DEVELOPMENT OF MULTIPLEX PCR ASSAY FOR THE DETECTION OF FIVE NON-HALAL SPECIES IN ISLAMIC FOODS

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ABSTRAK

Pemalsuan makanan adalah salah satu daripada isu sosio-ekonomi yang memberi kesan yang amat mendalam ke atas kesihatan, agama dan keewang. Baru-baru ini kes kontroversi berkaitan daging kuda di Eropah, daging tikus di China telah memberikan suatu kebimbangan dan pemikiran yang kritis untuk pengesanan, pembezaan dan mengenal pasti bahan-bahan, terutama barangan daging, dalam makanan, perubatan dan produk pengguna yang lain Pelbagai kaedah analisis berdasarkan lemak haiwan, protein dan penanda biologi-DNA telah dicadangkan untuk pengesahan spesies daging. Walau bagaimanapun, lemak dan ujian berasaskan protein kurang digemari kerana protein mudah terurai manakala tahap lemak boleh diubah suai dengan ketara melalui rawatan pemprosesan. Sebaliknya, kandungan maklumat sejagat dan kestabilan DNA yang luar biasa walaupun dalam keadaan tekanan ekstrim, memberikannya asas yang kukuh untuk berkhidmat sebagai penanda biologi yang boleh dikesan dalam semua siasatan forensik. Antara skim pengesanan berasaskan DNA, kaedah berasaskan tindak balas rantai polimerase (PCR) amat menjadi kegemaran kerana ia teknik ini dapat melipat-kali gandakan satu gen sasaran kepada beberapa salinan untuk kuantiti yang mudah dikesan. PCR multipleks sangat menarik kerana mereka membolehkan pengesanan sasaran pelbagai spesies dalam satu platform ujian tunggal, menjimatkan kos dan masa.

Kajian ini adalah usaha pertama untuk membangunkan satu sistem PCR multipleks untuk mengesan lima spesies daging haram yang berpotensi, iaitu spesies babi, anjing, kucing, tikus dan monyet, dalam satu platform ujian dimana bahan dalam keadaan mentah, diproses dan dicampur. Di sini kami mencipta lima set primer yang berbeza mensasarkan gen mitokondria ND5 untuk babi dan monyet; ATPase 6 gen untuk anjing dan tikus dan cytochrome b gen untuk spesies kucing. Primer ini khusus dikuatkan 172, 163, 141, 129 dan 108 bp serpihan kucing, anjing, babi, monyet dan tikus spesies dari matriks tulen dan kompleks. Ketepatan primer ini telah dibuktikan dengan menguji setiap

primer beserta dengan 21 jenis spesies haiwan daratan dan laut. Had pengesanan sistem multipleks yang dibangunkan adalah 0.01 ng untuk tikus, monyet dan anjing dan 0.02 ng untuk spesies kucing dan babi. Sistem multipleks yang dibangunkan jelas dapat dikesan samaada spesies daging sasaran di bawah sampel mentah mahupun dibawah tekanan tinggi dan bersuhu tinggi (autoklaf pada 121 ° C dan 45 psi untuk 2.5 h) tulen dan campuran. Saringan produk makanan komersial lagi disahkan kesahihan ujian di bawah matriks kompleks. Amplikon sasaran bersaiz pendek, kestabilan yang luar biasa serta sensitiviti sistem PCR multipleks yang maju mencadangkan ujian ini yang boleh digunakan oleh badan-badan kawal selia pengesahan makanan dan perlindungan hidupan liar.

ABSTRACT

Food forgery is one of the most concerning socio-economic issues having impact on health, religions and hard earned wages. The recent scandals on horse meat in Europe, rat meat in China have given consumers apprehension on the detection, differentiation and identification of ingredients, especially the meat items, in foods, medicine and other consumers' products. A range of analytical methods based on lipid, protein and DNA-biomarkers have been proposed for meat species authentication. However, lipid and protein-based examinations are less trustworthy since protein can be easily denatured and the level of lipids can be significantly modified through the processing treatments. On the other hand, universal information content and extraordinary stability of DNA even under compromised conditions have given it a strong foundation to serve as traceable biomarkers in all forensic investigations. Among the DNA-based detection schemes, polymerase chain reaction (PCR)-based methods are highly appreciated because of its extraordinary power of target amplification from few copies to easily detectable quantities. Multiplex PCR assays are especially interesting since they allow the detection of multiple species targets in a single assay platform, saving cost and time.

This study is the first endeavor to develop a multiplex PCR system for the detection of five potential "haram" meat species, namely pig, dog, cat, rat and monkey species, in a single assay platform under raw, processed, mixed and commercial matrices. We developed here five different sets of primers targeting mitochondrial ND5 gene for pig and monkey; ATPase 6 gene for dog and rat and *cytochrome b* gene for cat species. These primers specifically amplified 172, 163, 141, 129 and 108 bp fragment of cat, dog, pig, monkey and rat species from pure and complex matrices. Cross-species amplification was checked by performing species-specific PCR against 21 commercially important land and aquatic species and no cross-amplification was detected. The limit of detection (LOD) of the developed multiplex system was 0.01 ng for rat, monkey and dog and 0.02 ng DNA

for cat and pig species. In admixed samples and commercially processed foods, the tested LOD of 0.1% target meats. The developed multiplex system unambiguously detected target meat species under raw and heat-treated (autoclaved at 121 °C and 45 psi for 2.5 h) pure and admixed samples. Screening commercial food products further attested the assay validity under complex matrices. Short-sized target amplicons and extraordinary stability and sensitivity of the developed multiplex PCR system suggested that the assay could be used by regulatory bodies of food authentication and wildlife protection.

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Dedication

This thesis is dedicated to my beloved nephews and nieces

Saad, Safa**,** Muaz, Ubada, Damia, Maisarah,

Ariana, and Yasmin and to the children of Gaza.

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$	: dollar
%	: percent
′	: prime
°C	: degree Celsius
μg	: microgram
μl	: microliter
μM	: micro molar
=	: equal to
≥	: greater than or equal to
2D	: two dimensional
A	: adenine
A_{260}/A_{280}	: ratio of UV absorbance at 260 nm and 280 nm
ABI	: Applied Biosystems
AIDS	: Acquired Immune Deficiency Syndrome
ATP 6	: ATPase subunit 6
BLAST	: Basic Local Alignment Search Tool
bp	: base pairs
BSE	: Bovine Spongiform Encephalopathy
C	: cytosine
CEBAR	: Centre for Research in Biotechnology for Agriculture
Co.	: company
COI	: cytochrome c oxidase subunit I
Cq	: quantification cycle
Ct	: threshold cycle
CTAB	: Cetyl trimethylammonium bromide
cyt b	: cytochrome b
DBKL	: Dewan Bandaraya Kuala Lumpur
dH_2O	: distilled water
D-loop	: displacement loop
DMD	: duchenne muscular dystrophy
DNA	: deoxyribonucleic acid
dNTP	: deoxyribonucleoside triphosphate
ds-DNA	: double stranded- deoxyribonucleic acid

EC : European Commission

ELISA : enzyme-linked immunosorbent assay

E-nose : electronic nose

EtBr : ethidium bromide

fg : femto gram

FTIR : Fourier transformed infrared

Fwd : forward

g : gram

G : guanine

GC-MS : gas chromatography-mass spectrometry

GHR : growth hormone receptor

h : hour

HIV : human immunodeficiency virus

HPLC : High Performance Liquid Chromatography

IDT : integrated DNA technology

LINE : long interspersed nuclear element

LOD : limit of detection

Ltd : limited

MAG : 2-monoacylglycerol

MEGA5 : molecular evolutionary genetics analysis version 5

mg : milligram

MGB : minor groove binding

$MgCl_2$: magnesium chloride

min : minute

ml : mililitre

mM : milimolar

mt : mitochondrial

mtDNA : mitochondrial deoxyribonucleic acid

NCBI : national center of biological information

ND2 : NADH dehydrogenase subunit 2

ND5 : NADH dehydrogenase subunit 5

ND6 : NADH dehydrogenase subunit 6

ng : nanogram

nt : nucleotide

O.D. : optical density

PAGE : polyacrylamide gel electrophoresis
PCA : principal component analysis
PCR : polymerase chain reaction
PCR-RFLP : polymerase chain reaction- restriction fragment length polymorphism
PLS : partial least square
psi : pounds per square inch
RAPD : randomly amplified polymorphic deoxyribonucleic acid
Rev : reverse
rpm : rotations per minute
rRNA : ribosomal ribonucleic acid
s : second
SA : suitable amount
SINE : short interspersed nuclear element
T : thiamine
Ta : annealing temperature
TAG : triacylglycerol
Taq : *Thermus aquaticus*
TF-GB : target-function globotriaosylceramide
Tm : melting temperature
tRNA-Val : transfer ribonucleic acid- valine
US : United States
USA : United States of America
USD : United States Dollar
UV : Ultraviolet
w/w : weight/weight

CHAPTER 1. INTRODUCTION

Compliance of foods with individual health, religious rituals, budget and choice is a universal and long-term desire (Ghovvati et al., 2009; Cawthorn et al., 2013; Karabasanabar et al., 2014). To keep pace with the increasing work-volumes of the busier world, a growing number of people are being forced to spend more time at their work premises. They do not have enough time to cook their own meals and are thus being acquiesced to accept whatever they could manage from a nearby restaurant or grocery store. Thus the demands and prospects of restaurant business and ready-made foods, such as meatballs, burgers, frankfurters, pizzas, sandwiches, soups, cookies, candies, and creams are at the growing spree (Ali et al., 2012a; van der Spiegel et al., 2012). However, the consumers' concern over ingredients and quality of packaged and ready-made foods are not being abated due to the on growing threats of fraud labelling which poses the risk of zoonotic threats, allergens, ritually prohibited ingredients, and of course unfair trades and loosing personal budget (Dalvit et al., 2007; Nakyinsige et al., 2012a; Ali et al., 2012b). Ecological, environmental and wild-life protection are some of the other factors that have been added over the years (Opara & Mazaud, 2001).

In 2010, beef consumption in Europe has drastically fallen because of bovine spongiform encephalopathy (BSE), avian and swine influenza and contamination with toxic dioxin (Bottero & Dalmasso, 2011). Researchers believe that the most fatal and infectious disease, HIV/AIDS, has come to human race from African chimpanzee meat infected with Simian Immunodeficiency Virus (Fajardo et al. 2010). Religious rituals are also one of the prominent issues determining food avoidance, taboos and special regulation with respect to origins and processing of meats (Simoons, 1994). For instance, the presence of porcine derivatives in food products is a serious matter in Islam and Judaism (Ali et al., 2012c). While the global halal food turnover stood USD 661 billion

in 2011, it has now crossed US$2.1 trillion (Spring, 2011). The repeated amalgamation of prohibited food items such as pig, horse, dog, cat and rat meats with various dishes have put the Muslim consumers in red alert in determining the Halal status of the marketed foods (Mohamad et al., 2013; van der Spiegel et al., 2012). Experiments conducted on the restaurant industries to authenticate grouper (*Epinephelus marginatus*) demonstrated that only 9 out of 37 samples contained authentic species (Asensio, 2008a). Zha et al. (2010) demonstrated that fraud labelling is very prevalent in the deer products, especially heart, blood and antler products. Approximately 19.4% of meat products in the USA (Hsieh et al., 1995), 22% in Turkey (Ayaz et al., 2006), 15% in Switzerland and 8% in the United Kingdom were found to be mislabelled (Ballin et al., 2009). Market surveys on ground meat, sausages and cold nut expressed that 20% of labels were not accurate in terms of weight/weight (w/w) (Ballin et al., 2009). In Turkey, sausage sample labelled as 5% beef was found to contain no bovine DNA and meatball sample labelled as 100% beef was found to contain chicken and turkey (Ulca et al., 2013). A recent test on the British food industry for horse meat adulteration in beef pasta revealed 29 samples out of 2501 contained more than 1% horse meat merged with beef (Castle, 2013; Premanandh, 2013). More recently, in China rat meat was sold as lamb and Chinese police broke up a criminal ring and arrested 904 suspects involved in an alleged selling of fox, mink, rat and other meats after processing them with additives like gelatine and passed it off as lamb (Beijing, 2013). Another thunder bolt was fallen on Shaanxi province in China where police have seized over 20 tons of fake beef made up with chemically treated pig (Jeanette, 2013). The protection of endangered aquatic and wildlife in natural habitats is also relevant to meat authentication (Fajardo et al., 2010; Ali et al., 2012c). Further, the on-growing scientific innovation and technological breakthroughs in food processing and packaging along with the widespread globalization have made the task difficult to keep a check on

food ingredients and food manufacturing (Dalvit et al., 2007; McMillin, 2008; Ali et al., 2012c).

The above circumstances have raised concerns on the parameters needed to be measured, and the methods needed to be applied in determining the history and/or origin of meat species. European Commission legislation (178/2002) on food safety (European Commission, 2002) enables each stakeholder in a food supply chain to know the raw materials utilized in the manufacturing of any food products (Rodriguez-Ramirez et al., 2011). To ensure transparency in food manufacturing and food marketing, several countries have developed credible regulatory bodies to control the export and import of food products for years. For example, many countries such as Malaysia, Indonesia, China, Thailand, Singapore and Brazil have established trustworthy halal certification bodies to authenticate the halal status of marketed foods (Nakyinsige et al., 2012b). Surely, the enforcement of labelling regulations requires sensitive, reliable, and easily performable scientific methods to verify trace ingredients in processed and unprocessed foods, especially of animal origins.

For identification of meat species in the raw and processed foods, several molecular techniques based on lipids, (Szabó et al., 2007; Rohman et al., 2011) proteins (Chen et al., 2004; Ayaz et al., 2006) and DNA (Ali et al., 2011a, 2013; Karabasanavar et al., 2014) were proposed. Protein-biomarkers are fragile under physio-chemical shocks and both the type and amount of fats (lipid biomarkers) could be extensively modified during food processing (Ali et al., 2012a; Karabasanavar et al., 2014). On the other hand, DNA biomarkers, especially the shorter ones, are extraordinarily stable under compromised conditions (Arslan et al., 2006; Hou et al., 2014; Kitpipit et al., 2014; Ali et al., 2015a). So a myriad of DNA-based assays including species-specific PCR (Karabasanavar et al., 2014), PCR-RFLP (Dooley et al., 2005; Ali et al., 2011a; Chen et

al., 2014), PCR product sequencing (Ali et al., 2013), real-time PCR (Kesmen et al., 2013) and DNA barcoding (Di Pinto et al., 2013; Lamendin et al., 2015) have been documented for meat species authentication. Species-specific PCR seems to be the best and is considered as a robust method in comparison with other methods such as single nucleotide polymorphism (SNP) analysis, PCR-RFLP, PCR-RAPD and DNA barcoding (Ballin, 2010; Bottero & Dalmasso, 2011; Ali et al., 2014; Karabasanavar et al., 2014). Moreover, carefully designed species-specific PCR under optimized conditions is conclusive to detect and identify species, eliminating the need of restriction digestion and/or sequencing of PCR products (Rodriguez et al., 2004; Karabasanavar et al., 2014). However, current DNA identification schemes have also limitations in detecting multiple haram meat species in Halal foods.

Multiplex PCR assays with species-specific primers are greatly promising since they offer multiple target detection in a single assay platform, reducing both cost and time (Matsunaga et al., 1999; Zha et al., 2010, 2011; Bottero & Dalmasso, 2011; Ali et al., 2014). Therefore, here we developed a multiplex PCR assay suitable for detecting five most potential haram meats namely pig, dog, cat, rat and monkey meats and have thoroughly optimised it under commercial matrices and applied it for the screening of halal branded meat products, beef and chicken meatballs, burgers and frankfurters, which are popularly consumed across the world. Such an assay would find application in Halal Food industry, easing the halal authentication process in raw and processed meat products to safeguard consumers' health, religious believe, hard earned fortunes as well as to promote fair trades in the local and international markets.

1.1 Project Rationale

Current Halal food consuming population has crossed 1.8 billion and the market turnover is estimated to be US$ 2.1 trillion (Spring, 2011). Both consumption and markets are rapidly expanding. Specialised processing and supply chain requirements have made them costlier than ordinary counterparts and hence the fraudulent labelling of halal brand is frequently taking place.

The Islamic law prohibits Muslims from eating flesh and ingredients derived from pigs and animals having canine teeth or fangs such as dog, cat, monkey and rat. These animals are also potential carrier of anthrax, hepatitis, plague and some other dreadful diseases (Conly & Johnston, 2008; Fajardo et al., 2010; Rashid et al., 2015). However, in certain countries such as Vietnam, Switzerland, Tahiti, Mexico, South Korea, Taiwan and some parts of the United States, these animals have been consumed for ages (Ali et al., 2013). In certain regions, these animals could be obtained without any offered prices and hence there is a significant chance of mixing them in halal foods (Rahman et al., 2014). The recent horse meat scandal in Europe (Castle, 2013; Premanandh, 2013) and pig and rat meat scandal in China (Ali et al., 2014) have put the Muslim consumers in red alert in determining the presence of prohibited species-ingredients in marketed foods (van der Spiegel et al., 2012). Thus the verification of multiples species in a single assay platform is a timely need and would definitely improve consumer's perception and boost fair-trades in food business.

1.2 Problem Statements

Although several multiplex PCR assays have been documented, none of the granted or filed patents and documented PCR assays have targeted multiple haram meat species (Ali et al., 2015b). Additionally, the existing PCR-based methods involved longer amplicon-lengths which frequently breakdown during food processing treatments. To the best of our knowledge, for the first time we attempted here the development of multiplex PCR system with less than 200-bp amplicons for the unambiguous detection of five non-halal meat species, namely pig, dog, cat, rat and monkey species in halal foods.

1.3 Objectives

The purposes of this study are:

1. To design primer sets with closely matched annealing temperature for pig, dog, cat, rat and monkey species.
2. To develop, test and characterise multiplex PCR system for the detection of the above-mentioned species in raw and processed foods.

CHAPTER 2. LITERATURE REVIEW

2.1 History of Food Authentication

The initial methods of food identification were based on morphological characters such as flavour, colour, shape, taste and appearance (Winterhalter, 2006). The ruling bodies in ancient times used to verify weight for crop cereals and volume for drinks to ensure accurate measurements in the sale of food and drinks (Hargin, 1996). According to Winterhalter (2006), the initial method of fraud detection was very simple and based on physical inspections. For example, honey was physically examined for its purity by a duly appointed honey inspectors known as "Aletasters" in England or "Bierkiesers" in Germany. In the 19th Century, food verification methods was significantly improved and diversified and people started to identify alien substances using analytical balances and microscopes (Hahn, 1999; van Raamsdonk et al., 2007).

Germany and other European countries continued to apply microscopic methods until now to detect the presence of animal and plant derived materials in food and feeds (Ali et al., 2012c). However, the microscopic methods could not assign the exact origin of species in food and feedstuff ingredients in many instances (Ali et al., 2012a, c). Therefore, a number of molecular analytical tools based on lipid, (Szabó et al., 2007; Rohman et al., 2011), protein (Chen et al., 2004; Ayaz et al., 2006) and nucleic acid (Fumière et al., 2009; Ali et al., 2011a; Singh & Neelam, 2011) biomarkers have been documented. However, the appeal for lipid and protein-based methods have been dwindled since protein-based biomarkers can be easily denatured and the types and amount of lipids can be significantly modified through the processing treatments (Ali et al., 2012c; Ali et al., 2013). The major features and limitations of the most widely used food authentication techniques for better understanding have been summarised in Table 2.1.

