptaplex (cow, sheep, chicken, pig, buffalo, duck and goat) (Figure 4.10). The novel heptaplex PCR system so developed, amplified all targeted products (73, 106, 138, 161, 203, 236, and 263 bp from pork, beef, buffalo, chicken, duck, goat, and sheep, respectively) when DNA from seven targets was added. I did not use agarose gel electrophoresis for the separation and visualization of amplified products due to their narrow length difference. Agarose gel electrophoresis hardly separates nucleic acids differing by less than 50 bp. Moreover, this is a labour-intensive technique and requires longer time to separate. Consequently, a fully automated advanced multi-capillary electrophoresis system (QIAxcel, Germany) was used for this purpose. Given its built-in gel matrices in a simple gel cartridge, it is quite user-friendly requiring very low amount of sample. Being fully automated, it involves minimum handling interaction thereby resulting minimum human handling errors. Most importantly, it excludes the laborious and time-consuming gel preparation step. This device offered improved sensitivity and better resolution (even at ~5 bp length

difference), shortened analysis time, and minimized exposure chance to hazardous chemicals through the use of simple gel cartridge (Fajardo et al., 2010). The clearly separated multiplex PCR products corresponding to the seven targets were visualized both as gel images and electropherograms (Figure 4.10).

5.4 PCR Assay Specificity

Specific primers are prerequisite for PCR assays and the design of suitable primers always plays a vital role for successful amplification in species authentication. Therefore, oligonucleotide mismatch calculation should be cautiously considered while designing species-specific primers. Design of primers matching perfectly with the specific target species and mismatching considerably with non-targets would surely increase primer specificity minimizing chances of non-target amplifications. Before performing practical/experimental specificity, this study critically evaluated the theoretical specificity of the designed primers. Both intra-species (within species) and inter-species base mismatch was determined in the primer annealing regions (Tables 4.2 - 4.8). The primers were aligned against three individuals of the same species and 7–11 species of the same genus (depending on the availability of corresponding gene sequences from the NCBI database) as well as 30 different nontarget animal and plant species. Complete sequence matching (100 %) was observed only with the individuals of the target species. Moreover, when analyzed in silico specificity considering different species of the same genus, almost full sequence matching (maximum 1 base mismatch in few cases) was found. On the other hand, multiple mismatches of 3–18 nucleotides (13.63 - 76.19 %) were found with other non-target species (Tables 4.2 - 4.8). Thus, the *in silico* analysis results eliminated the chances of any non-target amplification during practical PCR experiments since even a single base pair mismatch at the primer annealing site might

interfere with PCR efficiency leading to false or unsuccessful amplification (Wu et al., 2009).

After optimization of the simplex PCR, the experimental specificity of the simplex PCR was ensured through a cross-amplification reaction using DNA templates from one target and 25 nontargets of terrestrial and aquatic animal and plant species (Figures 4.3 – 4.9). Specific PCR products [73 bp (ND5), 106 bp (ND5), 138 bp (ND5), 161 bp (cytb), 203 bp (ND5), 236 bp (ND5) and 263 bp (ND5)] were obtained only from the individual target species of pig, cow, buffalo, chicken, duck, goat and sheep, respectively. No product was found from any other non-target species (e.g., cat, dog, squirrel, rat, rabbit, crocodile, quail, pigeon, ostrich, tuna, sardine, tilapia, frog, turtle, onion, pepper, ginger, garlic and wheat). On the other hand, the universal eukaryotic primers amplified 99 bp-length products from all studied species. This reflects the presence of high-quality DNA in all reaction tubes, eliminating any possibility of false-negative detection. Thus, high specificity and fidelity of the developed simplex PCR assays were ensured.

After optimization of simplex PCR, the multiplex PCR system was sequentially developed through duplex, triplex, tetraplex, pentaplex, hexaplex and finally heptaplex, and cross specificity of the developed heptaplex assay was checked. The developed heptaplex system gave PCR products (73, 106, 138, 161, 203, 236 and 263 bp) only from the seven targets (pig, cow, buffalo, chicken, duck, goat and sheep) and none of the non-target species gave any PCR product (Figure 4.11) thereby indicating stringent specificity of the developed heptaplex PCR system in discriminatory detection of aforementioned seven targets. All the assays were carried out in three replicates on three different days, and the results were reproducible.

5.5 PCR Product Sequencing

Appropriately designed and optimized PCR assays usually offer successful and conclusive detection of specific species. Nevertheless, sequence analysis by validating the PCR products significantly improves the assay reliability and acceptability. Moreover, PCR products typically indicate whether the related species are present or absent. PCR products sequencing, on the other hand, ensures the absolute determination of the exact species (Bevan et al., 1992). The sequencing helps to determine the exact order of the nucleotides of the products. In this study, the PCR products of cow, buffalo, chicken, duck, goat, sheep and pork were sequenced bi-directionally. The sequencing result of PCR products of cow (ND5), chicken (cytb) and pig (ND5) showed 100% sequence homology with *Bos taurus*, *Gallus gallus* and *Sus scrofa* sequences available in GenBank, respectively. On the other hand, PCR products of buffalo, duck, goat and sheep gave the similarity score showing 98.55%, 99.38%, 99.01% and 99.5% homology with *Bubalus bubalis*, *Anas platyrhynchos*, *Capra hircus* and *Ovis aries* sequences, respectively (Table 4.16). All the similarity score values fell within the acceptable limit since at least 98% sequence similarity is required for potential species identification (Cawthorn et al., 2013). Thus, my result proved the reliability and authenticity of the amplified PCR products. Previously, Ahamad et al. (2017) demonstrated 99.18%, 98.35% and 98.14% sequence similarity with rabbit, squirrel and rat specific PCR products respectively. Natonek-Wisniewska et al. (2013) reported sequence similarity of 97.78% for bovine-species. A similarity score of 100% was obtained by Hossain et. al. (2019b) for chicken, cat, and dog products and by Dalmasso et al. (2004) for sheep, chicken, and pork species.