Table 2.1. Existing techniques and their limitations to identify animal species in foods and feeds

Methods	Major Features	Limitations	References
Physical Identification			
Label tracing	Product ingredients are identified based on the physical labelling given by the manufacturers	• Based on faith or trust does not have any scientific value • Wrongly labelled information is not verified • Labels may be lost during transport and storage	Hargin (1996); McKean (2001); McMillin (2008); Ballin et al., (2009)
Microscopic analysis	Microscopic biomarkers of different species are physically visualised using a microscope	• Microscopic biomarkers are frequently lost or modified during processing treatments • Cumbersome, costly and time consuming • Requires skilled microscopists	Hahn (1999); Damez and Clerjon (2008); Ali et al., (2012c)
Identification of lipids and volatile organic compounds			
Lipid Biomarkers	Species are identified based on the positional analysis of fatty acids in triacylglycerol (TAGs) and 2-monoacylglycerol (2-MAG). Fourier Transformed Infrared (FTIR), Gas Chromatography-Mass Spectrometry (GC-MS) and/or Electronic nose- GC-MS coupled with multivariate partial least square fit (PLS) or principal component analysis (PCA) are used as investigation tools.	• Less reliable since both the amount and type of fats and fatty acids could be modified during the processing treatments • Need complicated statistical analysis to draw a conclusion • Need expensive instrumentations and skilled manpower	Rohman et al., (2011); Ali et al., (2012c)

Table 2.1. Continued.

Methods	Major Features	Limitations	References
Volatile organic compounds	Identification of analytes in the headspace volatiles of a solid or liquid using Chromatography-Mass Spectrometry or Electronic nose- Chromatography-Mass Spectrometry	• Need solid phase microextraction to pre-concentrate volatile organic compounds prior to detection • Species identification and discrimination is often misleading under complex matrices • Need complicated statistical analysis to draw a conclusion • Need expensive instrumentations and skilled manpower	Fuh et al., (2004); Che Man et al., (2005); Nurjuliana et al., (2011); Ali et al., (2012a)
Protein Identification			
Cation exchange or reverse-phase HPLC	Detect and quantify species specific protein biomarkers such as histidine dipeptides	Cannot determine the exact source of animal proteins in a mixed background	Aristoy and Toldra (2004); Ali et al., (2012a)
Iso-electric focusing and 2D- Electrophoresis	• Provide information about the habitats, age, and health conditions of fish and animal species based on the analysis of structural proteins such as actin, myosin, and tropomyosin • The 2D-PAGE can resolve a complex mixture of hundred proteins	• Laborious, cumbersome and expensive • Need specialized skills • Not reliable for complex mixtures	Skarpeid et al., (2001); van der Spiegel et al., (2012)

Table 2.1. Continued.

Enzyme-linked Immunosorbent Assay (ELISA)	• Species specific antibody or antigen is detected • Can discriminate both the tissue source and type of animal proteins	• Raising antibodies against an analyte is a must for ELISA test • The sensitivity is compromised upon heat and pressure treatments which often alter epitopes' specificity • The sensitivity fluctuates under mixed background • Cross-species detection among closely related species is quite common	Macedo-Silva et al., (2000); Meireles et al., (2004); Asensio et al., (2008b); Fumière et al., (2009); Mecca et al., (2011);
Western Blotting	• Proteins and epitopes are effectively identified • Can map expressed proteins in cell cycle	• Non-quantitative • Need available primary antibodies against the protein of interest • Antibodies often exhibits off-target binding • Well trained staff is a must	Lucker et al., (2000); Sultan et al., (2004); Mollica et al., (2009)
Biosensors and Biochips			
DNA Sensors	• Portable or lab-based device able to detect specific target hybridization via changes in optical or electrochemical properties • Short DNA targets which survive under extreme treatments can be identified	• Cannot amplify target oligo-copy number that leads to poor sensitivity • Frequently detect cross-species	Ahmed et al., (2010); Ali et al., (2011b, 2011c); Ali et al., (2012d)
DNA Microarray Chips	• Portable or lab-based device that allows the identification of hundreds or even thousands of targets via changes in optical or electrochemical properties • Short DNA targets which survive under extreme treatments can be identified	• Cross-species detection between closely related species is frequent • Cannot amplify target oligo-copy number and thus poor sensitivity • Cannot provide quantitative information	Teletchea et al., (2008); Teletchea (2009); Iwobi et al., (2011)

The numerous problems of lipid and protein-based techniques have forced researchers, managers and regulators to pay attention towards the nucleic acid-based molecular approaches for the detection, quantification and monitoring of meat species (Fajardo et al., 2010; Ali et al., 2013; Kesmen et al., 2013; Ulca et al., 2013). Codon degeneracy, superior stability and universal traceability in all cells have made DNA based methods extraordinary in practical fields (Meyer & Candrian, 1996; Ali et al., 2011a). Currently, polymerase chain reaction (PCR) assays (Colgan et al., 2001; Ali et al., 2013), DNA barcoding (Haye et al., 2012), nucleic acid biosensor (Ahmed et al., 2010) and chips (Ali et al., 2011b; Ali et al., 2012d) have been proposed for the identification of meat species. However, PCR has been extensively used in biomedical, agriculture and forensic sciences for the tracing of diseases, gene targets, paternity, criminals, wildlife and meat species because of their inherent ability to amplify as low as single copy nucleic acid targets into multiple copies even from complex matrices (Alaeddini, 2012). In food manufacturing and food marketing, PCR has been used to decipher minute level of defilement in raw and processed meats, ushering a good prospect of transparency food business (Doosti et al., 2011; Herrero et al., 2011). Conventional PCR techniques (Mafra et al., 2007; Ali et al., 2013) which involve time consuming electrophoresis have been replaced by automated real-time PCRs including SYBER green (Soares et al., 2013), Eva green (Santos et al., 2012) and TaqMan PCRs (Kesmen et al., 2013; Ulca et al., 2013). Multiplex PCR assay (Matsunaga et al., 1999; Dalmasso et al., 2004; Köppel et al., 2011) which can detect many species in a single assay platform is the latest addition in PCR technology.

2.2 Multiplex PCR

In the classical PCR system, a species-specific oligonucleotide primer pair is used to amplify DNA targets which are then detected on agarose gel (Ha et al., 2006; Martin et al., 2007) and can be further confirmed by amplicon sequencing (Girish et al., 2004; Maede, 2006), restriction digestion (Ali et al., 2011a ; Amjadi et al., 2012) and RAPD analysis (Rastogi et al., 2007). In this format, template DNA of a single species is amplified in a single PCR run and thus several runs are needed to detect several target species resulting in additional cost and time (Köppel et al., 2011). On the other hand, a multiplex PCR does simultaneous amplification of multiple DNA targets in a single reaction vessel. Chamberlain et al. (1988) was the first to develop a multiplex PCR method for the analysis of several deletions mutations in Duchenne muscular dystrophy locus. Since then, multiplex PCR has got huge attention which has made it an outstanding multi-target detecting technique in a single assay platform. The success of a multiplex PCR depends on the ability of the primers to be selectively annealed with their respective targets under a single set of PCR conditions (reaction volume and cycling conditions) (Rojas et al., 2010). Thus it demands complicated primer design for multiple species and stringent reaction optimization. In fact, primer designing is the most crucial and critical step in the development of a multiplex PCR system. This is because of difficulties in optimizing melting, annealing and elongation temperatures as well as preventing the formation of secondary structures and primer-dimers. PCR efficiency can be affected by a little variation in melting temperature (*Tm*) of the primers (Matsunaga et al., 1999). Even 1% mismatching of bases in the primer binding regions results in the reduction of Tm by 1-1.5 °C (Sambrook et al., 1989). Usually, inter-species hyper variable and intra-species conserved regions are targeted for primer design (Ballin et al., 2009). Thus mtDNA ensures a lower limit of detection (LOD)

compared to single copy nuclear DNA targets (Mohamad et al., 2013). Some breakthroughs towards the development of multiplex PCR are presented in Table 2.2. For the simplicity of understanding, multiplex PCRs could be divided into end point and real-time categories which are discussed below under separate subheading.

Table 2.2. Some breakthroughs in multiplex and real-time PCRs.

Year	Breakthrough	Limitations	References
1. Multiplex (end point) PCR			
1988	The first Multiplex PCR for the detection of prenatal and postnatal deletion mutations in Duchenne muscular dystrophy (DMD) locus	Difficult and extensive optimization of PCR parameters; each pair of primers need parameter optimization	Chamberlain et al., (1988)
1996	Multiplex PCR was developed for the differentiation of four species of *Saccharomonospora* targeting16SrRNA gene	Cross-species detection was frequently encountered with multiple primers	Yoon et al., (1996)
1999	Multiplex PCR was developed for the identification of cattle, pork, chicken, sheep, goat and horse meats in raw and Italian sausages	Horse-specific DNA fragments could not be amplified from cooked (120 °C) meats due to template degradation	Matsunaga et al., (1999); Di Pinto et al., (2005)
2001	Multiplex PCR was documented for the identification of different shark species	Primer proximity encountered for the positive control and target species	Pank et al., (2001)
2004	Multiplex PCR was reported for the detection of ruminant, poultry and porcine derived materials in feedstuffs	Cross-species detection was frequented under complex matrices	Dalmasso et al., (2004); Ghovvati et al., (2009)
2008	Identification of 18 mammalian species in a single PCR assay	Sequencing was needed to confirm the authentic targets	Tobe and Linacre (2008)
2011	Identification of four deer (sika deer, wapiti, red deer and reindeer) species by one step multiplex PCR	Poor sensitivity; could not identify targets in deer products at low concentration	Eung Soo et al. (2011); Zha et al. (2011)

Table 2.2. Continued

Year	Breakthrough	Limitations	References
2. Real-Time PCR			
a) Intercalating Dye Based			
2003	SYBR Green (SG) and TaqMan Real-Time PCR assays were developed for the first time to detect and quantify porcine derivatives	• SYBR Green intercalating dye bound with any double stranded DNA, giving rise to non-specific detection • Multiplexing could not be done with the SG chemistry	Sawyer et al., (2003); Walker et al., (2003); Wang et al., (2006); Lopez-Andreo et al., (2006); Mao et al., (2007); Fajardo et al., (2008); Rojas et al., (2011)
2006	The first report of a duplex SG-PCR via melting curve analysis	Further verification of authentic target was needed due to non-specific fluorescent signal	Lopez-Andreo et al., (2006)
2012	• Ruminant and poultry derived materials were identified in feedstuffs using SYBR Green PCR • Hare meat was identified using EvaGreen Real-time PCR	Cumbersome melting curve analysis	Şakalar and Abasıyanık (2012); Santos et al., (2012)
2013	EvaGreen multiplex real-time PCR assays were developed for the first time to identify beef and soybean origin materials in processed sausages	Complicated primer development.	Safdar and Abasiyanik (2013)
b) TaqMan Probe Based			
2003	First report of TaqMan real-time PCR system for the semi-quantitative detection of beef and mammalian family in food and feeds	False positive detection under mixed matrices	Brodmann and Moor (2003)
2004	Mammalian and poultry species were detected using Multiplex TaqMan real-time PCR system	Sensitivity reduced upon multiplexing	Dooley et al., (2004)

Table 2.2. Continued

2005	The introduction of minor groove binding (MGB) fluorescent probe for horse and donkey species detection	Sequence homology between donkey and horse frequented cross-species detection	Chisholm et al., (2005)
2009	Heptaplex real-time PCR assay for the authentication of beef, pork, chicken, turkey, mutton and goat and horse meat	Seven TaqMan probes in one tube produced high level of background signal	Köppel et al., (2009)
2012	• Molecular Beacon real-time PCR assay for the detection and quantification of porcine, bovine, turkey, chicken and sheep DNA in meat mixtures. • Introduction of double quenching ZEN probe in TaqMan PCR for pork detection	Higher sensitivity but reduced specificity	Ali et al., (2012a); Cai et al., (2012); Cammà et al., (2012); Hazim et al., (2012)
2013	Multiplex real-time PCR for the detection and quantification of duck, goose, chicken, turkey and pork meats were developed	Assay validation needed matrix-adapted reference material	Köppel et al., (2013)

2.3 Multiplex end point PCR

Multiplex end point PCR is very similar to its archetypical counterpart with variation of mixing multiple primer pairs to amplify multiple oligo-targets in single reaction tube and product identification on agarose gel based on differences in amplicon lengths. Before running multiplex PCR the specificity of designed gene targets (primers and probes) should be tested using conventional simplex PCR and PCR-RFLP. Additionally, cross-species specificity is also performed through in-silico and in-vitro approaches. The various steps involving in the development of a multiplex PCR assay are schematically shown in Figure 2.1.

Over the time, multiplex PCR assays have been recognized as robust, cost effective, sensitive and reliable method for meat species detection. Matsunaga et al. (1999) were the first to develop a species detecting multiplex PCR assay. Using common forward but different reverse primers for mitochondrial cytb gene, they identified five meat species, namely, goat, cattle, sheep, pig, and horse. Multiplex PCR was also performed on processed industrial meat products to ensure the applicability of the assay and excellent results with good detection limit were obtained (Dalmasso et al., 2004; Di Pinto et al., 2005; Ghovvati et al., 2009). The most frequently targeted mitochondrial genes are cytochrome b (cyt b), 12S rRNA, 16S rRNA, D-loop gene, tRNA-Val, ND5, ND2 and ATPase6/ATPase8 and the most widely used nuclear genes are 18S rRNA, short interspersed nuclear element (SINE) and long interspersed nuclear element (LINE). A brief documentary on the development of multiplex PCR systems for various meat species detection is summarized in Table 2.3.

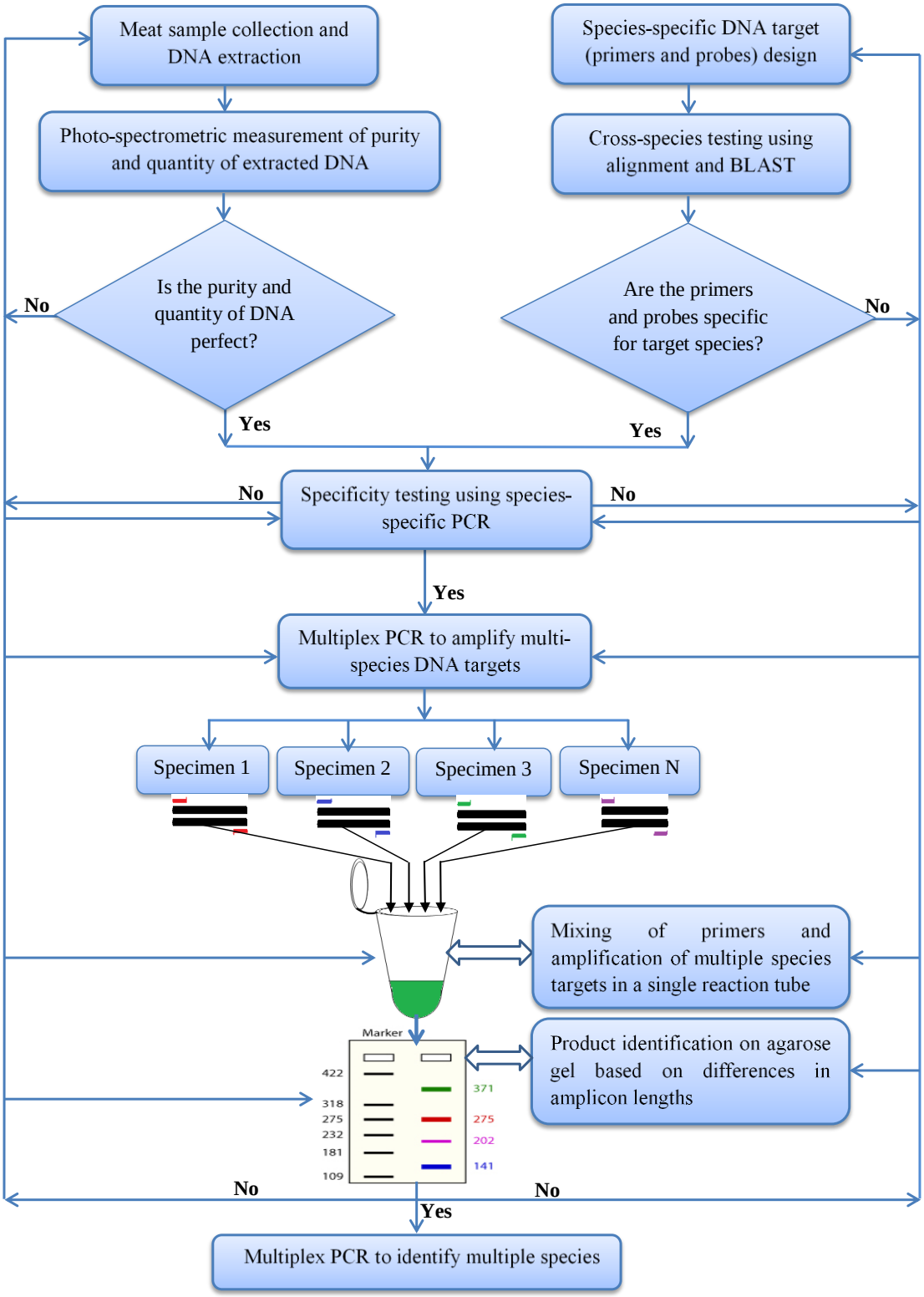

Figure 2.1. Schematic presentation of various steps in a multiplex PCR. In the diagram "**Yes**" denotes satisfactory results and "**No**" indicates unsatisfactory outcome that need optimization or repetition of earlier steps as shown by arrows.

Table 2.3. Identification of meat species using multiplex end point PCR.

Identified Species	Target Gene(s)	Product size (bp)	Detection limit (ng)	Reference
Cattle, Pork, Chicken, Goat, Sheep, Horse	cyt b	274, 398, 227, 157, 331, and 439	0.25	Matsunaga et al., (1999)
Ruminant (*Bos taurus, Capra hircus, Ovis aries*) Poultry, Fish and Pork	12S and 16S rRNA and tRNA-Val	104 – 106, 183, 220 – 230 and 290	0.0025-0.025	Dalmasso et al., (2004)
Horse and Pig	cyt b	439 and 398	0.25	Di Pinto et al., (2005)
Chinese alligator (*Alligator sinensis*)	cyt b	180	-	Yan et al., (2005)
Grouper, Nile perch and Wreck fish	16S rRNA	300, 230 and 140	-	Troota et al., (2005)
Mackerel (*Scomber scombrus*)	ND5	123	-	Infante et al., (2006)
Grouper, Wreck fish, Nile perch	5S rDNA	323, 471 and 185	-	Asensio (2008a)
Bonito (*Euthynnus pelamis, Euthynnus affinis, Auxis rochei, Auxis thazard, and Sarda orientalis*)	cyt b	236, 398, 143, 318, and 506	-	Lin and Hwang (2008)
Cattle, sheep, pig and chicken	16S rRNA	271, 274, 149, and 266	0.1-0.2	Luo et al., (2008)
18 common European mammal species	cyt b	89-362	0.00034	Tobe and Linacre (2008)
Yak and Cattle	mt 12S rRNA	290 (Yak), 290 and 159 (Cattle)	0.5	Yin et al., (2009)
Ruminant (*Bos taurus, Capra hircus, Ovis aries*), Poultry and Pork	12S and 16S rRNA	104–106, 183, 290	-	Ghovvati et al., (2009)
Bovine, Poultry, Ovine and Porcine	tRNA-Val and 16S rRNA	124, 183, 225 and 290	0.5- 5	Zha et al., (2010)

Table 2.3. Continued

Identified Species	Target Gene(s)	Product size (bp)	Detection limit (ng)	Reference
Mackerels (*Scomber japonicas, S. scombrus, S. australasicus, S. colias*)	D-loop, 5S rDNA, ND5	104, 123, 143 and 159	-	Catanese et al., (2010)
Wapiti, Sika deer, Tarim red deer, Red deer, Reindeer	D-loop and 16S rDNA	141, 230, 246, 272 and 307	0.02- 0.5	Zha et al., (2011)
Red deer, Sika deer, Wapiti and Reindeer	D-loop	199, 299, 245 and 375	0.05-1	Kim et al., (2011)
Chicken, beef, mutton, pork	cyt b	216, 263, 322, and 387	0.001	Zhang (2013)
Pork, lamb, chicken, ostrich, horse and beef	cyt b, t-Glu-cyt b, COI, 12S rRNA	100, 119, 133, 155, 253, and 311	7-21 fg	Kitpipit et al., (2014)
Poultry, donkey, camel, goat, and cattle	12S rRNA, ND2, cyt b	183, 145, 200, 157, and 274	0.05	Parchami Nejad et al., (2014)
Chicken, duck, goose	12S rRNA, cyt b, D-loop	131, 283, and 387	0.05	Hou et al., (2014)
Horse, soybean, poultry, and pig	cyt b, lectin, 12S rRNA, ATP 6	85, 100, 183, 212	0.012	Safdar et al., (2014)
Cow, sheep, goat, and fish	t-glu-cyt b, 12S rRNA, ATP 8, 18S rRNA	271, 119, 142, and 224	0.012	Safdar & Junejo, (2015)
Dog, cat, rat, pig, and monkey	ATP 6, cyt b, ND5	163, 172, 108, 141, and 129	0.01-0.02	Ali et al., (2015)

2.4 Multiplex Real-Time PCR

The failure of multiplex end point PCR to provide quantitative information of the target genes originally present in the sample (Tanabe et al., 2007; Ali et al., 2012a; Che Man et al., 2012; Hazim et al., 2012), has prompted scientists to develop real-time automated PCR. It effectively overcomes the limitations of end point PCR through the direct and independent monitoring of cycle-to-cycle amplification, offering a quantitative result based on the measurement of fluorescence intensity of a non-specific fluorescent dye such as SYBR Green (Chuang et al., 2012; Drummond et al., 2013; Soares et al., 2013) or a sequence-specific DNA probe called TaqMan probe (Ali et al., 2012a; Cai et al., 2012; Cammà et al., 2012; López-Andreo et al., 2012). Over the years, both the simplex (Kesmen et al., 2013) and multiplex (Köppel et al., 2011, 2013; Safdar & Abasiyanik, 2013) real-time PCRs have been documented for meat species detection and the development of a multiplex real-time PCR has schematically presented in Figure 2.2. The choice of intercalating dyes and fluorescent probes is a matter of investigation and has been described under separate subheading.

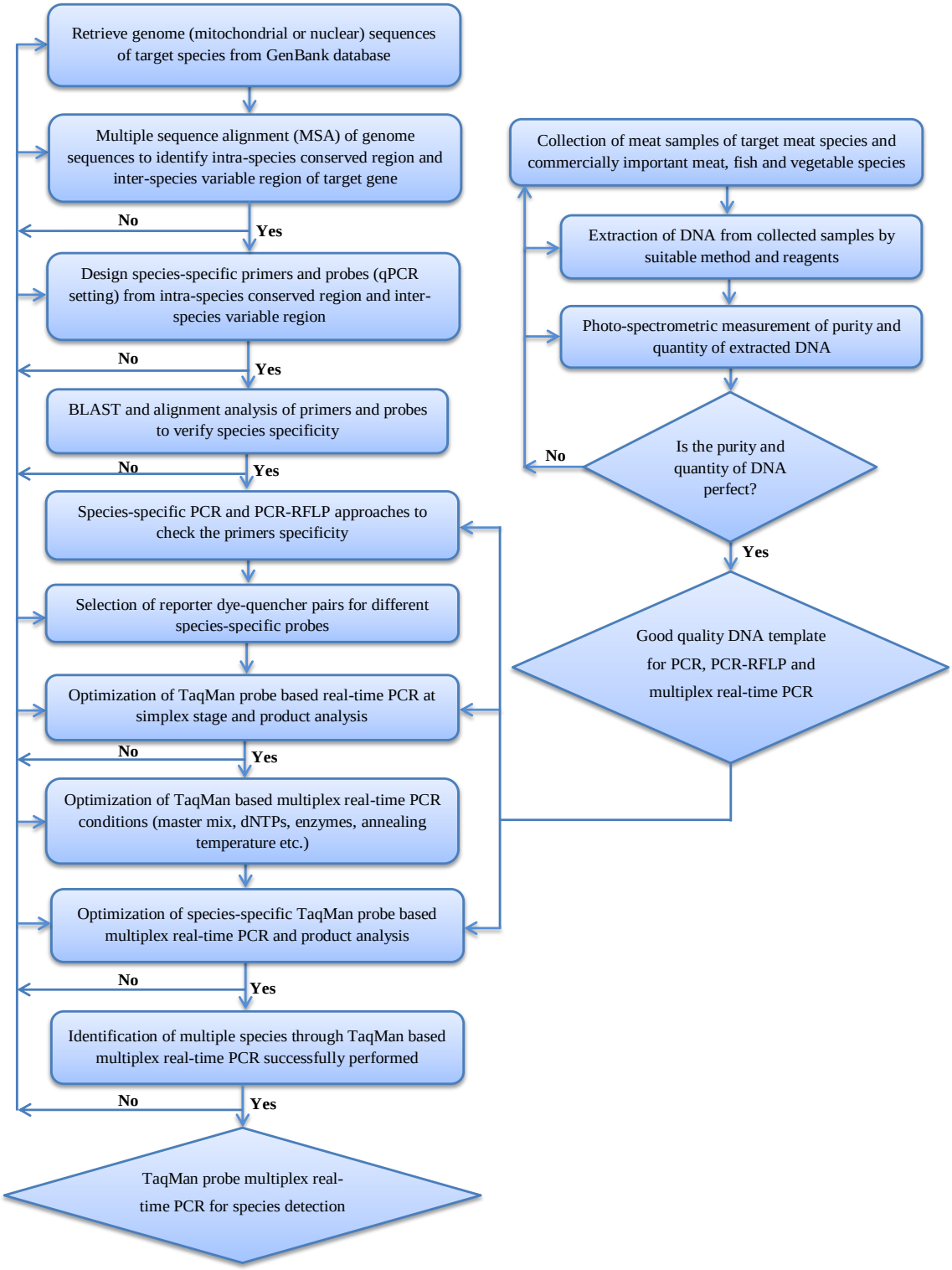

Figure 2.2. Steps in the development of TaqMan probe based Multiplex Real-Time PCR. In the diagram "**Yes**" denotes satisfactory results and "**No**" means unsatisfactory outcome that need optimization or repetition of earlier steps as shown by arrows.

The most important parameter of a real time PCR system is the threshold cycle (Ct) (Herrero et al., 2011) or quantification cycle (Cq) (Ali et al., 2012a,b) which is defined as the cycle at which fluorescence is first detected at a statistically significant level which is above the baseline or background signal (Figure 2.3) (Heid et al., 1996). The Ct value inversely correlates to the logarithmic value of the initial copy number and is set above the amplification baseline within the exponential phase. For PCR optimization, it is necessary to find the lowest Ct value and the highest final fluorescence by means of appropriate concentrations of primers and probes (Herrero et al., 2011). An early detection is indicative of more copies of target DNA templates present in the sample (Ali et al., 2012a; Mohamad et al., 2013). The detection limit of real-time PCR assays is variable but sufficient to detect adulterated materials (Lenstra, 2010). The principle of target copy number calculation using Ct or Cq values is demonstrated in Figure 2.3.

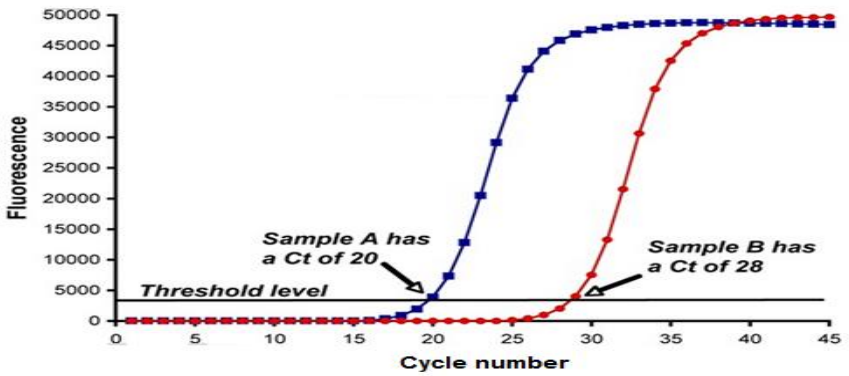

Figure 2.3. Threshold cycle (Ct) or quantification cycle (Cq) and calculation of target DNA copy number. The quantity of DNA doubles at each cycle of the exponential phase and can be calculated using the relative Ct values. If Ct value of sample A appears 8 cycles earlier than that of sample B, sample A will have $2^8 = 256$ times more copies of template DNA than that of sample B.

2.4.1 The Choice between SYBR Green and TaqMan Real-Time PCRs

The most widely used detectors in real-time quantitative PCR include SYBR Green dye and TaqMan probe. The first one binds non-specifically to the Minor Groove of double stranded DNA in the reaction mixture and the intensity of the emitted fluorescence increases with the increasing synthesis of double stranded amplicons (Fajardo et al., 2010; Hazim et al., 2012). SYBR Green dye chemistry is simple and cost-effective. It does not depend on complicated probe design but requires a complicated melting curve analysis to draw a conclusion (Fajardo et al., 2008). Simplicity and cost-effectiveness have encouraged researchers to develop SYBR Green real-time PCRs for the detection of cattle (Drummond et al., 2013), horse (Lopez-Andreo et al., 2006), deer (Fajardo et al., 2008), pork (Soares et al., 2013), lamb (Sawyer et al., 2003), wallaroo (Lopez-Andreo et al., 2006), chicken (Walker et al., 2003), ostrich (Rojas et al., 2011) and tuna species (Chuang et al., 2012) (Table 2.4). The sensitivity of the dye based real-time PCR is very high which allows the detection of even trace level contamination (0.000004 ng DNA) under pure states (Fajardo et al., 2008).

Despite several attractive features of SYBR Green PCR, it compromises with the specificity which has limited its applications in species authentication (Martin et al., 2009). SYBR Green dye also potentially inhibits PCR reactions at elevated concentrations (Mao et al., 2007). This forces the users to use low dye concentration which leads to insufficient redistribution of dyes in melting curve analysis. The non-specific binding of SYBR Green dye to any double stranded DNA compels one to perform additional verifications for authentic targets by electrophoretic separation or melting curve analysis (Lopez-Andreo et al., 2006). To avoid these problems, Mao et

al. (2007) suggested an alternative intercalating dye, named EvaGreen, which does not inhibit PCR amplification and thus can be used at high concentration which provides stronger signal in the analysis of melting curve. Additionally, EvaGreen is more stable than SYBR Green dye and can withstand intense PCR conditions, increasing sensitivity (Wang et al., 2006; Mao et al., 2007; Mohamad et al., 2013; Safdar & Abasiyanik, 2013). EvaGreen dye-based simplex and multiplex real-time PCRs were successfully applied in hare meat speciation (Santos et al., 2012) and beef and soybean authentication in processed sausages (Safdar & Abasiyanik, 2013).

The TaqMan probe which binds to the complementary sequence between the forward and reverse primers and is cleaved by the 5' exonuclease activity of *Taq* DNA polymerase is a better choice over SYBR Green and EvaGreen dyes (Ballin et al., 2009; Mohamad et al., 2013). Almost all commercially important meat producing species can be identified by TaqMan probe real time PCR and the targeting mitochondrial cytb, 12S-, 16S- and 18S-rRNA, ND2, ND5 & ATPase 6-8 and D-loop genes (Table 2.4). The TaqMan probe based real-time PCR for meat species identification can be designed by using one of the ways: (1) single probe for single species detection; (2) Multiple probes with a single reporter-quencher pair to detect multiple species and (3) multiple probes with a different reporter-quencher combination for each species. Family specific TaqMan probe based real-time PCR to detect several species of a certain family such as mammalian and poultry (Dooley et al., 2004; Lopez-Andreo et al., 2005) and fish (Benedetto et al., 2011) have been documented. However, family specific probe design requires some degree of base modifications to make them specific for various species of the same family (Dooley et al., 2004). In a single probe for single species detection platform, Ali et al. (2012a) documented short-amplicon length (109 bp) PCR assay targeting mt cytb gene to analyze pork adulteration in commercial burgers and

meatballs and obtained better target stability and sensitivity (0.001 ng DNA). Chisholm et al. (2005) and Cammà et al. (2012) described species-specific probes with single reporter-quencher combination for multiple species detection.

Although TaqMan probe based platform provides advanced specificity, high signal to noise ratio and shorter assay time, the probe designing protocol is entirely critical and required intensive monitoring of melting temperature, GC content and secondary structures, particularly for hairpin probe, self- and cross-dimerization of each primers and probe sets (Cammà et al., 2012). The melting temperature (Tm) of the probe must be 8-10 °C higher than that of the primers (Chisholm et al., 2005; López-Andreo et al., 2012; Kesmen et al., 2013). Besides TaqMan probe, couple probes such as molecular beacon probe and scorpion probe offer better specificity (Hazim et al., 2012). The great sensitivity of the molecular beacon probe might make it a future tool in species detection. The report of scorpion probe in meat speciation has not been described, probably due to its intricacy and complicated design (Whitcombe et al., 1999). The reported real-time PCR assays in meat species authentication is documented in Table 2.4.

Table 2.4. Simplex and Multiplex Real-Time PCR in species detection.

Identified Species	Target gene(s)	Limit of detection (ng)	Reference
1. Simplex real-time PCR			
TaqMan Chemistry			
Bovine (*Bos taurus*) and buffalo (*Bubalus bubalis*)	Cyt b, 16S rRNA	-	Drummond et al., (2013)
Seagull (*Larus michahellis*)	ND2	0.1	Kesmen et al., (2013)
Pork (*Sus scrofa*)	Not mentioned	0.1	Ulca et al., (2013)
Pork (*Sus scrofa*)	Cyt b	0.001	Ali et al., (2012a)
Pork (*Sus scrofa*), cattle (*Bos taurus*)	Repetitive elements	0.001	Cai et al., (2012)
Sheep, pork, beef, chicken, turkey	16S rRNA and Cyt b	0.00002-0.0008	Cammà et al., (2012)
4 tuna species (*Thunnus obesus, Thunnus orientalis, Thunnus maccoyii, Thunnus albacares*)	Cyt b, 16S rRNA, D-loop region	0.08	Chuang et al., (2012)
Pork (*Sus scrofa*)	Cyt b	-	Demirhan et al., (2012)
Beef (*Bos taurus*), pork (*Sus scrofa*)	Cyt b, *t*-Glu gene	0.001-0.3	López-Andreo et al., (2012)
Chicken, turkey, duck and goose	D-loop region and 12S rRNA	-	Pegels et al., (2012)
Fish species	12S rRNA	0.0002	Benedetto et al., (2011)
Atlantic Salmon (*Salmo salar*)	Internal transcribed spacer (ITS) 1	0.01	Herrero et al., (2011)
Beef, Pork and Goat	D-loop region	0.1	Pegels et al., (2011)
Ostrich (*Struthio camelus*)	12S rRNA	-	Rojas et al., (2011)
Donkey (*Equus asinus*), pork (*Sus scrofa*) and horse (*Equus caballus*)	ND2, ND5 & ATPase 6-8	0.0001	Kesmen et al., (2009)
Cattle, pork, chicken, lamb, goat, turkey	Cyclic guanosine monophosphate, Phosphordiesterase, ryanodine receptor, interleukin-2 precursor and myostatin	-	Laube et al., (2007a)

Table 2.4. Continued

Identified Species	Target gene(s)	Limit of detection (ng)	Reference
Cattle, pork, chicken, lamb, goat, duck, turkey	Cyclic guanosine monophosphate, Phosphordiesterase, ryanodine receptor, interleukin-2 precursor, myostatin	-	Laube et al., (2007b)
Cattle, pork	$tRNA^{LYS}$ ATPase 8	-	Fumiere et al., (2006)
Horse, donkey	Cyt b	0.001	Chisholm et al., (2005)
Mallard and Muscovy duck	Cyt b	-	Hird et al., (2005)
Cattle, pork, lamb, chicken, turkey, and ostrich	Cyt b, *t*-glu, ND5, nuclear 18S rRNA gene	0.000006-0.0008	Lopez-Andreo et al., (2005)
Pork	12S rRNA	0.05	Rodriguez et al., (2005)
Beef, pork, lamb, chicken, turkey	Cyt b	0.01- 0.1	Dooley et al., (2004)
Haddock	Transferrin	-	Hird et al., (2004)
Cattle	Growth hormone	0.02	Brodmann and Moor (2003)
Cattle, pork	Phosphodiesterase, ryanodine gene	-	Laube et al., (2003)
Molecular Beacon Chemistry			
Pork	Cyt b	0.0001	Hazim et al., (2012)
SYBR Green Chemistry			
Tuna species (*Thunnus obesus*)	ATPase 6, 16S rRNA	0.08	Chuang et al., (2012)
Bovine (*Bos taurus*) and buffalo (*Bubalus bubalis*)	Cyt b, 16S rRNA	-	Drummond et al., (2013)
Pork	Cyt b,	0.01	Soares et al., (2013)
Ostrich (*Struthio camelus*)	12S rRNA	0.0000245 and 0.00023	Rojas et al., (2011)
Pork	12S rRNA	0.002	Martin et al., (2009)
Red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*)	12S rRNA	0.000004	Fajardo et al., (2008)

Table 2.4. Continued

Identified Species	Target gene(s)	Limit of detection (ng)	Reference
Pork, cattle, horse, wallaroo	3′ end of ND6 and the 5′end of cyt b gene	0.00004-0.0004	Lopez-Andreo et al., (2006)
A number of mammalian and avian species	Short interspersed nuclear element (SINE), long interspersed nuclear element (LINE)	0.0001-0.1	Walker et al., (2004)
Cattle	16S rRNA	-	Sawyer et al., (2003)
Ruminant, cattle, pork, chicken	Bov-tA2 SINE, 1.711B bovine repeat, PRE-1 SINE, CR1 SINE	0.00001-0.005	Walker et al., (2003)
EvaGreen Chemistry			
Hare (*Lepus* species)	Cyt b	0.001	Santos et al., (2012)
2. Multiplex real time PCR			
TaqMan Chemistry			
Duck, goose, chicken, turkey and pork	12S rRNA and Cyt b	-	Köppel et al., (2013)
Beef, pork, horse and sheep	Prolactin receptor gene, growth hormone receptor(GHR), Beta-actin-gene	0.32	Köppel et al., (2011)
Beef, pork, turkey, chicken, horse, sheep, goat	Beta-actin-gen, Prolactin receptor, Target-Function Globotriaosylceramide (TF-GB3), Cyt b	0.32	Köppel et al., (2009)
SYBR Green Chemistry			
Ruminant (*Bos taurus*), Poultry (*Gallus gallus*)	16S rRNA-tRNA, 12S rRNA	0.0000245	Şakalar and Abasiyanik (2012)
EvaGreen Chemistry			
Beef and Soybean	ATPase 8, Lectin	0.0027 and 0.0009	Safdar and Abasiyanik (2013)

2.5 Probability and Prospect

The verification of food components is a must to safeguard consumers' health, religious rituals, hard-earned opulence, wildlife and endangered species (Fajardo et al., 2010; Ali et al., 2011a; Doosti et al., 2011; Hazim et al., 2012). The bottom line of this verification process is a precise, unique, efficient and universal authentication technique that can detect and assign the original meat species present even in trace amount in any forms in the finished and/or raw food products. In this context, DNA-based molecular techniques poise huge potentials because of some interesting features such as universality, codon degeneracy, thermal-stability and polymorphism of the molecule itself (Lockley & Bardsley, 2000; Aida et al., 2005; Ballin, 2010). The issues that need to be taken into account in designing a DNA based technique include (1) choice of target gene; (2) ease of DNA extraction and (3) detection sensitivity (Bottero & Dalmasso, 2011). PCR-based methods have taken a central position among the DNA based investigation schemes. This is because of its extraordinary power to ensure target availability through the amplification of little targets, even a single copy, into multi-copies (Tanabe et al., 2007; Köppel et al., 2011). Compared to conventional single species PCR systems, multiplex PCR is a technique to save costs and time since it offers multiple target detection in a single assay within short time (Tobe & Linacre, 2008).