5.6 Heptaplex (multiplex) PCR Assay

5.6.1 Limit of Detection of Heptaplex (multiplex) PCR Assay

The sensitivity of the heptaplex PCR assay was determined by using serially diluted DNA of all target species starting from higher to lower concentrations (10.0, 1.0, 0.1, 0.01 and 0.005 ng/μL). The seven distinct bands corresponding to the seven targets for as low as 0.01 ng DNA template were distinctively visible in both the gel images (Figure 4.12 a) and electropherograms (Figure 4.12 b), reflecting amplification of all the targets (pig, cow, buffalo, chicken, duck, goat and sheep) at 0.01 ng DNA concentration. Again, when I used DNA template at a lower concentration of 0.005 ng/μL, it showed bands for products from pig (73 bp), buffalo (138 bp), duck (203 bp), goat (236 bp) and sheep (263 bp) but no band was observed for cow (106 bp) and chicken (161 bp) (Figure 4.12 a). This indicates that the detection limit for pig, buffalo, duck, goat and sheep was lower (0.005 ng) than that for cow and chicken which was 0.01 ng. But for DNA below 0.005 ng, the gel images and electropherograms became very faint making them almost undetectable. Thus, the obtained superior sensitivity might be attributed to the short-length amplicons (≤263 bp) used in this study (Ali et al., 2015a). We know that most of the intentional adulteration is economically motivated and the LOD at this lower level carries significant importance in detecting any commercial fraud committed for profit making purposes. Thus, the detection of 0.005 ng/0.01ng DNA may be considered sufficient to prove the sensitivity and reliability of any multiplex PCR assay. A detection limit of 0.005 ng was also achieved in simplex and multiplex assays for duck, cow and lamb using amplicons ranging from 99 to 204 bp (Qin et al. 2016). Again, detection limits of 0.001 ng and 0.01 ng were demonstrated by Ahmad Nizar et al. (2018) in a double gene targeting PCR assay using 77–127 bp PCR products to detect crocodile species. In different mPCR assays, Hossain et al. (2017b) detected 0.02 ng DNA for cow, buffalo and pig (90 to 146 bp amplicons), whereas Hou et al. (2015) detected 0.05 ng for chicken,

duck and goose (131–387 bp products). Thus, the variation in detection sensitivity is a common phenomenon in multiplex assays and sensitivity often varies with different PCR assays depending on target genes, target species, amplicon sizes and quality of the source materials (Hou et al., 2015). Therefore, my developed method showed satisfactory efficiency with respect to sensitivity compared to those of other published reports.

5.6.2 Stability and Sensitivity Test of Heptaplex (multiplex) PCR Assay

Since heat or other processing treatments usually result in DNA degradation, assay validation in terms of stability is essential for heat-treated samples prior to applying the technique to analyze commercially processed food products (Qin et al., 2016). The detection efficiency of our developed technique has been evaluated under thermally treated meat samples (Hossain et al., 2017b). Figure 4.13 shows that the developed assay could successfully amplify all the seven targets under all heat treatments, including microwaving at 700 W for 30 min and autoclaving at 121°C and 15 psi for 20 min. Microwaving at 700 W causes extreme thermal effect making the samples burnt and dried out. Autoclaving is also a severe thermal treatment with high temperature and pressure and was applied to mimic canning and steaming process. It is clear that the multiplex method that I developed is highly stable even for degraded samples undergoing harsh cooking methods. Earlier reports showed that PCR assay stability largely depends on amplicon sizes; small-length targets offer higher stability over the longer ones (Ali et al., 2015a). Cautiously addressing this point, this study kept amplicon lengths between 73 and 263 bp targeting mitochondrial cytb and ND5 genes. Thus, the short length of our targets probably contributed to successful outcome.

Processed meat products like frankfurters, burgers, meatballs etc. are susceptible targets for fraudulent adulteration often perpetrated by introducing cheaper meats as partial or total replacements for high-priced ones. Therefore, the developed heptaplex

PCR assay was validated by analysing very popular meat products, meatballs, frankfurters and burgers under raw and heat-treated conditions (processed states) to evaluate the reliability, sensitivity and applicability of the method. The developed assay positively detected each of the seven targets of beef, buffalo, goat, sheep, chicken, duck and pig from down to 0.5 % (w/w) adulterated and severely autoclaved model beef, chicken and pork meatballs and frankfurters (Figures 4.14 and 4.15). Fraudulent adulteration is mostly economically motivated and the mixing of less than 0.5% of low-priced meats does not make any significant profit. Thus, the detection of 0.5% adulteration was considered sufficient to prove high sensitivity and applicability of any multiplex PCR assay. Recently, a limit of detection of 0.5 % was also determined by Hossain et al. (2019b) for dog, cat, pig, buffalo, cow, chicken, and fish in meatball products involving the amplicon sizes ranging from 73-198 bp. Some other studies documented a higher sensitivity level of 1% for cat, dog, pig, monkey and rat using 108–172 bp amplicon targets in model meatball formulation (Ali et al., 2015b), for Chicken, pig and duck (76–212 bp) (Cheng et al., 2014) and for pork (276 bp) (Mane et al., 2013) in admixed states. Again, a lower detection level of 0.1 % was documented by Hossain et al. (2016) for beef, buffalo, and pork (73–146 bp) in frankfurters and by Razzak et al. (2015) for dog, pig, rat, cat, and monkey (108–172 bp) in model burger, meatball and frankfurters. Thus, detection sensitivity frequently varies in different PCR assays depending on target species and genes as well as amplicon sizes and our developed method showed satisfactory sensitivity comparable with others' findings.

5.6.3 Commercial Product Analysis Using Heptaplex PCR Assay

The increasing demand for commercial meat products often leads to deliberate adulteration along the food chain. Compared to raw meat, adulteration in processed meat products could be more competently manipulated and thus, these products are susceptible

targets for such fraud (Soares et al., 2013). Since meatballs, burgers and frankfurters are popular food items and widely consumed worldwide, I applied the method for the analysis of 26 meatballs (beef, chicken and pork), 50 burgers and frankfurters (beef, chicken and pork) and 20 curry samples (beef and chicken) of different brands available in the local food market. The results are summarized in Table 4.18. Mislabelling could be determined in two ways, if meat species stated on the product label fails to be detected and/or species not declared on the label is found to be detected. The highest incidence of adulteration was observed in tested beef samples (meatballs, frankfurters, burgers and curries). Screening of commercial meatballs revealed that 100% of the analyzed beef meatballs were adulterated with buffalo; both beef and buffalo were detected in 80% of samples and remaining 20% was totally replaced by buffalo, indicating species substitution as well as mislabelling. Among the tested commercial burger and frankfurter samples, the incidence of mislabelling was observed in only beef products of which 84% were buffalo adulterated (Table 4.18). Moreover, undeclared buffalo DNA was detected in 90% of the tested beef curry samples. The above findings imply that adulteration of beef items in Malaysia is rampant. It would be noteworthy that no buffalo meatballs, burgers and frankfurters were available in Malaysian markets; maybe, all have been falsely labeled as beef products. Although buffalo meat is also acceptable from the religious point of view, beef is preferred as tastier over buffalo by most South Asian people. The tendency of buffalo meat inclusion in processed beef products is most likely due to economic reasons; in the global market as well as in Malaysia, buffalo meat is cheaper than beef. On the other hand, among all the avian (chicken) products tested, only chicken meatballs (30%) were found to be positive for undeclared species (beef and/or buffalo) which could be attributed to accidental cross-contamination from common food processing equipment. However, none of the porcine products tested were found contaminated, revealing the integrity of these items. Since majority of the Malaysian population is Muslim, halal

authenticity is vital for food, especially for meat products. Although there is a previous incidence of porcine DNA detection in halal food items in Malaysia (Farouk et al., 2006), however, because of the strict surveillance and monitoring, halal certified meat products of all categories were found pure without containing any non-halal materials, such as porcine residuals. Moreover, Malaysia is one of the largest halal food exporting countries and has been committed to building up a reliable halal hub through ensuring continuous surveillance. Mislabelling incidences of meat products were also documented by some other researchers. However, most of the previous studies analyzed mostly model meat products like patty, kabab, meat block etc. applying simplex assays that incurred increased cost and time requiring separate assays for individual species and analyzed mostly model meat products (Mane et al., 2012a; Mane et al., 2012b). Hossain et al. (2017b) reported 100% mislabelling in beef meatballs. Other reports revealed ≥80% (Chuah et al., 2016) and 68% (Cawthorn et al., 2013) species substitution in processed meat products. Despite continuous surveillance of regulatory authorities, the exact levels of food fraud occurrence or mislabelling crimes in Malaysia as well as across the globe remain unclear.

5.7 Authentication of PCR Products by RFLP Analysis

Species-specific PCR assay, although often provides conclusive results, may be proved unsuitable to be considered a definitive analytical tool due to certain features (Yang et al., 2005). For instance, it sometimes may produce artifacts because of contamination by alien DNA at a very small level and may cause non-specific target amplification (Doosti et al., 2014). However, to eliminate such ambiguities, amplified product verification is effective that certainly increases assay reliability. Verification of PCR products could be performed through at least one of the techniques, namely, probe hybridization, DNA sequencing, and PCR-RFLP (Maede, 2006). Probe hybridization

provides multiple species detection simultaneously in a single run (Abd Mutalib et al., 2015). However, the technique is labor-intensive and needs high-quality DNA and thus is less feasible to work with heat-treated DNA from processed meats or meat products (Mutalib et al., 2012). on the other hand, DNA sequencing is highly promising and reliable, but it demands expensive laboratory settings and skilled manpower which make it difficult for the routine meat authentication (Girish et al., 2004). In contrast, the PCR-RFLP assay is free from the above shortcomings and has attracted researchers' attention worldwide because of its low cost, simplicity and reliability (Hashim & Al-Shuhaib, 2019). It authenticates the PCR product amplified from a selected gene fragment by generating species-specific fragments through digestion with restriction enzymes (Pereira et al., 2008) followed by separation and visualization of the DNA fragments by gel electrophoresis. Thus, it enables the distinction of the artificial PCR product from the original with the help of the restriction fingerprints (Ali et al., 2018).

In this study, the PCR products of the targets (pig, cow, buffalo, chicken, duck, goat, and sheep) were digested with appropriate restriction enzymes and clearly distinctive fingerprints were obtained (Figure 4.16 and Table 3.8). Three restriction enzymes (*Fat*I, *Bfa*I and *HPY1*88I) were used for the digestion of seven PCR products. *Fat*I enzyme digested three products [Pig (73 bp), cow (106 bp) and Sheep (263 bp)] producing six restriction fragments (two for each target): 52 and 21 bp (pig), 87 and 19 bp (cow) and 153 and 110 bp (sheep). On the other hand, *Bfa*I RE digested chicken (161 bp), Duck (203 bp) and goat (236 bp) resulting in six fragments (93 and 68 bp, 141 and 62 bp and 130 and 106 bp, respectively). Again, buffalo (138 bp) upon digestion with *HPY1*88I endonuclease generated 70 and 68 bp fragments. However, due to very short length difference (only 2 bp) between the fragments from buffalo, the two generated bands were not distinctively visible; 70 bp band being merged with 68 bp, appeared as a single thicker band since the electrophoretic technique has the limitation to resolute ≤5 bp difference in

fragment length. Restriction digestion generated fragments of the same size as those obtained in theoretical RFLP analysis using NEBcutter software. Thus, the experimental findings were in accordance with those of theoretical RFLP analysis, reflecting that the developed systems unambiguously amplified the exact target species.