In the monarchy of meat species identification, the first multiplex end-point PCR was documented by Matsunaga et al. (1999) to identify six meat species with a detection limit of 0.25 ng raw DNA. The second breakthrough came from Dalmasso et al. (2004) who designed species-specific primers based on mitochondrial 12S- and 16S-rRNAs and tRNA-Val genes to detect ruminant (*Bos taurus, Capra hircus, Ovis aries*), poultry, fish and pork with a detection limit of 0.005 ng for fish and 0.0125 ng

for others. The works of Dalmasso et al. (2004) were verified by Ghovvati et al. (2009) and Zha et al. (2010) and brilliant results were obtained. The third breakthrough was from Yan et al. (2005) who documented a multiplex PCR for the differentiation of Chinese alligator (*Alligator sinensis*). The fourth breakthrough was from Asensio (2008a). He used multiplex PCR for the identification of grouper, wreck fish and Nile species. The fifth contribution was from Lin and Hwang (2008) who differentiated documented five bonito species. The sixth breakthrough was again from Yin et al. (2009) who identified yak and cattle meats with a detection limit of 0.5 ng DNA using a multiplex platform. The seventh contribution was provided by Zha et al. (2011). They verified the fraudulent labeling of medicinally important deer species using a multiplex PCR assay targeting mitochondrial D-loop and 16S rDNAs with a detection limit of 0.02-0.5 ng.

All the previous researchers annotated multiplex end point PCR as a robust, prompt, extremely sensitive and prominently suitable tool for species identification. However, the questions arose from the qualitative information of template DNA and higher length of amplicons which encounter higher rate of DNA target fragmentation under severe heat and other processing treatments, reducing PCR efficiency and sensitivity (Arslan et al., 2006; Ilhak et al., 2007; Martin et al., 2007; Yin et al., 2009). For example, Matsunaga et al. (1999) could not detect 439 bp fragment of heat treated horse meat. Similarly, Ali et al. (2011c) failed to amplify a 411bp fragment of 12S rRNA gene from extensively autoclaved pork meat. Additionally, the difference in lengths among the amplified fragments should be 40–50 bp to permit adequate resolution of various PCR products by agarose gel electrophoresis (Bottero & Dalmasso, 2011; Ali et al., 2015a) and agarose cannot discriminate PCR products smaller than 10 bp difference in length and staining of DNA or RNA on agarose using

dyes such as ethidium bromide (EtBr) needs at least 20 ng of PCR products. Moreover, some fragments disappeared from a multiplex PCR product when more efficiently amplified loci negatively influence the yield from the less efficiently amplified loci (Zha et al., 2011). Thus the possibility of detecting lower levels of DNA in commercial food items was interesting in theory, but did not work in practice using a multiplex end point PCR (Zha et al., 2010, 2011).

To overcome the limitations of multiplex end point PCR, multiplex real-time PCR was invented. Using a fluorescent-labeled signaling probe or reporter dyes (Chuang et al., 2012), this self-automated PCR system meritoriously provided quantitative detection with great sensitivity without the need of any laborious agarose- or polyacrylamide-gel electrophoresis (Köppel et al., 2011; Sakai et al., 2011). The species authenticating multiplex real-time PCR based on SYBR Green (Şakalar & Abasiyanik, 2012), EvaGreen (Safdar & Abasiyanik, 2013) and TaqMan probe (Köppel et al., 2009, 2011, 2013) have been documented. Since the SYBR Green and EvaGreen based protocol is non-specific, inhibits PCR reaction at high dye concentration and unequally redistribute dye at low concentration, also providing poor signal in melting curve analysis as well as encounters primer complexity, TaqMan probe based real-time PCRs have got preference.

TaqMan based multiplex real-time PCR offers double screening of species through species specific primers and probes and Köppel et al. (2009) was the first to detect seven meat species with a detection limit of 0.32 ng using the platform. In 2011, Köppel et al. again reported a multiplex real-time PCR assay for the detection of four meat species in reference food samples. More recently, Köppel et al. (2013) performed

quantitative detection of duck, goose, chicken, turkey and pork using a multiplex real-time PCR. In all cases, the assay was found to be automated, reliable and time saving.

There are also some challenges in developing multiplex real-time PCR such as the emission maxima of reporter dyes should be separated by at least 15 nm and the absorption spectrum of the quencher must overlap with the fluorescence spectrum of the reporter to avoid the re-absorption of emitted light. Consequently, the probe concentrations should be less than 0.5 µM (Johansson, 2006). Thus, the intensive optimization of reporter-quencher fluorescence, absorbance and concentration are greatly required. Additionally, the use of several TaqMan probes in a single tube produce high level of background signal (Köppel et al., 2009). To reduce the background signal, the smallest possible concentration of each probe should be titrated (Köppel et al., 2009) and a second quencher (such as ZEN probe) in the middle of probe could be used to increase signal to noise ratio (Ali et al., 2012a, b; Cai et al., 2012). Nevertheless, the choice of reporter-quencher combination must be compatible with the instrumentation used to read fluorescence (Lopez-Andreo et al., 2005; Johansson, 2006). Additionally, the authentication technique should detect a broad spectrum of analytes and the PCR systems has to be cost effective to keep laboratory expenses at a reasonable level (s). Since TaqMan probe based multiplex real-time PCR system ensures high sensitivity and convenient Ct analysis, it would get more popularity in near future to identity several species in a single assay platform. The illustrative presentation of multiplex end point (qualitative analysis) and real-time (quantitative analysis) PCRs are presented in Figure 2.4.

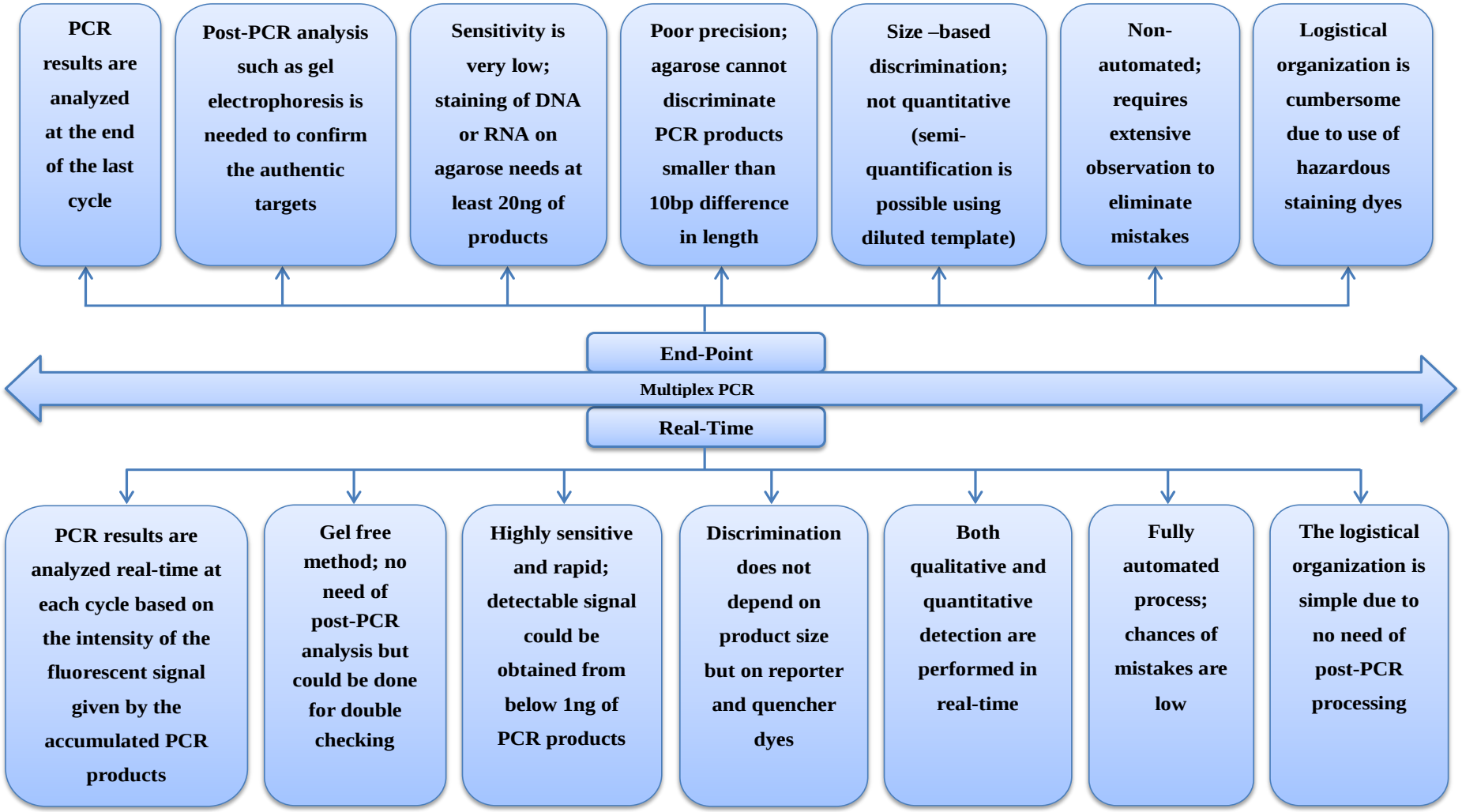

Figure 2.4. Illustrative presentation of Multiplex End Point and Real-time PCRs.

CHAPTER 3. METHODOLOGY

3.1 Meat Samples Collection

Among the five target meat species (Dog: *Canis lupus familiaris*, Cat: *Felis catus*, Rat: *Rattus rattus*, Pork: *Sus scrofa*, and Monkey: *Macaca fascicularis*) dog, cat and rat meats were collected in triplicates from the Faculty of Veterinary Science, University of Putra Malaysia and Dewan Bandaraya Kuala Lumpur, Malaysia. Monkey meats were obtained from Wildlife Malaysia. Pork was purchased in triplicates from three different vendors from Chinese wet market in Seri Kembangan, Selangor, Malaysia. The most commonly used commercial meat (beef (*Bos taurus*), chicken (*Gallus gallus*), goat (*Capra hiscus*), lamb (*Ovis aries*), buffalo (*Bubalus bubalis*), venison (*Odocoileus virginianus*), duck (*Anas platyrhychos*), and pigeon (*Columba livia*)), expensive fish species (salmon (*Salmo salar*), cod (*Gadus morhua*), tuna (*Thunnus orientalis*), and carp (*Cyprinus carpio*)) were purchased in triplicates on three different days from the various wet and supermarkets across Malaysia. In case of purchasing commercially available meat products, three salient features were considered. First of all, popularity of products; secondly, labelled with halal logo; and thirdly, the products are whether local or imported? I found three meat products namely meatball, burger, and frankfurter as the most popular food items in Malaysia and all over the world (Rohman et al., 2011; Ali et al., 2012a; Ali et al., 2013; Rahmania et al., 2015). Based on the selection criteria, five different branded beef and chicken meatballs, eight burgers, and seven frankfurter items were procured from different supermarkets of Kuala Lumpur, Petaling Jaya, Malaysia. All meat samples and products were transported under ice-chilled condition (4 °C) and stored frozen at -20 °C until use to prevent natural and enzymatic decompositions of meats and DNAs.

3.2 Preparation of Dummy Meat Products

The most popular and consumed ready to eat meat products namely meatball, burger and frankfurter of both beef and chicken were prepared to validate the reliability of the developed assay. To simulate commercial meat products, meatballs were prepared following Rohman et al. (2011) and Rahman et al. (2014); burgers were prepared according to Ali et al. (2012a) and the formula of Ali et al. (2013) was followed to prepare frankfurters with balanced amount of grinded beef, chicken and target meat species individually and together and an approximate recipe of meat products preparation is given in Table 3.1. Prepared ready to eat meat products were subjected to autoclave at 121 °C under 45-psi pressure for 2.5 h to simulate extensive cooking and boiling effects. All samples were prepared in triplicate on three different days by three independent analysts and were stored at -20 °C prior to DNA extraction.

Table 3.1. Formulation of ready to eat model meat products.

Ingredients	Meatball (≥35 g/piece)		Burger (≥85 g/piece)		Frankfurter (≥70 g/piece)	
	Beef	Chicken	Beef	Chicken	Beef	Chicken
Minced meat	23 g[a]	23 g[a]	60 g[a]	60 g[a]	45 g[a]	45 g[a]
Soy protein	3 g	3 g	10.8 g	10.8 g	7.5 g	7.5 g
Starch/breadcrumb	5 g	5 g	8 g	8 g	6.5 g	6.5 g
Chopped onion[b]	1 g	1 g	3 g	3 g	2.5 g	2.5 g
Chopped ginger[b]	0.1 g	0.1 g	0.2 g	0.2 g	0.15 g	0.15 g
Cumin powder[b]	0.75 g	0.75 g	1 g	1 g	0.75 g	0.75 g
Garlic powder[b]	0.5 g	0.5 g	0.75 g	0.75 g	0.5 g	0.5 g
Black pepper[b]	0.14 g	0.14 g	0.25 g	0.25 g	0.23 g	0.15 g
Tomato paste	1.5 g	1.5 g	2.5 g	2.5 g	2 g	2 g
Butter	1.5 g	1.5 g	2.5 g	2.5 g	2.5 g	2.5 g
Egg			1 g	1 g		
Salt	SA	SA	SA	SA	SA	SA
Others[c]	SA	SA	SA	SA	SA	SA

[a] 1%, 0.5%, and 0.1% of all target meats (pig, dog, cat, monkey, and rat) were added both individually and collectively with a balanced amount of minced chicken and beef to make ≥ 35g, 85g, and 70 g specimen of each meatball, burger, and frankfurter preparation, respectively.

[b] Amounts are in approximate values and some items were taken in tea-spoon measurement.

[c] Enhancer materials, flavouring agents.

SA- Suitable amounts.

3.3 DNA Extraction from Raw Meats and Meat Products

Total DNA of all meat and fish samples were extracted using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan) from 20 mg of muscle tissues following manufacturer's instruction. DNA from dummy and commercial meat products was extracted from 100 mg specimen. Firstly, sample was grinded with Micropestle in a

1.5 ml micro-centrifuge tube to make a pulp followed by the addition of 20 µl of Proteinase K. The mixture was incubated at 60 °C for 30 min to lyse the sample. After adding 400 µl of lysis buffer, sample mixture was incubated again at 60 °C for 20 min to ensure the clarity of sample lysate. Subsequent steps followed the instructions given by the kit manufacturer (Yeastern Biotech Co., Ltd. Taipei, Taiwan). DNA from plant species (wheat (*Triticum aestivum*), tomato (*Solanum lycopersicum*), garlic (*Allium sativum*), onion (*Allium cepa*), and pepper (*Capsicum annuum*)) was extracted using CTAB method according to Rahman et al. (2014). As DNA might be loss during aqueous and organic phase separation (Karabasanavar et al., 2011), the spin column based extraction technique was used to increase yield of DNA. Similarly, the DNA of raw and heat treated meat products is supposed to be highly degraded so 100 mg samples have been taken for DNA extraction. The quantity of total extracted DNA was estimated by taking optical density (O.D.) readings at 260nm and the purity of DNA was checked by taking ratio of O.D. readings at 260 nm and 280 nm using Biochrom Libra UV-Vis Spectrophotometer (Biochrom Ltd., Cambridge, England).

3.4 Gene Selection and Primers Designing

3.4.1 Salient Features of Selected Genes

Carefully designed species-specific PCR under optimised conditions is conclusive to detect and identify species, eliminating the need of restriction digestion and/or sequencing of PCR products (Rodriguez et al., 2004; Karabasanavar et al., 2014). Here species-specific primers were designed by targeting mitochondrial genes since they are well protected by mitochondrial membrane, maternally inherited and presence in multiple copies per cell (Xin et al., 2006). Among the mitochondrial genes, NADH dehydrogenase subunit 5 (ND5) and ATPase subunit 6 offer appropriate target length, sufficient degree of intra-species conserved regions and interspecies polymorphism, and available

sequence database for most animals and plants (da Fonseca et al., 2008; Kitpipit et al., 2014). On the other hand, moderate evolutionary rate and clear evolutionary patterns have made cytochrome b (cyt b) gene a suitable candidate to study phylogenetic evolution at the intra- and inter-species levels and target for specific primers and probes (Brown et al., 1979; Xin et al., 2006). These features build our interests to design species-specific primers targeting ND5 gene for pig and monkey, ATPase 6 for dog and rat, and cyt b for cat species.

3.4.2 Major Criteria of Primers

Primers should have some unique criteria to be considered as ideal for PCR. First of all, the length as the specificity and annealing to the target templates are significantly affected and determined by the length of primers. Non-specific amplification and low specificity may occur due to very short primers whereas, decreasing the template-binding efficiency at normal annealing temperature may take place to extremely long primers due to the higher probability of forming secondary structures such as hairpins. Ideally primers are 18-28 nucleotides in length and can be longer in case of necessity.

Since annealing in a PCR occurs for both forward and reverse primers simultaneously, pairs of primers should have similar melting temperatures (*Tm*). A little difference of *Tm* among the primer pairs (2-5 ^{o}C differences tolerated) would affect the efficiency of multiplex PCR. If *Tm* (melting temperature) of a primer is significantly higher than the reaction's annealing temperature (*Ta*) may lead to mis-hybridization and can extend at an incorrect location along the DNA sequence, while failure to anneal and extend may happen with *Tm* significantly lower than the annealing temperature.

The approximate melting temperature (*Tm*) of primers containing less than 25 nucleotides can be calculated using the equation: $Tm = 4\ (G + C) + 2\ (A + T)$, where G,

C, A, T – number of respective nucleotides in the primer. If the primer contains more than 25 nucleotides specialized computer programs e.g., Reviewer, *Tm* calculator from Promega, applied Biosystems, Oligo etc. are recommended to account for interactions of adjacent bases and the effect of salt concentration. *Tm*'s of forward and reverse primers must be similar (2-5 ^{o}C differences tolerated). In case of real-time PCR, the *Tm* of probe must be 8-12 degree higher than the *Tm* of the primers (Cammà et al., 2012; Ali et al., 2014).

Specificity of primers to target species and non-specificity to non-target species totally depend on mismatching between primers and template DNA. Because each 1% mismatching of the bases in a double-stranded (ds) DNA reduces melting temperature (*Tm*) by 1-1.5 °C (Sambrook et al., 1989; Matsunaga et al., 1999; Köppel et al., 2013; Zhang, 2013). The ratio of mismatching in designing primers for multiplex PCR should be more than 15% between a species-specific primer and the other species sequences. The mismatching of more than 15% decreases *Tm* more than 15 °C making the primer pairs anneal only to the species-specific sequences in the multiplex PCR. As the 3' end mismatching is fatal for PCR amplification so the primers should be designed to mismatch with different species at 3' end or next nucleotides.

A minimum of intra-molecular or inter-molecular homology is important in designing primers. This would result to secondary structure formation such as hairpins or primer dimerization (Figure 3.1). Usually intra-primer homologies of 3 bp or more should be avoided. The worst situation is when the 3' ends of the primers anneal; this leads to "primer-dimer" formation (Figure 3.1b). So the internal inter-molecular interactions should also be minimized.