The developed mPCR-RFLP assay was subsequently evaluated to screen commercial frankfurters under raw and heat-treated states. Deliberately adulterated model beef, chicken and pork frankfurters were subjected to boiling and autoclaving and their restriction digestion patterns were analysed (Figure 4.17). The digests of all samples including raw and heat-treated clearly presented their signature fingerprints of total 14 fragments (Figure 4.17). Notably, the restriction digestion profiles of various heat-treated (boiled and autoclaved) samples were found similar to those obtained from the raw samples justifying that any variation in food processing treatments does not affect the stability and efficiency of the developed biomarkers. In other words, our developed assay experimentally proved its stability, efficiency, and sensitivity in detecting beef, buffalo, goat, sheep, chicken, duck, and pork in processed and heat-treated foods containing degraded DNA.

Recently, Rahat et al. (2020) documented a PCR-RFLP assay to identify cattle and buffalo by using mitochondrial cytb gene of 359 bp target. Previously, Girish et al. (2005) proposed a PCR-RFLP assay of 12 s rRNA gene for the simultaneous detection of buffalo, goat, sheep, and cattle using 456 bp amplicon length. Another RFLP pattern was documented by Kumar et al. (2014) with a 609 bp target to differentiate cattle, buffalo, sheep, goat, and pig. In addition, Haider et al. (2012) documented a PCR-RFLP assay with 710 bp amplicon and used common primers to detect chicken turkey, cow, pig, buffalo, sheep, camel and donkey. However, these studies are based on longer amplicons (359–710 bp). Several studies suggest that the stability of the PCR system largely relies

on the amplicon sizes; longer targets are more susceptible to degradation under food processing and thus, the assay certainly loses its efficiency and applicability in processed meat analysis (Rashid et al., 2015). Conversely, herein, I reported a short amplicon length (between 73 and 263 bp) targeted PCR-RFLP technique and systematically proved its sensitivity and reliability under raw, boiled and autoclaved states for differential detection of beef, buffalo, goat, sheep, chicken, duck and pork in pure, admixed and commercial food (frankfurter) formulation.

5.8 Multiplex TaqMan Probe-based Real-time PCR System

TaqMan-based assays require the effective binding of specific probes in addition to primers binding. In developing mqPCR system, design of primers and probes is especially critical since multiple primers and probes interact simultaneously with multiple templates across a range of temperature and it is essential to ensure that the melting temperatures (T_m) of all concerned primers and probes must be similar or very close to each other to facilitate their annealing to their specific sites in DNA template under the same set of PCR settings (Cheng et al., 2014). All the primers and probes used in this study were critically evaluated for T_m, GC and sequence mismatching. In this assay, primers (T_m values 57.8–61.7°C) annealed at 60°C. The probes' Tm values (67.4–70.7°C) were 8.0 - 12.0°C higher than those of primers and this facilitated favourable probe binding before the primers were annealed to the template which necessarily happens in TaqMan chemistry (Arya et al., 2005). The probes' Tm values were kept minimum 8ºC higher than that of the primers. The Tm values differences among all primers were maintained very narrow (less than 2ºC). Similarly, the Tm values differences among all probes were kept less 2ºC. Moreover, the GC content of all primers and probes were within the acceptable range. Thus, multiple amplicons were clearly detected and discriminated in both mqPCR assays (beef, buffalo, goat, sheep and IAC in mqPCR-1 and, chicken, duck,

pig and IAC in mqPCR-2) through the use of five different fluorescent reporter dyes namely TAMRA, HEX, CY5, FAM and TEXAS RED (Section 3.8.1 and Table 3.10). The mean Ct values for the mqPCR-1 assay were 15.775, 16.612, 18.490, 16.195 and 14.211 for bovine, buffalo, goat, sheep and IAC respectively. Again, for mqPCR-2, the mean C_t values were obtained 21.034, 19.999, 14.982 and 17.321for chicken, duck, pig and IAC, respectively. (Figure 4.18). The use of endogenous system as an IAC in this assay ruled out the chances of false negative detection as well as helped to identify the effects of reagent components and probable inhibitors if any, in the reaction mixture (Rojas et al., 2011). Additionally, the use of endogenous control is necessary particularly, for processed food analysis since total DNA extracted from these samples might be in degraded states or of inferior quality. Moreover, the endogenous system helps in checking any change in amplification caused by any variation of PCR components or by PCR inhibitors. Thus, the error between the unknown sample and standard could be effectively minimized by comparing the endogenous system with the specific signals generated from the target sample.

5.8.1 Specificity of Multiplex qPCR Systems

Previous reports confirm that primers significantly matching the target species and containing huge mismatches with non-targets, contribute to greater chances of specific PCR assay and eliminate non-target amplification possibilities (Murugaiah et al., 2009). Considering this point, the designed primers and probes used in this study was critically evaluated regarding sequence matching among targets and other related non-target species. Alignment of primers and probes with targets and several non-target species using MEGA 7 software revealed absolute sequence matching (100 %) only with the target species. On the other hand, sufficient mismatches (3–18 nucleotides in primers and 3-20 nt in probes) were found with other non-target species (Tables 4.19–4.25). Thus, the

in silico analysis results eliminated the chances of any non-target amplification during practical PCR experiments since even a single base pair mismatch at the primer annealing site might interfere with PCR efficiency leading to false or unsuccessful amplification (Wu et al., 2009). Finally, both the mqPCR systems (mqPCR-1 and mqPCR-2) were checked for practical specificity taking DNA from seven target species (cow, buffalo, goat, sheep chicken, duck and pig) along with 23 nontarget species (dog, rabbit, turtle, rat, pigeon, quail, monkey, donkey, ostrich, crocodile, frog, tortoise, cat, salmon, tuna, sardine, rohu, cod, tilapia, wheat, onion, garlic and pepper) on three different days in triplicates. The amplification profile (Figure 4.19 a,b) clearly shows that the mqPCR systems successfully amplified only the target species (cow, buffalo, goat and sheep in mqPCR-1 and, chicken, duck and pig in mqPCR-2) with the Ct values 18.16, 19.58, 20.37, 18.31 for bovine, buffalo, goat and sheep respectively (mqPCR-1) and 24.16, 23.13 and 17.68 for chicken duck and pig respectively (mqPCR-2) (Tables 4.26 and 4.27) whereas, other non-target species provided only the background fluorescence during 40 cycle assay eliminating the possibilities of cross-amplifications. On the other hand, this study used the endogenous system (IAC) (Eukaryotic 18s rRNA) to avoid any false negative detection. The endogenous system with the Ct values of 16.41-16.90 (for mqPCR-1) and 19.83-20.71 (for mqPCR-2) (Tables 4.26 and 4.27) successfully amplified eukaryotic target from each of the targets and non-target species giving significant detectable signals and this indicated the presence of good quality of extracted DNA in all tubes (Figure 4.19 a, b). Thus, the developed mqPCR systems proved their superior specificity by amplifying only the target species and showing no cross amplifications.

5.8.2 Limit of Detection of Multiplex qPCR Systems

The LOD of an assay is the minimum detectable quantity of an analyte in a given sample. The LOD of the present mqPCR systems were determined by using serially

diluted (10-fold) mixed genomic DNA (60 to 0.006 ng) of the target species. Both the systems successfully generated detectable fluorescence signals for all targets from all concentrations of DNA mixtures. Thus, the assays were sensitive to detect and quantify down to 0.006 ng of target DNA. RSD for all concentrations of DNA were found to be less than 1.0 (0.07–0.7) (Table 4.28) reflecting that there was minimum variation among the replicates in this assay system. Recently, Ahamad et al. (2019) documented a tetraplex qPCR assay for identifying cat, rabbit, rat, and squirrel in processed food products and detected 0.003 ng DNA from each species. A detection limit of 0.15 ng of DNA was demonstrated by Cheng et al. (2014) in a mqPCR system for the identification of pig, duck and chicken in blood curd samples. On the other hand, an LOD of 0.32 ng was achieved by Koppel et al. (2008) for beef, pork, chicken, and turkey. Thus, LOD might be varying for different species and samples, maybe, depending on certain factors like degree of decomposition, nature of processing treatments, background matrices etc. Thus, the detectable limit of 0.006 ng of these mqPCR systems made it sensitive enough to be conveniently applied for authentication purposes of the target species in processed food products.

5.8.3 Quantification of Target Species by Multiplex qPCR Systems

To quantify the targets, individual standard curves were constructed for all the target species by plotting Ct values against logarithmic value of DNA concentrations. The five-point dilutions (60 to 0.006 ng) were applied for cow, buffalo, goat, sheep (mqPCR-1) and for chicken, duck, pig (mqPCR-2) and the minimum quantification of 0.006 ng DNA that was attained is considered sufficient to detect any commercial fraud perpetrated for profit gaining purpose. It is notable that good linear regressions were achieved for all the standard curves in all measurements as evidenced by the satisfactory regression coefficient (R^2) of 0.9993, 0.9987, 0.9987, 0.9974 and 0.9992 for cow, buffalo, goat,

sheep and IAC (mqPCR-1) and 0.9991, 0.9966, 0.9981 and 0.9888 for chicken, duck pig and IAC (mqPCR-2), respectively. The corresponding slopes were attained –3.447, –3.501, –3.49, –3.525 and –3.391 (for cow, buffalo, goat, sheep and IAC in mqPCR-1) and, –3.411, –3.542, –3.39 and –3.106 (for chicken, duck, pig and IAC in mqPCR-2), respectively. The PCR efficiencies were recorded to be 95.03%, 93.03%, 93.43%, 92.17% and 97.20% (mqPCR-1) and 96.41, 91.57%, 97.24% and 109.84 (mqPCR-2) respectively. The obtained efficiency values are within the limit of recommended values (90−110%) (Ali et al., 2012a). Thus, the developed mqPCR systems along with the generated standard curves could be effectively used for quantitative determination of trace amounts of target species. Ahamad et al. (2019) demonstrated the PCR efficiencies of 106.87%, 109.36%, 106.06% and 106.53% for rat, squirrel, cat and rabbit, respectively. Similarly, Hossain et al. (2017a) achieved the efficiencies of their mqPCR system for cow, pig, buffalo and IAC at 108.73%, 94.68%, 107.82% and 104.03%, respectively.

5.8.4 Sensitivity and Validity of the Multiplex qPCR Systems under Model Meat Products

Sensitivity of the developed mqPCR assays was evaluated by preparing deliberately adulterated model meat products (10, 5 and 1%). All the seven species could be successfully detected down to 1% adulteration by the two mqPCR systems. For minimally detectable quantity (1%), the C_t values were obtained between 15.29 and 24.47 (Table 4.29) in mqPCR-1 (for cow, buffalo, goat and sheep) and 15.67–28.27 (Table 4.30) in mqPCR-2 (for chicken, duck and pig). It is notable that, IAC constantly maintained mean Ct values for both the systems ranging from 13.26 to13.62 in qPCR-1 and 18.43 to 18.68 in qPCR-2 for all percentages of adulteration. This reflects that any variation in the level of adulteration was unable to make significant change in endogenous target since all adulterants were of eukaryotic origin. Thus, the developed systems were promising in terms of sensitivity and specificity to authentically detect all the targets from 1%

contaminated samples. Cheng et al. (2014) documented an mqPCR system to detect chicken, duck and pig and got the sensitivity of 1% in admixed samples. Similarly, Cai et al. (2012) detected 1% beef and porcine gelatins in admixed gelatin samples by a qPCR assay. A sensitivity of 0.5% was shown in another mqPCR for the binary admixture of pork and beef (Iwobi et al., 2015). A similar sensitivity (0.5%) was reported by Dooley et al. (2004) in a qPCR assay for detecting bovine, porcine, chicken, turkey and lamb.