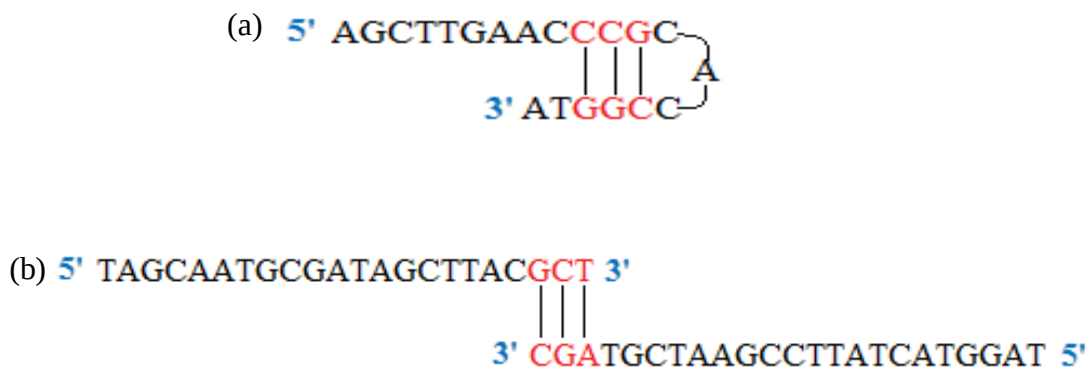

Figure 3.1. Formation of secondary structure (a) hairpin; (b) primer-dimer.

Secondary structures negatively affect primer-template binding, leading to poor or no amplification. Some web-based softwares are available to detect secondary structure of primers such as Oligo 4.0, Primer Express 3.0 (Applied Biosystems), AutoDimer, IDT OligoAnalyzer 3.0, PUNS, NCBI BLAST, UCSC In-Silico PCR etc.

Ideally the primer will have a near random mix of nucleotides. The presence of G or C within the last 4 bases from the 3' end of primers is desirable to increase yield and to prevent mis-priming. However, primers with long polyG or polyC stretches need to avoid because of possibility to promote non-specific annealing. Although having one or two G and/or C at 3' is allowed but adding Gs or Cs may adversely influence the overall specificity of the primers.

3.4.3 Design of Primers

Primers for mulitplex PCR can be designed either by primer designing software or by multiple sequence alignment (Matsunaga et al., 1999). There are two publicly available multiplex PCR primer designing softwares namely MultiPLX (http://bioinfo.ebc.ee/ multiplx/) and PrimerStation (http://ps.cb.k.u-tokyo.ac.jp/index .html). Although MultiPLX software can design and analyze primer properties and compatibilities but it has very poor online documentation; whereas, PrimerStation can

design higly specific and accurate multiplex genomic PCR primer only for human genome. So these softwares are not available in practice. Primer designing with multiple sequence alignment by ClustalW software is the easiest and most practising procedure.

In the present study, the mitochondrial gene sequences of pig: AF034253.1; monkey: FJ906803.1; dog: NC_002008.4; rat: NC_012374.1; cat: NC_001700.1; cow: V00654.1; buffalo: NC_006295.1; yak: AY684273.1; goat: GU229279.1; sheep: HM236175.1; deer: DQ985076.1; horse: X79547.1; donkey: X97337.1; chicken: X52392.1; duck: EU009397.1; pigeon: NC_013978.1; salmon: KF792729.1; tuna: GU256524.1; cod: AM489716.1; shad: AP011596.1; tilapia: AF015020.1; carp: KJ511883.1; turtle: NC_014769.1); wheat: X02352.1; and garlic: AF356823.1; were retrieved form NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned using ClustalW multiple sequence alignment tool (Thompson et al., 1994) to select the inter-species hyper-variable and intra-species conserved regions. Regions of the gene sites were used to design species-specific primer pairs for pig, dog, rat, monkey and cat (Table 3.2).

The further checking of mismatches to all other species either at the 3′ position or where possible, for both forward and reverse primers was performed by MEGA5 software (Tamura et al., 2011) (Appendix B, Table 1S-5S). The designed primers were also screened for unique specificity to eliminate cross-species binding with other animal or plant species using the online BLAST local alignment tool in NCBI data base (http://blast.ncbi.nlm.nih.gov /Blast.cgi). The BLAST results showed 100% identity with the target species and eliminate the probability of primer binding with non-target species DNAs. Finally, the sequences of target meat species were retrieved from online Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) software and were used to compute the pairwise distances among the studied species

using the maximum composite likelihood method (Tamura et al., 2011) (Appendix C, Table 6S-10S). In this method, lower distance value indicates higher similarity, whereas, higher value indicates lower similarity (Ali et al., 2013). The pairwise distances among the studied species in this study were in the range of 0.43 to 1.18, which represents high inter-species variability of the amplicon regions and no probability of cross-species amplification. All designed primers were purchased from IDT, USA.

Table 3.2. Species-specific oligonucleotide primers for five target meat species.

Species	Genes	Primers	Primer sequence (5'-3')	Amplicon size (bp)
Cat	Cyt b	Fwd Rev	GGAATAATGTTTCGACCACTAAGC TGCCTGAGATGGGTATTAGGAT	172
Dog	ATP 6	Fwd Rev	TGGCTCTAGCCGTTCGATTA AAGGCAACAGCAAATTCTAGG	163
Pig	ND5	Fwd Rev	CCATCCCAATTATAATATCCAACTC TGATTATTTCTTGGCCTGTGTGT	141
Monkey	ND5	Fwd Rev	TGAGACCTCCAACAAATACTAGC CTCTATGGCAGAAGGTAGTCAG	129
Rat	ATP 6	Fwd Rev	ATCATCAGAACGCCTTATTAGC AGGTTCGTCCTTTTGGTGTA	108

3.5 Simplex PCR Optimization

In a preliminary phase, species specificity of the designed primers was assessed separately using DNA extracted from cat, dog, pig, monkey and rat muscle tissues. PCR amplification was accomplished in a 25 µl total volume containing 0.5U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 5 µl of 5X GoTaq Flexi Buffer, 200 µM each of dNTP, 1.5 mM $MgCl_2$, 0.2 µM primers and 0.5 µl (20 ng/µl) of total DNA in ABI 96 Well verity thermal cycler (Applied Biosystems, Foster city, CA) and the cycling parameters were initial denaturation at 94 °C for 3 min following 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C, 62 °C, 60 °C, 62 °C, and 61 °C for cat, dog, pig, monkey and rat primers, respectively, for 30 s and elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min.

The amplified products of species-specific simplex PCR were visualized firstly on 2% agarose gel stained with Florosafe DNA stain (1st base Laboratories, Selangor, Malaysia) using a gel image documentation system (AlphaImager HP, California, USA) and secondly on gel-mage and electrochromatograms of Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Inc.) using Experion DNA 1K Analysis Kit (Bio-Rad Laboratories, Inc.). Typically, 1µl of diluted (50 ng/µl) PCR products was applied with 5µl of specialized buffer into each of the 11 sample wells and 9µl of gel-stain into gel-stain and priming wells of Experion DNA Chip. One microliter of DNA 1000 ladder containing 1500, 1000, 850, 700, 500, 400, 300, 200, 150, 100, 50, 25, and 15-bp marker DNAs was applied into the ladder well. The samples were vortex-mixed for 1 min at 2000 rpm and were immediately run on the bioanalyzer.

3.6 Multiplex PCR optimization

In the development of multiplex PCR for the detection of all five meat species targeted in this study, a duplex PCR was optimised for dog and rat, two triplex PCRs for dog, pig, rat; and cat, pig and rat, respectively. Then tetraplex PCR was carried out for cat, dog, monkey, and rat and finally multiplex PCR to detect cat, dog, pork, monkey and rat. All assays were performed in ABI thermal cycler (Applied Biosystems, Foster city, CA) in a 25 µl total volume containing 1U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 5 µl of 5X GoTaq Flexi Buffer, 200 µM each of dNTP, 1.5 mM $MgCl_2$, 2.5 µl (0.5 µl of 20 ng/µl for each species) of total DNA. The primer concentration and the cycling parameters are listed in Table 3.3. Negative control (PCR reaction mixture without template DNA) was included to ensure the purity of the PCR reaction mixture from contaminating DNA.

Table 3.3. Primer concentration and cycling parameters for multiplex PCR.

Target species	Primer concentration	Initial denaturation	35 cycles			Final elongation
			Denaturation	Anneal*	Elongation	
Dog and rat	0.1-0.2µM	94 °C for 3 min	94 °C for 30s	60 °C for 30s	72 °C for 30s	72 °C for 5 min
Dog, pork and rat	0.1- 0.4µM	94 °C for 3 min	94 °C for 30s	59 °C for 30s	72 °C for 30s	72 °C for 5 min
Cat, pork and rat	0.1- 0.3µM	94 °C for 3 min	94 °C for 30s	59 °C for 30s	72 °C for 30s	72 °C for 5 min
Cat, dog, monkey and rat	0.2-0.3µM	94 °C for 3 min	94 °C for 30s	60 °C for 30s	72 °C for 30s	72 °C for 5 min
Cat, dog, pork, monkey and rat	0.2-0.4µM	94 °C for 3 min	94 °C for 30s	59 °C for 30s	72 °C for 30s	72 °C for 5 min

* Similar annealing temperature was required for the proper amplification efficiency of all primers in the multiplex PCR.

CHAPTER 4. RESULTS

4.1 DNA Extraction

The OD_{260}:OD_{280} ratios of extracted DNA (Table 4.1) were between 1.7 and 2 which indicated a high quality of DNA in all specimens (Parchami Nejad et al., 2014).

Table 4.1. Concentration and purity of extracted DNA.

Name of species and food items	**Concentration of DNA (ng/μL)**	**Purity of DNA (OD_{260}:OD_{280})**
Target and non-target species at raw state:		
Dog (*Canis lupus famiiaris*)	133.6	1.8
Cat (*Felis catus*)	92.6	1.9
Rat (*Rattus rattus*)	145.1	1.8
Pig (*Sus scrofa*)	106.7	1.8
Monkey (*Macaca fascicularis*)	103.7	1.8
Beef (*Bos taurus*)	114.4	1.8
Chicken (*Gallus gallus*)	714.8	2.0
Goat (*Capra hiscus*)	192.1	1.8
Lamb (*Ovis aries*)	103.9	1.7
Buffalo (*Bubalus bubalis*)	94.82	1.8
Venison (*Odocoileus virginianus)*	139.4	1.8
Duck (*Anas platyhychos*)	251.8	1.9
Pigeon (*Columba livia*)	319.3	1.7
Salmon (*Salmo salar*)	234.1	1.9
Cod (*Gadus morhua*)	204.9	1.8
Tuna (*Thunnus orientalis*)	197.5	1.8
Carp (*Cyprinus carpio*)	124.1	1.7
Wheat (*Triticum aestivum*)	333.1	1.8
Tomato (*Solanum lycopersicum*)	159.3	1.7
Garlic (*Allium sativum*)	116.1	1.6
Onion (*Allium cepa*)	110.0	1.7
Pepper (*Capsicum annuum*)	103.6	1.7
Target species at heat treated state:		
Dog (*Canis lupus famiiaris*)	47.3	1.7
Cat (*Felis catus*)	66.8	1.6
Rat (*Rattus rattus*)	42.4	1.8
Pig (*Sus scrofa*)	55.1	1.6
Monkey (*Macaca fascicularis*)	21.3	1.7
Dummy ready-to-eat meat products:		
Beef meatball spiked with dog meat	416.0	1.67
Beef meatball spiked with cat meat	315.0	1.8
Beef meatball spiked with rat meat	250.0	1.8
Beef meatball spiked with pig meat	327.0	1.8
Beef meatball spiked with monkey meat	159.0	1.7
Beef meatball spiked with all target meats (raw)	232.0	1.8

Table 4.1. Continued.

Name of species and food items	Concentration of DNA (ng/μL)	Purity of DNA (OD_{260}:OD_{280})
Beef meatball spiked with all target meats (heat treated)	49.3	1.7
Chicken meatball spiked with dog meat	344.0	1.9
Chicken meatball spiked with cat meat	267.0	1.9
Chicken meatball spiked with rat meat	364.0	1.9
Chicken meatball spiked with pig meat	281.0	1.9
Chicken meatball spiked with monkey meat	130.0	1.8
Chicken meatball spiked with all target meats (raw)	293.0	2.0
Chicken meatball spiked with all target meats (heat treated)	64.5	2.0
Beef burger spiked with dog meat	416.0	1.9
Beef burger spiked with cat meat	351.0	1.8
Beef burger spiked with rat meat	181.0	1.7
Beef burger spiked with pig meat	883.0	1.8
Beef burger spiked with monkey meat	157.0	1.8
Beef burger spiked with all target meats (raw)	331.0	1.8
Beef burger spiked with all target meats (heat treated)	97.0	1.7
Chicken burger spiked with dog meat	421.0	1.8
Chicken burger spiked with cat meat	768.0	1.8
Chicken burger spiked with rat meat	955.0	1.7
Chicken burger spiked with pig meat	752.0	1.8
Chicken burger spiked with monkey meat	959.0	1.8
Chicken burger spiked with all target meats (raw)	394.0	1.8
Chicken burger spiked with all target meats (heat treated)	82.2	1.7
Beef frankfurter spiked with dog meat	798.0	1.7
Beef frankfurter spiked with cat meat	519.0	1.7
Beef frankfurter spiked with rat meat	491.0	1.7
Beef frankfurter spiked with pig meat	690.0	1.7
Beef frankfurter spiked with monkey meat	681.0	1.7
Beef frankfurter spiked with all target meats (raw)	275.0	1.7
Beef frankfurter spiked with all target meats (heat treated)	131.0	1.68
Chicken frankfurter spiked with dog meat	1269.0	1.7
Chicken frankfurter spiked with cat meat	392.0	1.7
Chicken frankfurter spiked with rat meat	495.0	1.7
Chicken frankfurter spiked with pig meat	373.0	1.69
Chicken frankfurter spiked with monkey meat	826.0	1.7
Chicken frankfurter spiked with all target meats (raw)	737.0	1.9
Chicken frankfurter spiked with all target meats (heat treated)	190.0	1.6

Table 4.1. Continued.

Name of species and food items	Concentration of DNA (ng/μL)	Purity of DNA (OD_{260}:OD_{280})
Commercial meat products:		
Beef meatball		
BM 1	473.0	1.6
BM 2	310.0	1.8
BM 3	278.0	1.8
BM 4	315.0	1.8
BM 5	176.0	1.6
Chicken meatball		
CM 1	337.0	1.9
CM 2	266.0	1.9
CM 3	291.0	1.9
CM 4	280.0	1.9
CM 5	135.0	1.9
Beef burger		
BFB 1	886.0	1.9
BFB 2	97.0	1.7
BFB 3	287.0	1.8
BFB 4	270.0	1.8
BFB 5	435.0	1.9
BFB 6	310.0	1.8
BFB 7	244.0	1.8
BFB 8	371.0	1.9
Chicken burger		
CKB 1	224.0	1.8
CKB 2	254.0	1.8
CKB 3	225.0	1.8
CKB 4	324.0	1.8
CKB 5	338.0	1.8
CKB 6	210.0	1.8
CKB 7	381.0	1.8
CKB 8	272.0	1.8
Beef frankfurter		
BFF 1	761.0	2.0
BFF 2	871.0	1.9
BFF 3	1042.0	1.7
BFF 4	1576.0	1.9
BFF 5	796.0	1.9
BFF 6	857.0	1.9
BFF 7	720.0	1.8
Chicken frankfurter		
CFF 1	221.0	1.7
CFF 2	253.0	1.7
CFF 3	284.0	1.7
CFF 4	241.0	1.7
CFF 5	209.0	1.7
CFF 6	287.0	1.7
CFF 7	249.0	1.7

4.2. Cross-Species Specificity of Designed Primers

In the elementary phase of this research, simplex PCRs were performed on DNA extracted from muscle tissues of each target species to verify the specificity of the designed primers. The species-specific primers amplified 172 bp, 163 bp, 141 bp, 129 bp, and 108 bp fragments from cat, dog, pig, monkey and rat DNA template, respectively. Each set of primers was checked against the meat extracted DNAs of twelve common meat-providing animal and fish species (beef, buffalo, goat, lamb, venison, chicken, duck, pigeon, cod, salmon, tuna, and carp) and five plant species (wheat, tomato, garlic, onion, pepper) commonly used for meat products preparation and no cross-species amplification was detected (Figures 4.1a-e). All assays were done in triplicates on three different days by three independent analysts to avoid any biasness. The results indicated no cross amplification even on repetition in blind experiments.

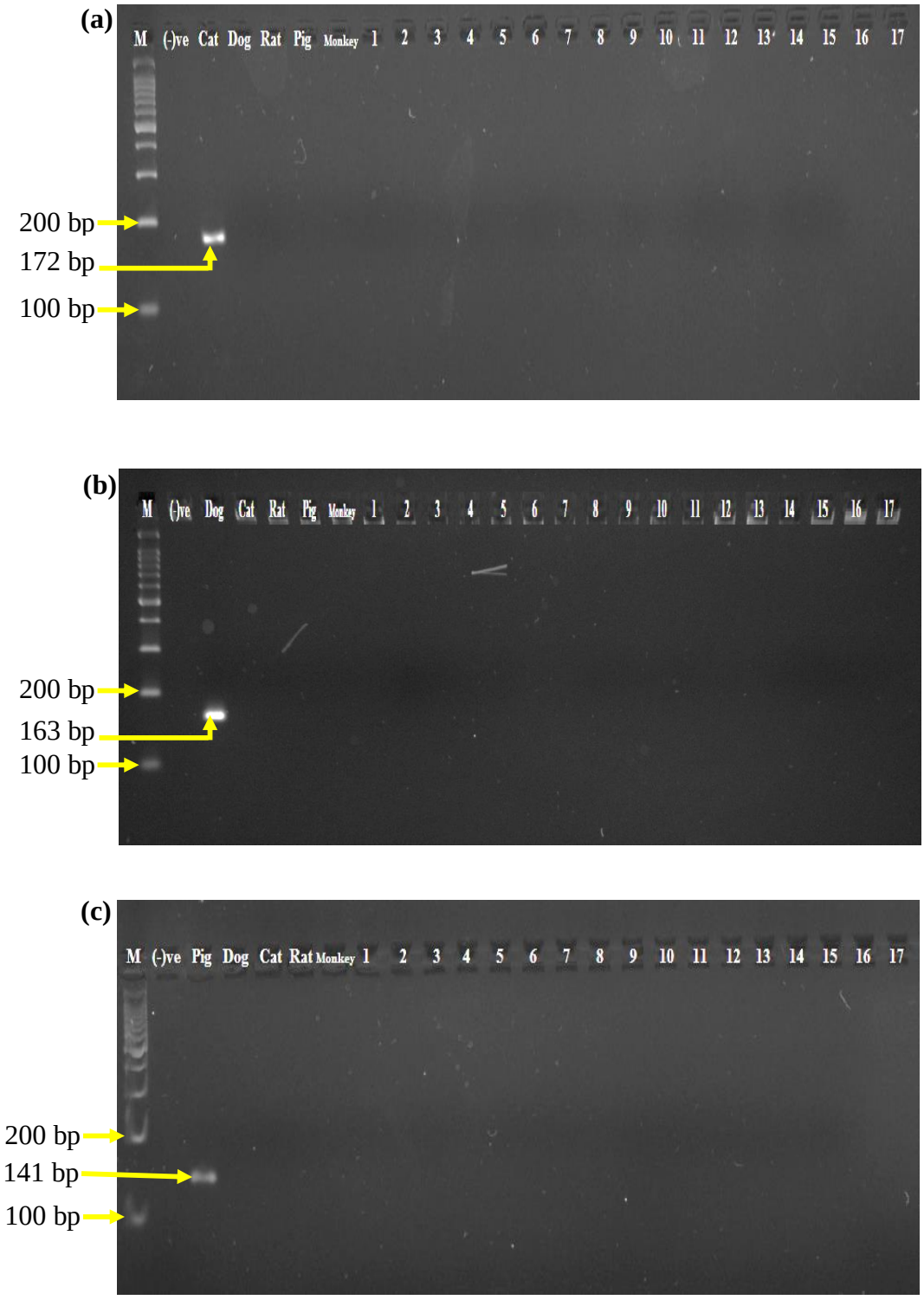

Figure 4.1. Cross-species specificity test of designed primers for cat **(a)**, dog **(b)**, pig **(c)**, monkey **(d)**, and rat **(e)** with non-target species. In the gel image **(a-e)**, M, 100bp DNA marker; lanes of respective target species are labelled specifically, and lanes 1-17, beef, buffalo, goat, lamb, venison, chicken, duck, pigeon, cod, salmon, tuna, carp, wheat, tomato, garlic, onion, and pepper respectively.