The validity of both mqPCR systems was checked by analysing processed meat products (frankfurters and meatballs). The analysis results of three different days for all the spiking levels (10, 5 and 1%) were recorded for both the systems (Tables 4.31 and 4.32). The findings from mqPCR-1 showed the target recoveries between 88.43% and 112.20% along with systematic errors between −11.57% and +12.20% and RSDs 0.47−13.54%. The maximum recovery (112.20%) was observed from 5% sheep adulterated goat meatball and a minimum (88.43%) from beef meatball spiked with 10% sheep. RSD value was maximum for 5% contaminated goat meatball and minimum for 5% sheep adulterated goat meatball. On the other hand, the analysis results of mqPCR-2 revealed the target recoveries from 87.33 to 113.72% along with systematic errors from –12.67 to +13.72 and RSDs 1.29–14.0. The maximum and minimum recoveries were obtained from 5% adulterated chicken frankfurter and 1% pig contaminated chicken frankfurter respectively. RSDs were calculated maximum for 5% pig adulterated chicken meatball and minimum for 1% duck adulterated chicken frankfurter. Druml et al. (2015) obtained 40.9% systematic error and 12.9% RSD for 2% adulteration, and 23.10% systematic error and 1.69% RSD were calculated by Asing et al. (2016) for 0.1% adulteration. Thus, the systematic errors and RSD of my assays were in consistent with those of other published report. The significance test to compare the experimental and actual quantities of the target species for different model meat products shows that the mean experimental values obtained from the mqPCR assays were very close to the mean

actual values that were originally used during their preparation in the laboratory (Table 4.33). This implies that the developed mqPCR systems can precisely and reliably determine down to 1% adulteration of target species in meat products.

5.8.5 Analysis of Commercial Meat Products by Multiplex qPCR

The ready-to-eat modern food products such as burgers, meatballs, frankfurters, sausages etc., are usually prepared from minced meats and the morphological features and other physical attributes are completely destroyed due to processing treatments. Compared to raw meat, adulteration in these processed products could be manipulated more competently and their increasing demand made them most susceptible to such fraud (Soares et al., 2013). Therefore, the developed assays were finally tested for screening of commercially available popular food products of various meat species which are consumed worldwide. Cattle, buffalo, goat, sheep, poultry and porcine adulterations in food chain have an enormous importance from health, culture, religion, and business viewpoint. Therefore, a total of 49 meat products including 17 burgers (beef, chicken and lamb), 17 frankfurters (beef, chicken and pork), 4 cocktails (pork), 4 sausages (pork) and 7 cooked whole muscle meats (lamb) were purchased from different Malaysian outlets, and these were analyzed using the developed mqPCR assays. The screening results (Table 4.34) revealed considerable species substitution rate with the highest incidence in beef products. The analysis results showed that 85%, 100%, 100%, and 100% of beef burgers, frankfurters, meatballs and cocktails were adulterated with buffalo of which 9% contained no beef at all indicating species substitution in addition to mislabelling. This suggests that buffalo substitution in beef products is rampant in Malaysia. The inclusion of buffalo meat in processed beef products is most likely to achieve economic gain because in international market, buffalo meat is cheaper than beef and particularly in Malaysia, buffalo meat is priced approximately half compared to beef (MoA, 2015). Buffalo

substitution was also observed in chicken frankfurter (3/9) and lamb cooked whole meat (1/7). Pork products (frankfurter, sausage and cocktail) contained chicken adulteration in 50% of tested samples. The inclusion of chicken in pork products and buffalo in chicken products might be occurring through unintentional cross contamination during processing since most manufacturers share the same equipment (grinders) for different species. Whether deliberate or incidental, meat product misdescription carry similar impacts on consumers. Thus, presence of traces of meat (e. g., of species prohibited in certain religion) maybe, due to cross contamination, might also have serious religious impacts. It is notable that, in our study, all tested halal meat products were negative for the non halal porcine material, signifying the halal sanctity of commercially processed meat products sold in Malaysia. This may be due to the strict monitoring of halal status by the Malaysian government. Since, more than half of Malaysian population is Muslim, halal authenticity is vital for meat products and Malaysian authority is striving hard to represent Malaysia as an important global halal hub.

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CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

It is a long-cherished consumers' desire that foods should comply with individual health, religious belief, affordability, and choice. Consumers, nowadays, demand more detailed disclosure on the origin, composition, and safety of the food they purchase. However, unfortunately, food fraud is a global problem raising increased concerns over the past decades and food authenticity is a burning issue of the present day. These malicious practices must be stopped, and it is important to have reliable analytical tools able to verify trace ingredients whether they match the attributes and features declared by the manufacturer or distributor. An authentic and cost-effective analytical technique capable of detecting multiple species on a single platform has the potential to remarkably cut analysis cost and time and thus can significantly contribute to overseeing the situations of species substitution in foods. Cattle, buffalo, pig, goat, sheep and poultry are the most common meat source obtained from animal species and have enormous importance from health, culture, religion, and business perspectives. Given the growing demand, these species often become the target of fraudulent substitution/adulterations in food products that raised increased questions related to health safety (e. g., allergy concerns), religious sensitivity (restriction on beef and pork consumptions in some religions) and lifestyle issues (vegetarians and vegans avoid all kinds of animal materials) apart from economic loss. Although several PCR assays have been documented to detect different animal species individually or parallelly, until now, no study is available that included these seven commonly consumed species to detect in a single platform. However, most of the previously reported assays used longer amplicons that are more prone to break down by food processing treatments making the assays less trustworthy and often inconclusive.

Addressing the above knowledge gap, this study has developed and validated for the first time, a reliable, cost-effective heptaplex PCR system involving the short-length targets of cow, buffalo, chicken, duck, goat, sheep, and pig. The short-length amplicon ensured extra stability even under high processing treatments that degrade DNA. The detection of multiple targets in a single platform is highly promising since it remarkably saves analysis cost and time, making the authentication procedure easy. The assays were evaluated for specificity, sensitivity, stability and applicability under pure, admixture and commercial meat products, including meatballs, frankfurters, burgers, and curry samples.