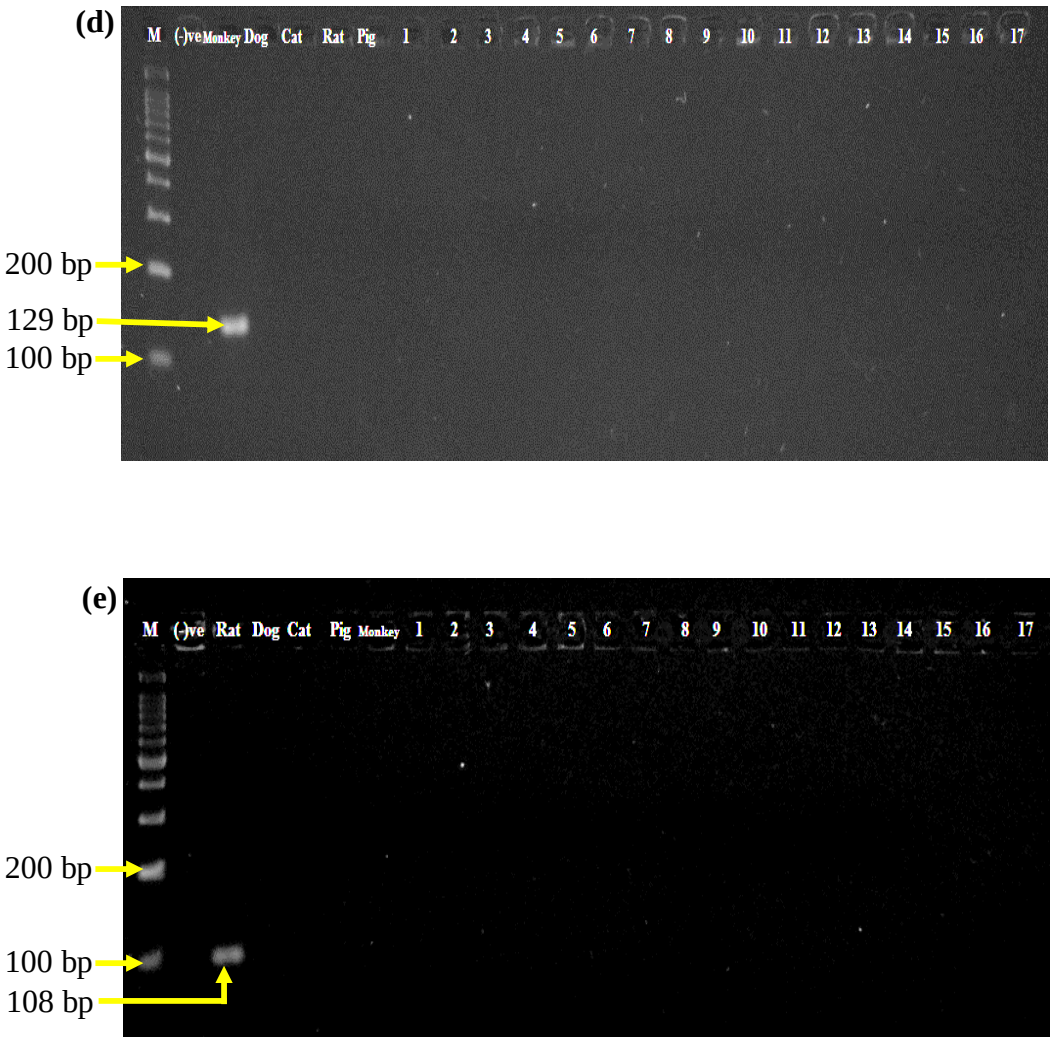

Figure 4.1. Continued.

4.3 Simplex and Multiplex PCR

The specificity of designed primers was assessed using simplex PCR assays for rat, monkey, pig, dog, and cat species (Figure 4.2, lanes 7-11). For the simultaneous detection of all species, a one-step-multiplex PCR was developed using five primer sets previously designed for the simplex PCR. The result of which might be seen in Figure 4.2. In the gel image lane 2 represents a duplex PCR for dog and rat; lane 3 and 4, two triplex PCRs for dog, pig, rat; and cat, pig and rat, respectively and tetraplex PCR was carried out for cat, dog, monkey, and rat (lane 5) and finally lane 6 showed multiplex PCR to detect cat, dog, pork, monkey and rat.

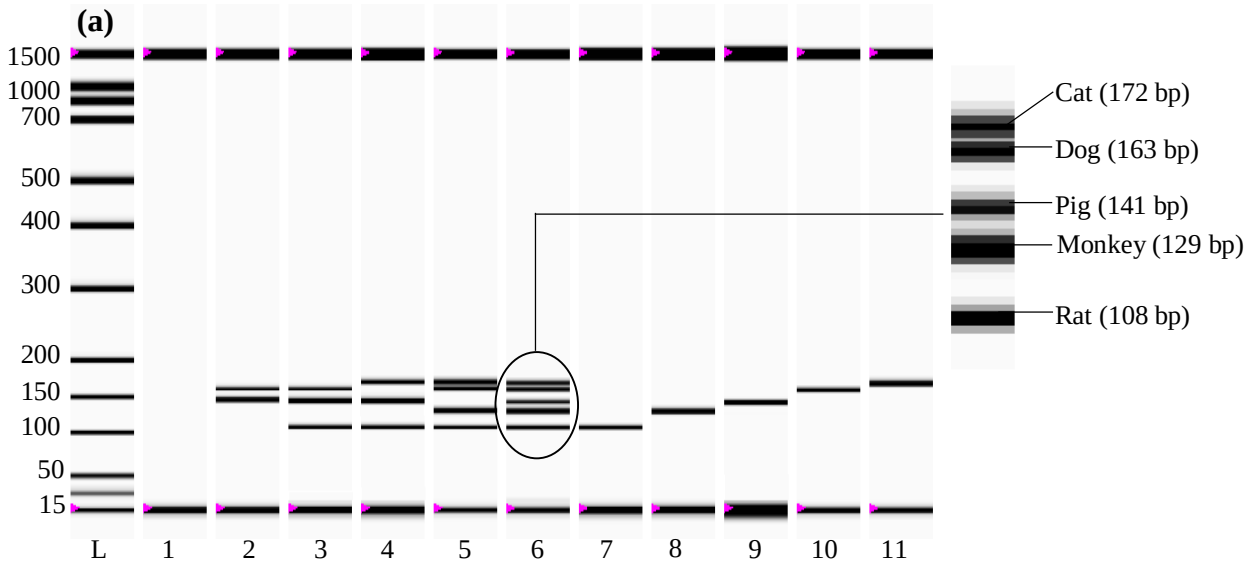

Figure 4.2. The gel image (**a**) and the electropherogram (**b**) of multiplex PCR. In the gel image: L, Ladder; lane 1, Negative control; lane 2, duplex PCR of dog (*Canis lupus familiaris*) and pig (*Sus scrofa*); lane 3, triplex PCR of dog (*Canis lupus familiaris*), pig (*Sus scrofa*), and rat (*Rattus rattus*); lane 4, triplex PCR of cat (*Felis catus*), pig (*Sus scrofa*), and rat (*Rattus rattus*); lane 5, tetraplex PCR of cat (*Felis catus*), dog (*Canis lupus familiaris*), monkey (*Macaca fascicularis*), and rat (*Rattus rattus*); lane 6, multiplex PCR of cat (*Canis lupus familiaris*), dog (*Canis lupus familiaris*), pig (*Sus scrofa*), monkey (*Macaca fascicularis*), and rat (*Rattus rattus*); lane 7, rat (*Rattus rattus*); lane 8, monkey (*Macaca fascicularis*); lane 9, pig (*Sus scrofa*); lane 10, dog (*Canis lupus familiaris*); and lane 11, cat (*Felis catus*). Corresponding electropherograms are demonstrated by respective labels.

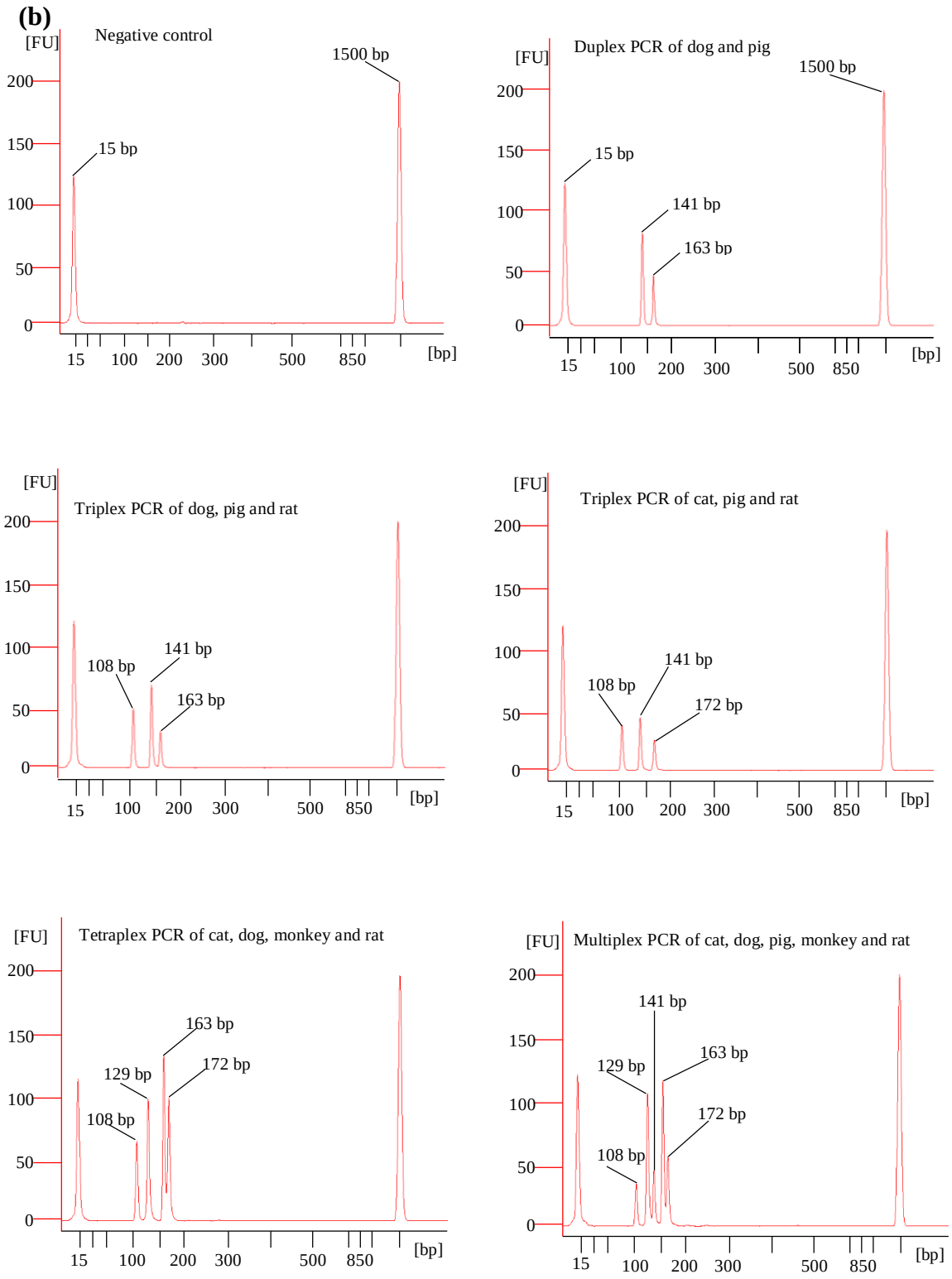

Figure 4.2. Continued.

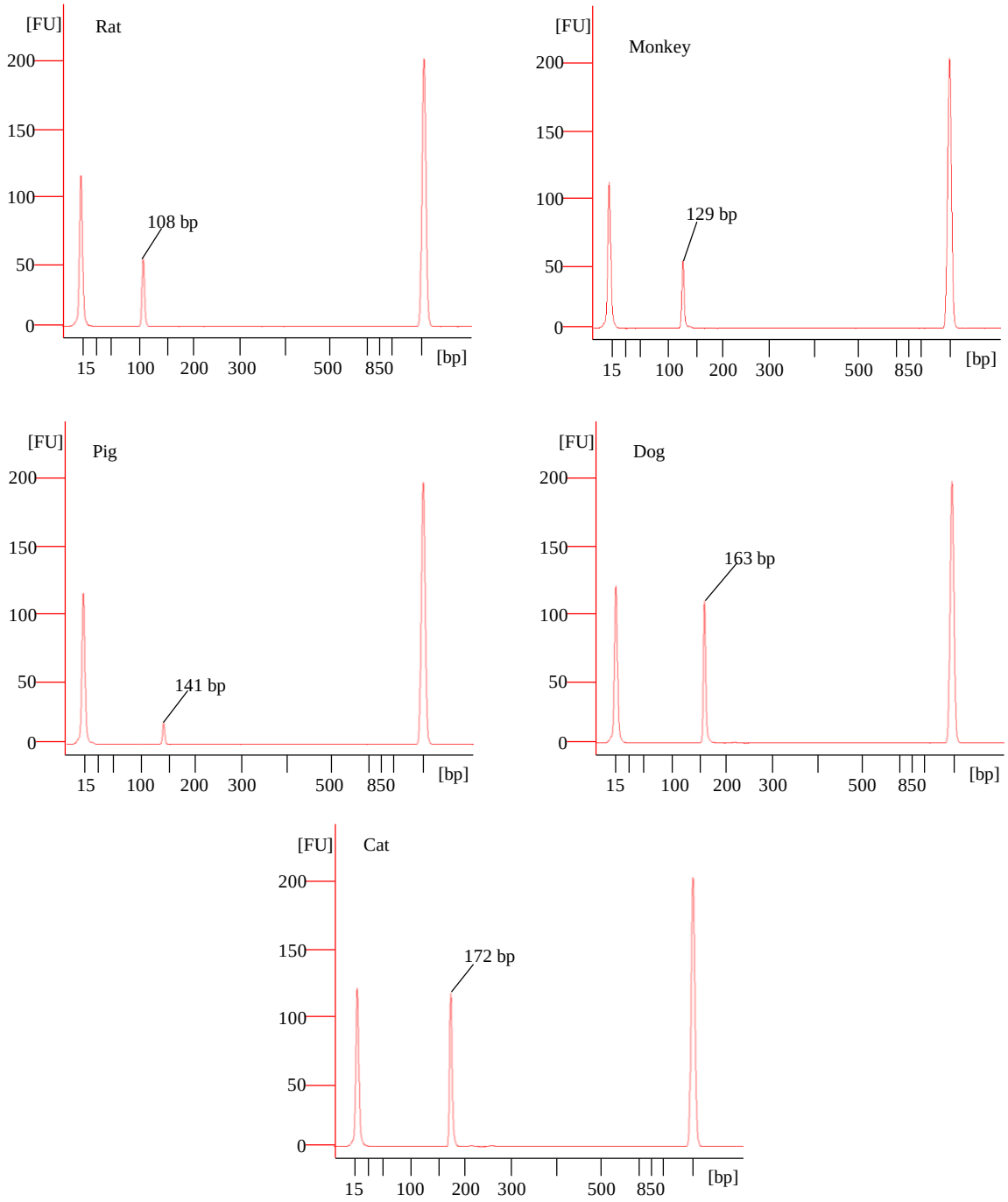

Figure 4.2. Continued.

4.4 Sensitivity of the Multiplex PCR

The sensitivity of the multiplex PCRs were performed in two methods. Firstly, by adding 10, 5, 1, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng of DNA template of each species in a common reaction mixture, and secondly by mixing target meat species in a portion of 1%, 0.5% and 0.1% with ready to eat beef and chicken meatballs, burgers and frankfurters (Safdar & Junejo, 2015). The DNA band patterns (Figure 4.3a; lanes 2-9) showed five bands corresponding to the five species. In lane 10, the bands for cat and pig were extremely faded but those for dog, monkey and rat were clearly observed. However, the electropherograms reflected only three peaks corresponding to dog, monkey and rat. So the limit of detection for cat and pig in dilution method was 0.02 ng and that for dog, monkey and rat was 0.01 ng. In case of second category sensitivity test, it is possible to detect 0.1% of target meat species (Figures 4.4-4.6; lane 9) in meat products formulations.

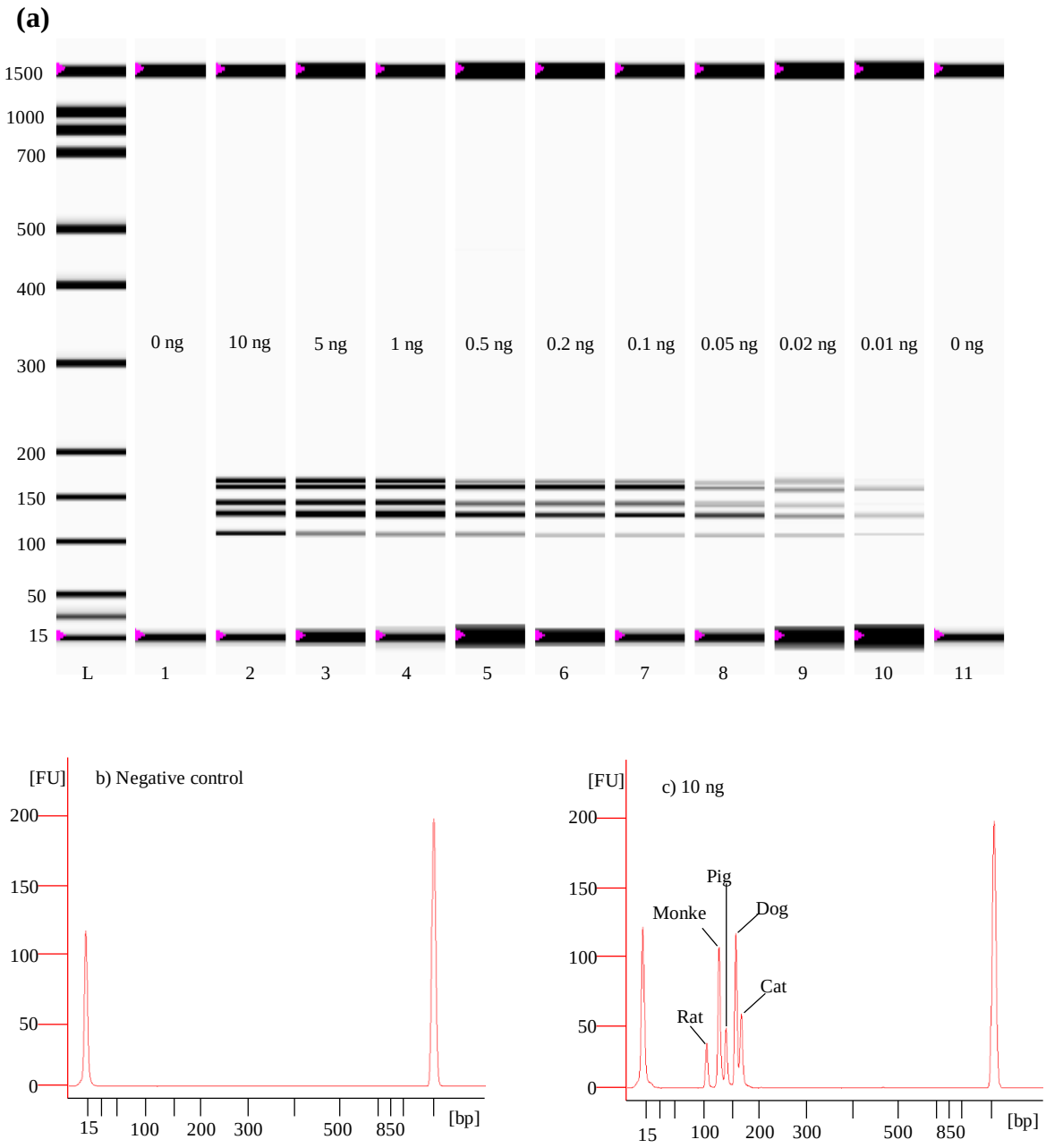

Figure 4.3. Sensitivity test of multiplex PCR. Shown are (**a**) gel image of PCR products (Lanes 1-11) obtained from 0, 10, 5, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 and 0 ng of DNA from five meat species (cat, dog, pig, monkey and rat). L for ladder and (b-l) are electropherograms for Lanes 1-11, respectively.

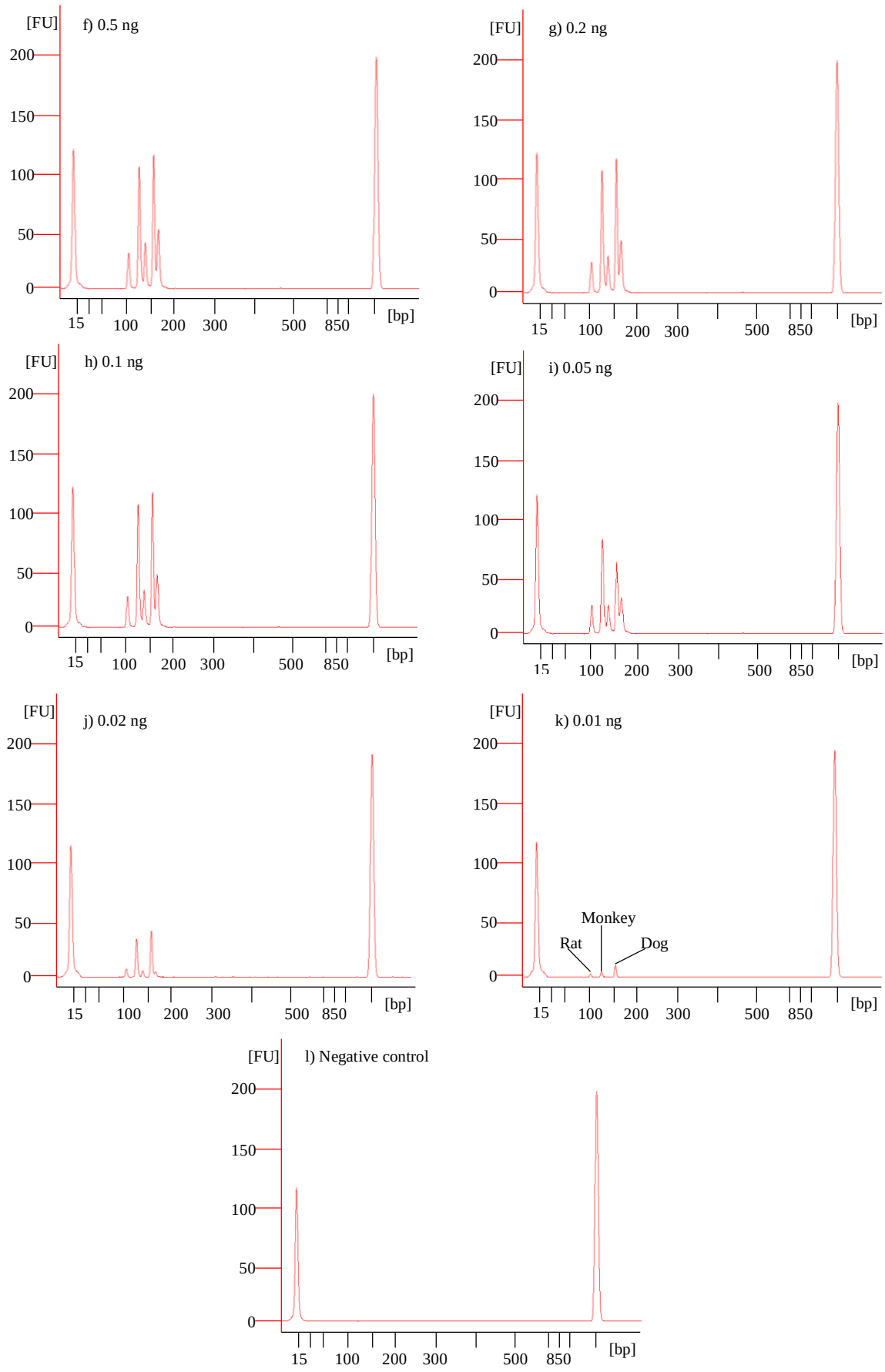

Figure 4.3. Continued.

4.5 Specificity and Sensitivity under Food Matrices

Primer specificity to other species was also examined through multiplex PCR optimized with DNA isolated from other non-target species and binary meat mixtures prepared in a form of beef and chicken meatballs, burgers and frankfurters as discussed in Section 3.2. The separation of multiplex PCR products was achieved within 30 min by the application of high voltage in the sieving polymer and specialized buffer in the microfluidic channels through independent electrodes for each well and the automated electrophoretic patterns as shown in Figures 4.4-4.6 of lanes 1-5 showed a single band of targeted PCR products from cat, pig, pig, monkey and rat meat species, respectively, without producing any fragment of non-specific amplification, indicating high species-specificity of the primers towards the target species.

(a)

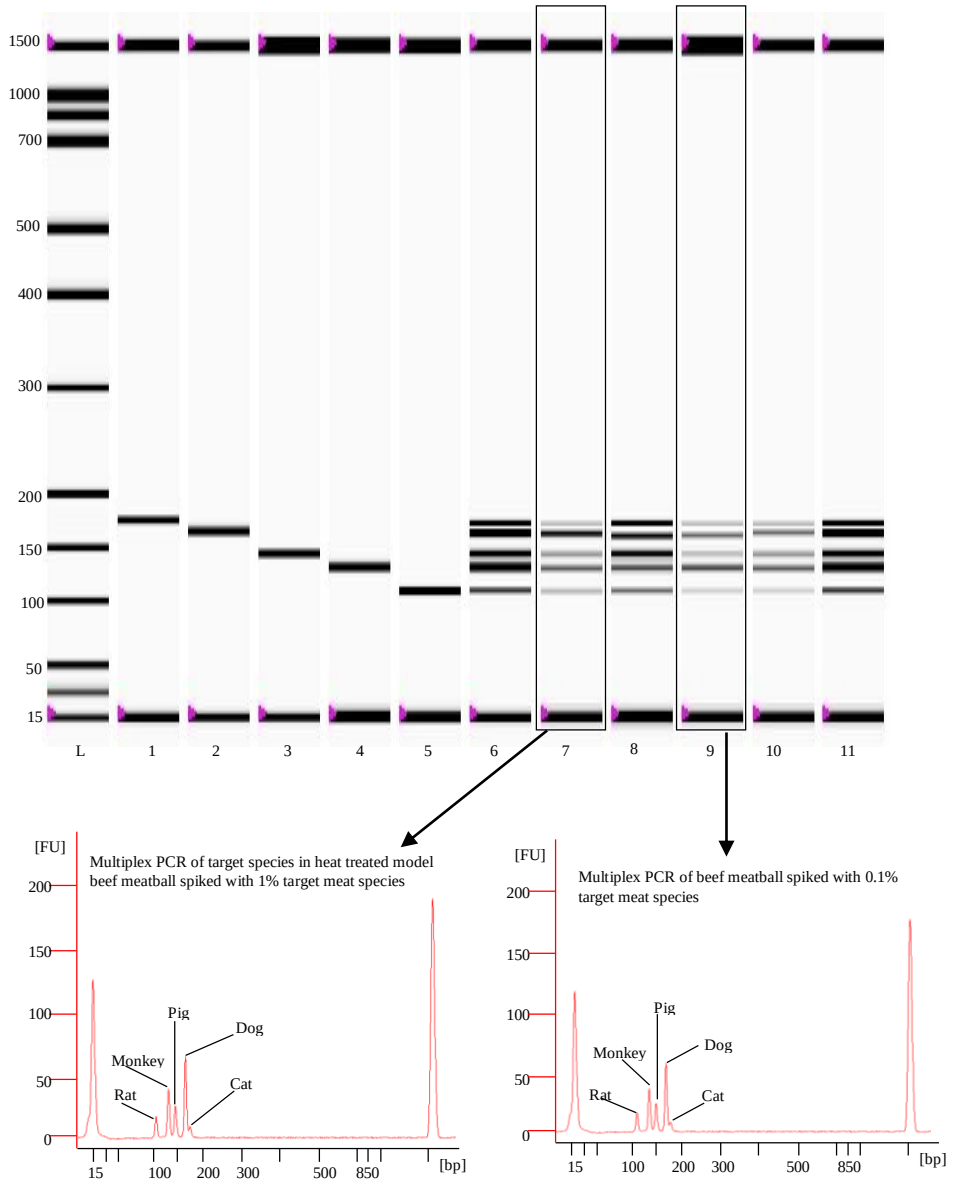

Figure 4.4. The gel image and the electropherograms of multiplex PCR (M-PCR) of beef **(a)** and chicken **(b)** meatball with sensitivity. In the gel images; *L*, Ladder; *Lanes 1-5*, species-specific PCR with multiplex optimization to detect cat (*Felis catus*), dog (*Canis lupus familiaris*), pig (*Sus scrofa*), monkey (*Macaca fascicularis*), and rat (*Rattus* rattus) DNA respectively, spiked with in beef and chicken meatball; *Lane 6*, multiplex PCR to detect five target species in beef and chicken meatball in raw state and *Lane 7*, in heat treated (121 °C for 2.5 hours) states; *Lanes 8-9*, M-PCR of beef and chicken meatball spiked with 0.5 and 0.1% target meat species respectively; *Lane 10 of figure **(a)***, M-PCR of heat-treated (121 °C for 2.5 hours) target meat species and ; *Lane 10* of figure **(b)** and *11* of figure **(a)**, positive control (M-PCR of beef and chicken meatball in raw state); *Lane 11* of figure **(b)**, negative control. Electropherograms of *Lane 7* and *9* show the detection

of five meat species in heat-treated state and from 0.1% spiked target meat species, respectively.

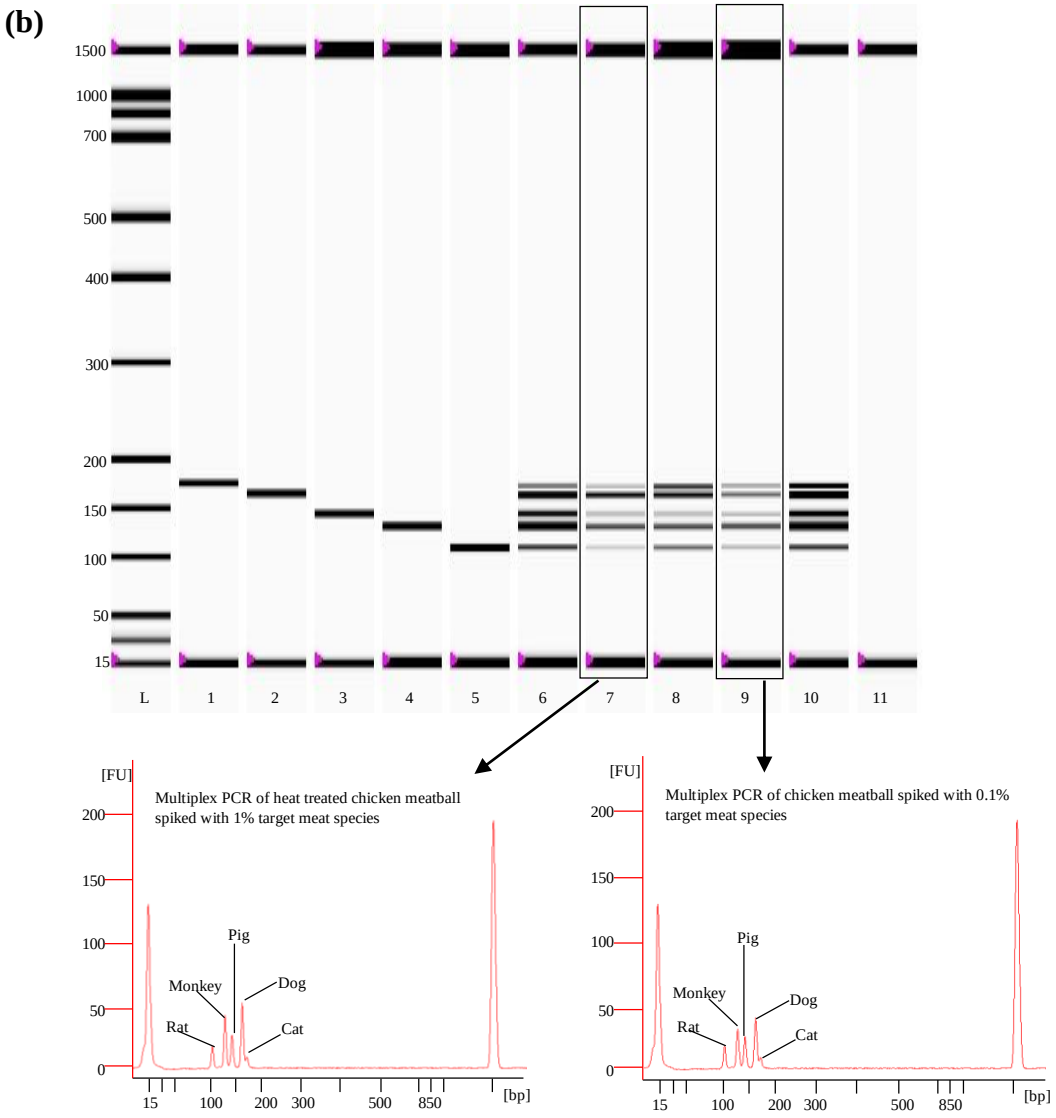

Figure 4.4. Continued.

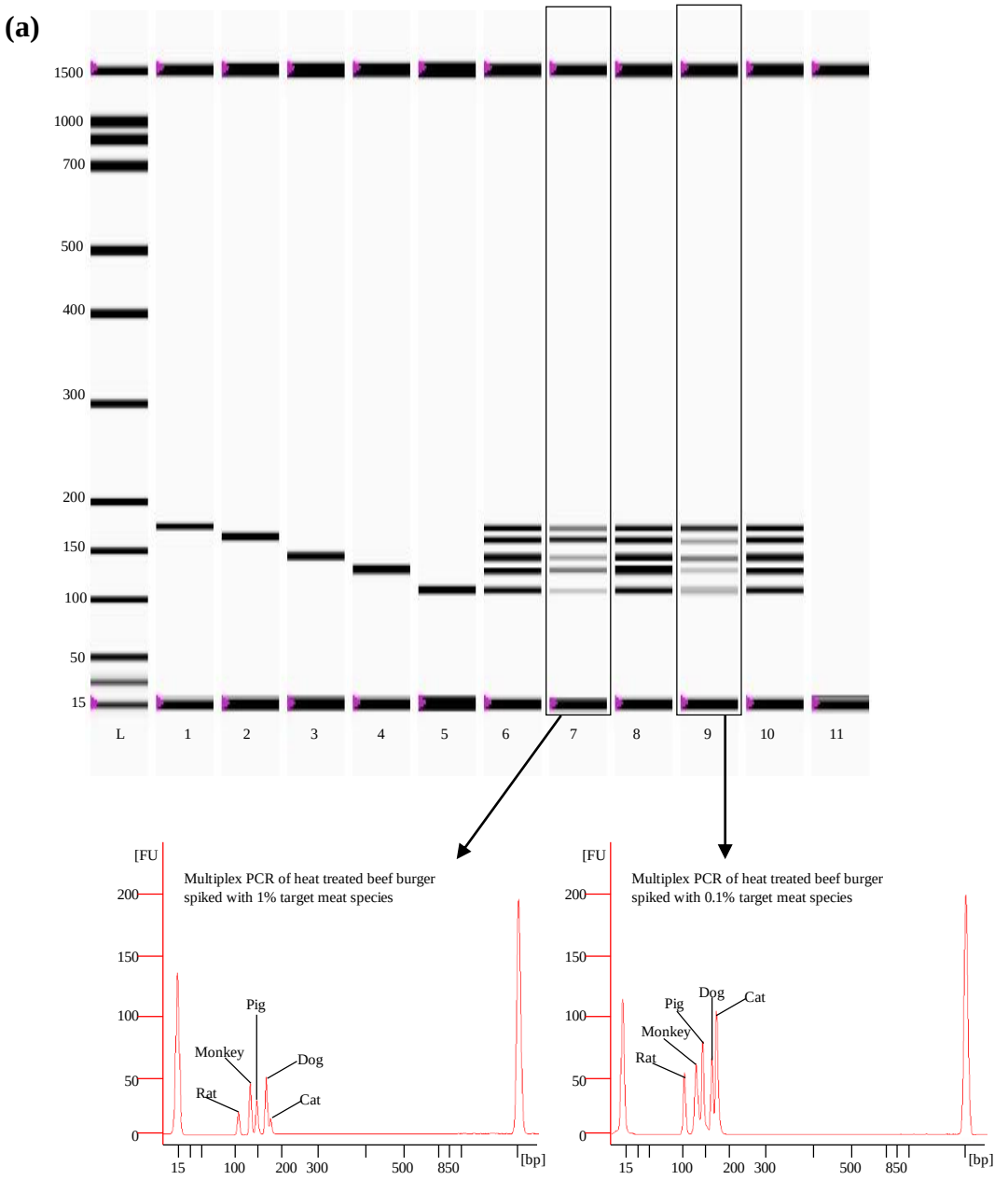

Figure 4.5. The gel image and the electropherograms of multiplex PCR (M-PCR) of beef **(a)** and chicken **(b)** burger with sensitivity. In the gel image: *L*, Ladder; *Lanes 1-5*, species-specific PCR with multiplex optimization to detect cat (*Felis catus*), dog (*Canis lupus familiaris*), pig (*Sus scrofa*), monkey (*Macaca fascicularis*), and rat (*Rattus* rattus) DNA respectively, spiked with 1% target meat individually in beef and chicken burger; *Lane 6*, multiplex PCR to detect five target species in beef and chicken burger in raw state and *Lane 7*, in heat treated (121 °C for 2.5 hours) state; *Lanes 8-9*, M-PCR of beef and chicken burger spiked with 0.5 and 0.1% target meat species respectively; *Lane 10*, positive control (M-PCR of beef and chicken burger in raw state); *Lane 11*, negative control (M-PCR with no template). Electropherograms of *Lane 7* and *9* show the detection

of five meat species in heat-treated state and from 0.1% spiked target meat species, respectively.