Good quality DNA was extracted from all raw as well as processed meat and commercial meat products, and a number of commercially available kits had to be used depending on the type of samples to get optimum yield. Since commercial products, unlike raw meat, contain some added ingredients like spices, food color, additives, vegetables etc., they required extraction kit different from that for raw meat to extract DNA of high quality. Seven pairs of species-specific primers were carefully designed targeting mitochondrial genes to use in singleplex and multiplex PCR assays. All the primers were critically evaluated for Tm, GC and they were within the recommended range. Intraspecies and interspecies evaluation of base mismatch and other *in silico* studies, unveiled remarkable genetic distances between the species studied and thus ruled out the chances of any cross-target detection. The primer specificities were practically confirmed through simplex and later heptaplex PCR assays taking DNA from seven targets and 19 non-target animal and plant species. A universal eukaryotic primer set was included that amplified 99 bp product from all studied species eliminating any possibility of false-negative detection.

Simplex PCR assays were optimized initially for each of the target species using a specific primer set so that each target amplification could be ensured. Following

confirmation of the simplex PCR, multiplex PCR assays were developed stepwise, starting from duplex to heptaplex for simultaneous detection of all target species. All the PCR assays (including the simplex and multiplexes) could successfully amplify the target gene (cytb and ND5) sites producing 73, 106, 138, 161, 203, 236 and 263 bp products from pig, cow, buffalo, chicken, duck, goat and sheep, respectively, reflecting full uniformity with the simplex assay. The stability and sensitivity of the assay were tested under raw meat and mixed matrices. The developed multiplex assay was validated with various adulterated and heat-treated (boiled, microwaved and autoclaved) meatball and frankfurter products and were found to show high stability and sensitivity under all processing conditions. There was successful amplification of all the markers from the samples under all heat treatments reflecting high assay stability even under harsh cooking conditions. It is noteworthy that the short length (73-263 bp) of our targets offered additional stability since short targets are more stable than the longer ones even under the state of DNA degradation in processed samples. The assay was sensitive enough to detect 0.01–0.005 ng of DNA from raw meat and 0.5% (w/w) adulterated meat in mixed matrices. The method was further validated for screening of commercial meatballs, frankfurters and burgers and a good applicability and operational feasibility were observed in detecting traces of cattle, buffalo, goat, sheep, chicken, duck and porcine materials in the food chain.

A market survey of commercial meat products revealed that 100% of the analyzed beef meatballs were buffalo adulterated; among them 80% samples contained both beef and buffalo and remaining 20% was totally replaced by buffalo. Commercial beef burgers and frankfurters were found to be adulterated with buffalo in 84% of tested samples. Moreover, undeclared buffalo DNA was detected in 90% of the tested beef curry samples. The above findings imply rampant substitution of beef with buffalo in Malaysian meat products maybe, due to the fact that buffalo meat is cheaper than beef in Malaysia. On

the other hand, pork meatballs, frankfurters and burgers were not found contaminated. None of the above halal beef and chicken products contained any non-halal porcine materials indicating their halal sanctity.

In order to increase assay reliability and acceptability, authentication of PCR products is very effective to ensure that authentic targets have been properly amplified. In this study, two types of authentication protocols have been applied: sequencing and RFLP analysis. Upon sequencing bi-directionally, the amplified PCR products from the targets showed more than 98% (98.5-100%) sequence similarity with specific target sequences available in GenBank and this was within the acceptable range for successful species identification purposes. Secondly, PCR products were digested by three restriction enzymes known as *Fat*I, *Bfa*I and *HPY1*88I that confirmed the authentic molecular fingerprints from the seven target species. The developed PCR-RFLP assay was subsequently evaluated to screen deliberately adulterated commercial beef, chicken and pig frankfurters under raw, boiled and autoclaved states and distinctive signature fingerprints were obtained for all the samples, including the heat-treated ones reflecting that the assay was stable under various heat treatments. Thus, this PCR-RFLP assay is sensitive, authentic and efficient for discriminatory detection of beef, buffalo, goat, sheep, chicken, duck, and pork in processed and heat-treated foods.

The developed conventional multiplex (heptaplex) PCR and PCR-RFLP assays in this study could successfully detect bovine, buffalo, goat, sheep, chicken, duck and porcine materials in food chain, but these are just qualitative approaches; they cannot tell how much the target species is present in the real-world specimens. Therefore, short amplicon length TaqMan probe- based mqPCR assays were developed for the quantitative detection of the said species. The systems were highly promising and reliable for the quantitative determination of the target species in any processed and unprocessed foodstuffs since the

use of species-specific primers, in addition to TaqMan probes, offered double-checking opportunities in specific targets detection. Short-length amplicons offered extra stability even under state of DNA degradation. Species-specificity was ensured through cross checking against 23 non-target species. The chances of false negative detection were successfully eliminated by the use of an internal positive control (141 bp site of eukaryotic 18S rRNA). The assays were valid under pure, processed and admixed states at 10 - 1% (w/w) adulteration with target recoveries of 87.33−113.72%. The systems were sensitive to detect and quantify down to 0.006 ng of target DNA in raw samples as well as 1% meat in adulterated samples. The results of the applicability test on commercial samples prove the suitability of the assay for the analysis of real-world samples.

The above findings have demonstrated that our developed novel assays possess the potential to be used as a practical tool for routine analysis of meat products even in the state of degraded specimens. In addition to multiplexing, the assays could be applied in simplex formats for individual identification of all the seven targets depending on needs and laboratory facilities. Most importantly, law enforcement agencies may include the system into their supervision framework in order to prevent or at least, minimize meat fraud. A summary of the present research in line with the objectives is stated below:

1. Design and development of biomarkers targeting mitochondrial genes

During designing the primers, this study perfectly evaluated their GC content (37.5 – 50.0%) and Tm values (58.6^{o} – 61.7^{o}C) that were optimum for successful PCR assay. This study also kept the amplicon sizes of the designed sets of primers for each of cow, buffalo, goat, sheep, chicken, duck and sheep between 73 and 263 bp [ND5 of cow (106 bp), ND5 of buffalo (138 bp), ND5 of goat (236 bp), ND5 of sheep (263 bp), cytb of chicken (161 bp), ND5 of duck (203 bp) and ND5 of pig (73 bp)]. Short biomarker targets of ≤263 bp length offered extra stability making the system especially advantageous for

target amplification even under diverse food processing treatments. Sequence alignment of the designed primers was performed in silico within species (intra- species) and against the similar sites of 30 non-target species (interspecies) and complete sequence matching (100%) was observed only with the individuals of the target species and multiple mismatches of 3–18 nucleotides (13.63-76.19%) were found with other non-target species. This reflects that the primers would bind only with the target DNA while avoiding any non-target annealing. In addition, determination of pairwise distance and construction of the phylogenetic tree with other 30 non-target animal and plant species revealed enough genetic distances among the species studied, thereby eliminating the possibility of any cross-target detection. Finally, to confirm absolute specificity, theoretical findings were validated experimentally by running practical PCR assays with the target and 25 different nontarget species and only the target amplification was observed.

2. Development of multiplex conventional and real-time PCR systems

A highly specific multiplex (heptaplex) PCR assay was developed targeting short length amplicons (73-263 bp in length) using seven pairs of species-specific primer sets targeting mitochondrial cytb and ND5 genes. Seven different targets (73 bp for pig, 106 bp for cow, 138 bp for buffalo, 161 bp for chicken, 203 bp for duck, 236 bp for goat and 263 bp for sheep) were successfully amplified simultaneously from raw, boiled, microwaved and autoclaved meat under pure and mixed matrices. The assay was sensitive enough to detect 0.01–0.005 ng of DNA from raw meat and 0.5% (w/w) adulterated meat in mixed matrices. For authentication, the PCR products were digested by three restriction enzymes namely *Fat*I*, Bfa*I, and *HPY188*I and distinctive restriction fingerprints were obtained for each of the seven targets under raw and different heat-treated samples. The authenticity of RFLP findings was further confirmed through satisfactory sequencing of

PCR products. I also present here multiplex real-time PCR assays with TaqMan probes to discriminate and quantify the contributions from bovine, buffalo, goat, sheep, chicken, duck and porcine materials in foodstuffs. The use of multiple fluorescent dyes in the probes allowed simultaneous detection of multiple species. The use of internal amplification control (141 bp site of eukaryotic 18S rRNA) eliminated the chance of any false negative amplification. Specificity evaluation against 23 nontarget species reveals no cross-reactivity. Analysis of reference samples reveals satisfactory target recovery, high PCR efficiency and good detection limit under mixed matrices. The developed mqPCR assays were sensitive enough to detect and quantify down to 0.006 ng of target DNA in pure meat and 1% (w/w) meat in mixed and commercial matrices. The results of the applicability test under commercial samples proved the suitability of the mqPCR assays for the analysis of real-world samples.

3. Assay validation under commercial meat products

The developed novel methods were tested for screening of commercially available and popular processed foods (meatballs, frankfurters, burgers, sausages etc.) and meat curries. A market survey reveals that 100% of tested beef meatballs were adulterated with buffalo. Beef burgers and frankfurters were found to be adulterated with buffalo in 84% (16/19) of tested samples among which 70% (7/10) were beef burgers and 100% (9/9) were beef frankfurters. Moreover, undeclared buffalo DNA was detected in 90% of the tested beef curry samples. This reflects that adulteration of beef with buffalo is commonplace in Malaysia. On the other hand, porcine meatballs, frankfurters and burgers were found to be 100% pure and no porcine materials were detected in any of the halal meat products tested. Furthermore, screening of meat products through qPCR revealed that 91% of beef items were buffalo adulterated. Undeclared DNA was detected in 23% of chicken burgers and frankfurters, whereas 50% of pork products contained both pork and chicken.

Moreover, lamb burgers and cooked whole lamb meat contained either beef or buffalo in 15% of tested samples.

6.2 Recommendations for Future Research

Given some advantageous features including stringent specificity, exceptional stability and superior sensitivity, the developed short amplicons targeted multiplex DNA-based approach could discriminatorily detect and quantify the target species even in severely processed and heat-treated specimens that contain degraded DNA. It possesses the potential to be used as a practical tool for routine analysis of meat products and thus might contribute to preventing or at least reducing food falsification and misdescription practices.

Due to time constraints, some other types of samples such as dairy and pharmaceutical products could not be included for analysis. I believe the developed authentication schemes could be conveniently applied in detecting species origin in dairy food, pharmaceutical capsule shells and cosmetics as well since bovine, chicken, buffalo and porcine are the most common sources of capsule shell gelatin. Among these, bovine and porcine have religious unacceptability to a large group of people worldwide. However, extraction of DNA from capsule shells, dairy and cosmetics items seems challenging and the procedure is yet to be perfectly optimized; therefore, an authentic and rapid DNA extraction protocol might be developed.

A newer approach in qPCR technology is droplet digital PCR (ddPCR) which offers accurate quantification with improved precision and sensitivity and eliminates the effect of complex matrices thereby providing an absolute measurement of DNA concentration without the use of standard curves. Herein, low concentrated DNA templates in the mixture of higher numbers of non-target DNA can be detected and hence, a direct relative

quantification is possible. This novel technology might be incorporated in future qPCR system for target quantification.

Currently, researchers have paid more attention to nanotechnology-based biosensor systems for the detection of DNA and these approaches have received enormous responses as they are fast, cost-effective, do not require expensive laboratory and instruments set up, and skilled personnel. Moreover, on-field delivery of results is possible. Although sensor-based detection still faces some challenges, there is scope to use these devices for routine food authentication purposes in near future.

6.3 Limitations of this Study

This study could not perform a multiplex real-time PCR (mqPCR) system to detect all the seven targets simultaneously in one reaction tube due to the unavailability of the qPCR instrument and reporter dyes that could give distinctive fluorescence signals in a single platform for eight targets (seven targets and IAC) at a time. Therefore, mqPCR assays had to run in two separate tubes.

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