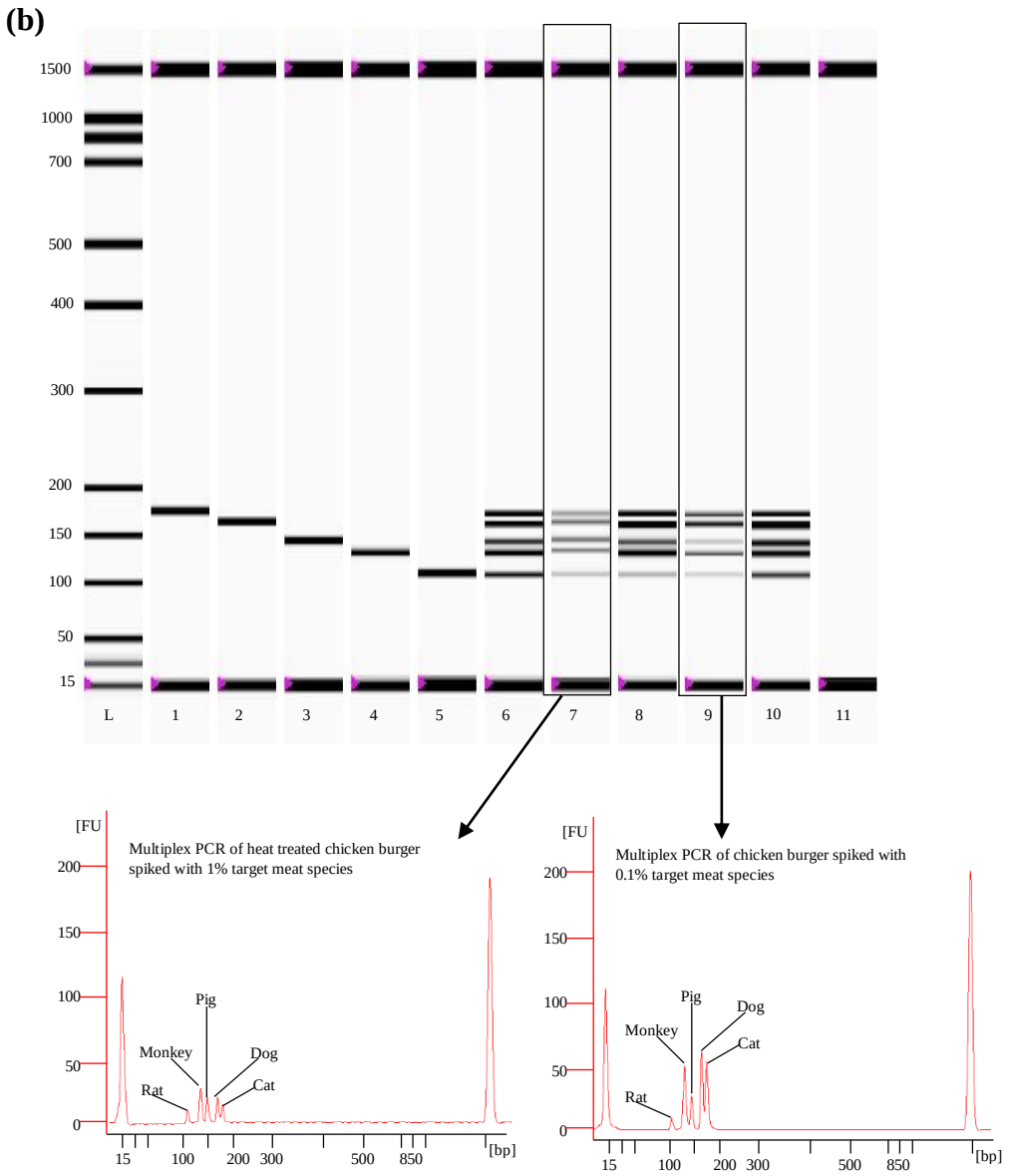

Figure 4.5. Continued.

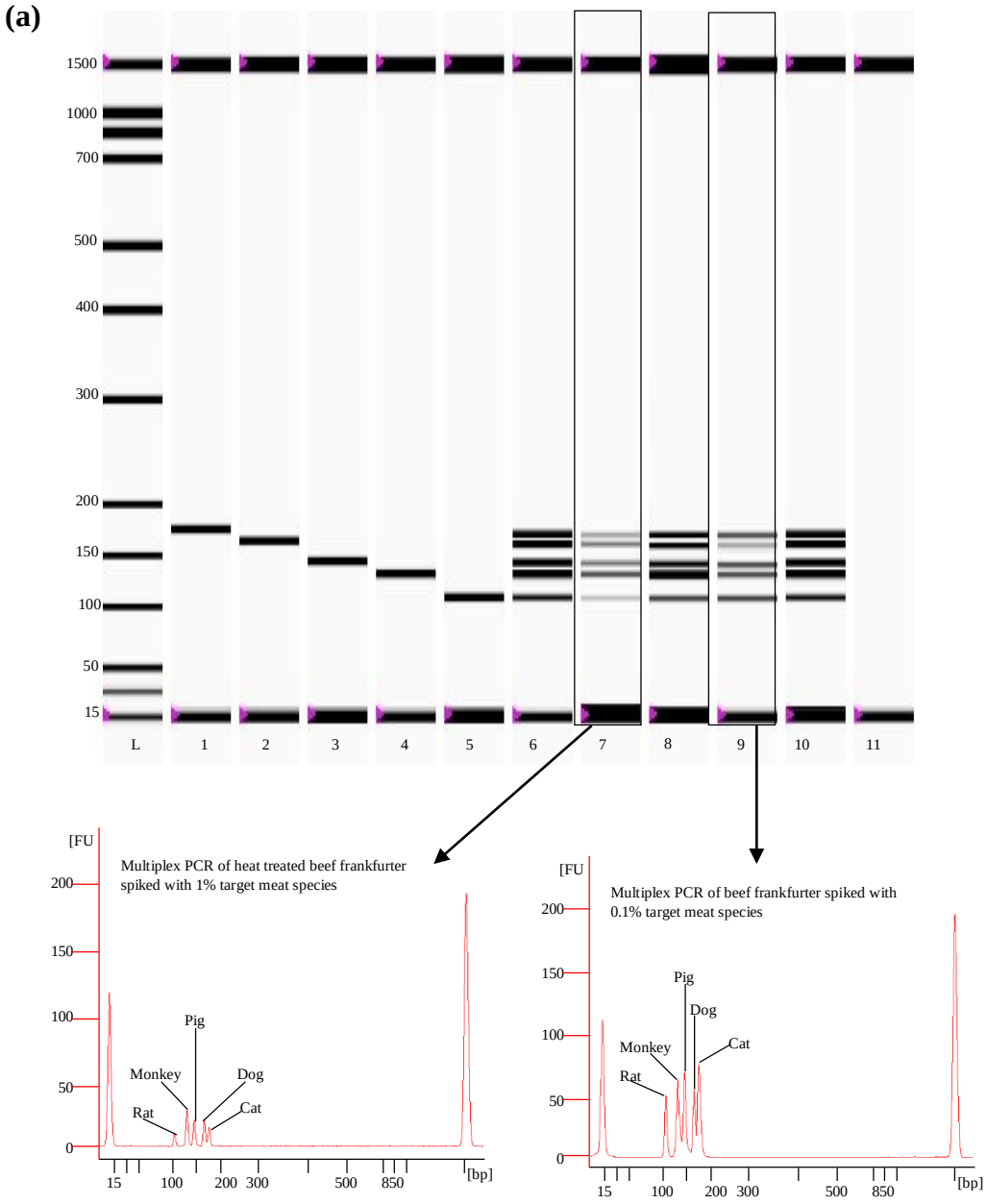

Figure 4.6. The gel image and the electropherograms of multiplex PCR (M-PCR) of beef **(a)** and chicken **(b)** frankfurter with sensitivity. In the gel image: *L*, Ladder; *Lanes 1-5*, species-specific PCR with multiplex optimization to detect cat (*Felis catus*), dog (*Canis lupus familiaris*), pig (*Sus scrofa*), monkey (*Macaca fascicularis*), and rat (*Rattus* rattus) DNA respectively, spiked with 1% target meat individually in beef and chicken frankfurter; *Lane 6*, multiplex PCR to detect five target species in beef and chicken frankfurter in raw state and *Lane 7*, in heat treated (121 °C for 2.5 hours) state; *Lanes 8-9*, M-PCR of beef and chicken frankfurter spiked with 0.5 and 0.1% target meat species respectively; *Lane 10*, positive control (M-PCR of beef and chicken frankfurter in raw state); *Lane 11*, negative control (M-PCR with no template). Electropherograms of *Lane*

7 and *9* show the detection of five meat species in heat-treated state and from 0.1% spiked target meat species, respectively.

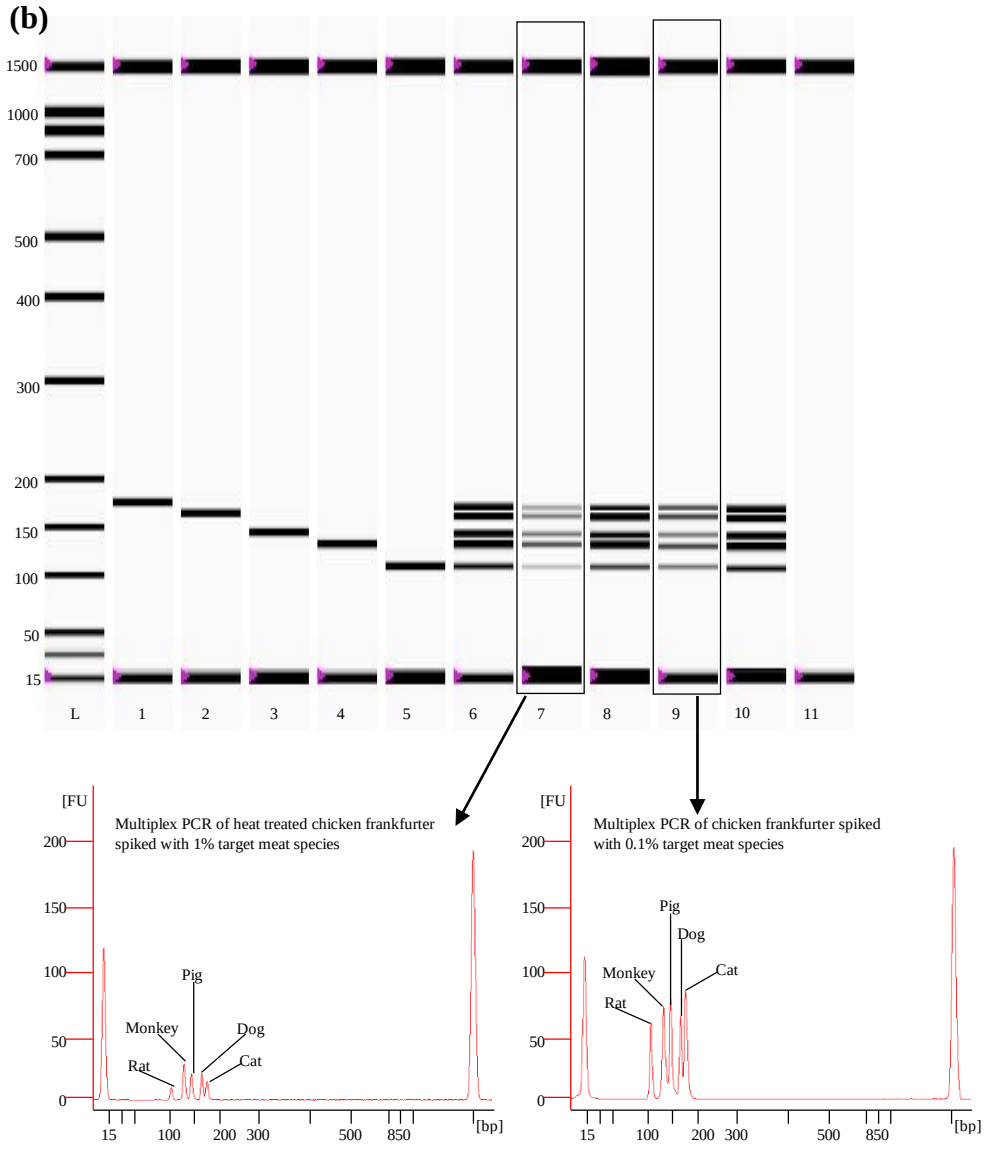

Figure 4.6. Continued.

4.6 Reliability of Multiplex PCR

Although different multiplex PCR assays were previously proposed for meat species detection (Matsunaga et al., 1999; Di Pinto et al., 2005; Ghovvati et al., 2009; Zha et al., 2010, 2011; Hou et al., 2014; Kitpipit et al., 2014; Safdar & Junejo, 2015) none of them have been tested for multiple commercial meat products. Here, I analysed the developed assay performance under dummy chicken and beef meatball, burger, and frankfurter formulation. The statistical data of tested samples are given in Table 4.11 and experimental results of both raw and heat-treated (121 °C, 45 psi for 2.5 h) states (electrophoretic gel image and electropherograms) are presented in Figures 4.4-4.6. In case of heat treated meat detection, firstly species-specific PCR with multiplex optimization has been performed (Figure 4.7, lane 7-11), secondly, multiplex PCR assay has carried out to detect five heat treated meat species simultaneously (Figure 4.3a, lane 10), and finally heat treated dummy meat products have analysed to detect the said species from meatballs, burgers and frankfurters formulations (Figure 4.4-4.6, lane 7). While all model meatballs with deliberate adulterations were positively detected target species in blind experiment, all commercial samples were found with negative targets. All of these experiments have done in triplicates, by three different analysts in three different days to avoid biasness.

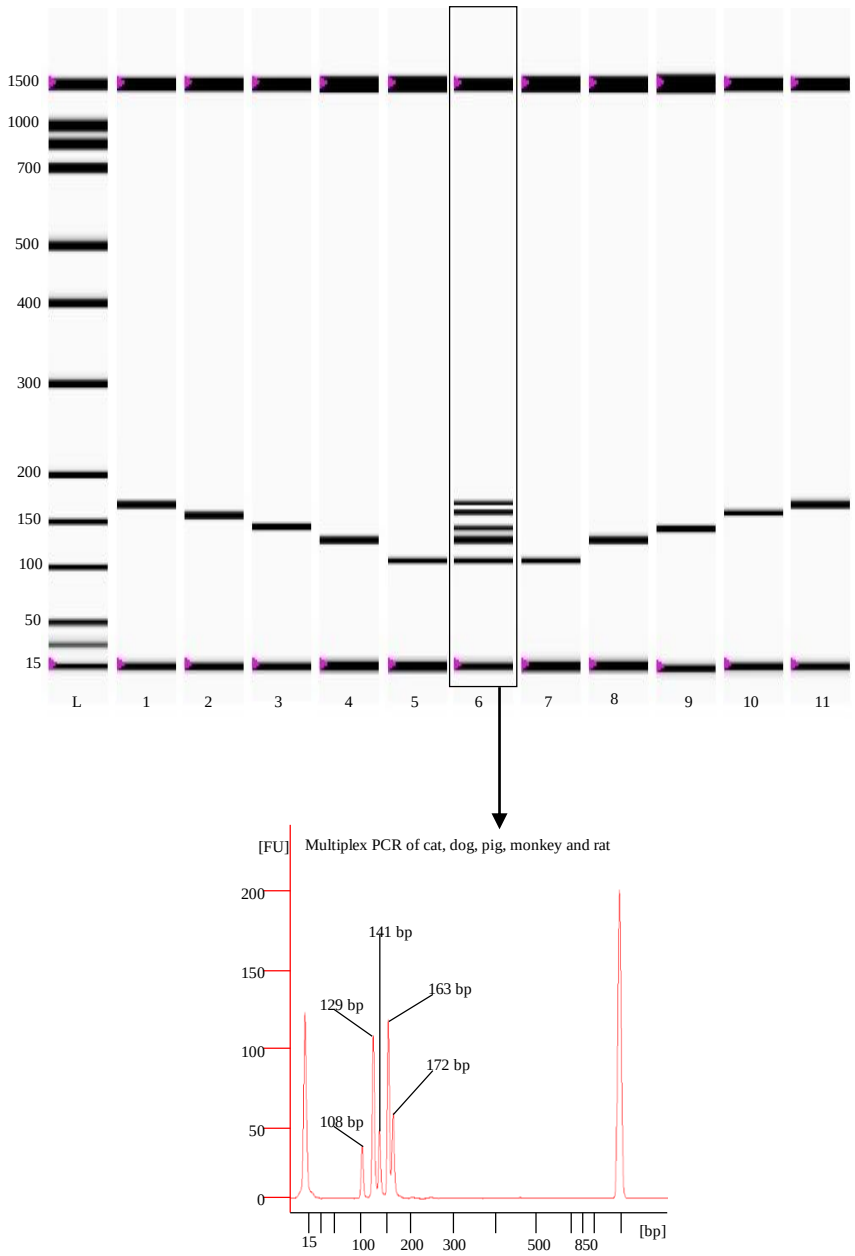

Figure 4.7. The gel image and the electropherograms of species-specific simplex and multiplex PCR. In the gel image: *L*, Ladder; *Lane 1-5*, species-specific simplex PCR of cat (*Felis catus*), dog (*Canis lupus familiaris*), pig (*Sus scrofa*), monkey (*Macaca fascicularis*), and rat (*Rattus* rattus) DNA from raw meat; *Lane 6*, multiplex PCR for five target meat species; *Lane 7-11*, species-specific simplex PCR of heat treated (121 °C for 2.5 h) rat (*Rattus* rattus), monkey (*Macaca fascicularis*), pig (*Sus scrofa*), dog (*Canis lupus familiaris*), and cat (*Felis catus*) meat DNA, respectively. The electropherogram of *Lane 6* has demonstrated by respective labels.

Table 4.2. Wide screening of model and commercial ready to eat meat products sold in markets using developed multiplex PCR.

Meat products	Detected species					PCR accuracy (%)
	Pig	Dog	Cat	Monkey	Rat	
Different meat products spiked with						
Pig meat	54/54	-	-	-	-	100
Dog meat	-	54/54	-	-	-	100
Cat meat	-	-	54/54	-	-	100
Monkey meat	-	-	-	54/54	-	100
Rat meat	-	-	-	-	54/54	100
Beef meatballs spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100
Beef meatballs spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Chicken meatballs spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100
Chicken meatballs spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Beef burgers spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100
Beef burgers spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Chicken burgers spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100
Chicken burgers spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Beef frankfurter spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100

Table 4.2. Continued

Meat products	Detected species					PCR accuracy (%)
	Pig	Dog	Cat	Monkey	Rat	
Beef frankfurter spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Chicken frankfurter spiked with all target meat species (raw)	9/9	9/9	9/9	9/9	9/9	100
Chicken frankfurter spiked with all target meat species (heat-treated)	9/9	9/9	9/9	9/9	9/9	100
Commercial meat products*						
Beef meatball	0/9	0/9	0/9	0/9	0/9	100
Chicken meatball	0/9	0/9	0/9	0/9	0/9	100
Beef burger	0/9	0/9	0/9	0/9	0/9	100
Chicken burger	0/9	0/9	0/9	0/9	0/9	100
Beef frankfurter	0/9	0/9	0/9	0/9	0/9	100
Chicken frankfurter	0/9	0/9	0/9	0/9	0/9	100

* 5 different brands of each beef and chicken meatballs, 8 burgers, and 7 frankfurter items were analysed to validate the developed multiplex PCR assay. The numerator and denominator of each fraction denote the number of positive detection and total number of samples analysed using the multiplex PCR assay.

CHAPTER 5. DISCUSSIONS AND CONCLUSION

Since the short-length nucleic acid targets are extraordinarily stable under food processing conditions and mitochondrial genes are present in multiple copies (Ali et al., 2012a, Rahman et al., 2014; 2015), the assay increased the chances of target detection even in the degraded and extremely processed meats and food products (Zha et al., 2011). In this study, five pair of species-specific primers targeting the intra-species conserved and interspecies hyper variable regions of mitochondrial ND5, ATP 6 and cyt b genes were designed to amplify short-length amplicons of the range 108-172 bp. The primer specificity and melting temperature (*Tm*) are very crucial in the development of multiplex PCR since its success depends on the ability of the primers to be selectively annealed with their respective targets under a single set of PCR conditions, including reaction volume, cycling and annealing (Matsunaga et al., 1999; Ali et al., 2014). Thus the primer design is very significant step in multiplex PCR development and it must contain adequate intra-species conserved sequences and inter-species polymorphism with closely related *Tm*. It has been quoted and implicated in several reports that the efficiency of the PCR assays might reduce or end up with failure in amplification in presence of single mismatches in the primer binding regions (Smith et al., 2002; Wu et al., 2009; Ali et al., 2013; Rahman et al., 2014, 2015). Similarly, having 15% mismatch between designed primers and non-target species is sufficient in multiplex PCR (Sambrook et al., 1989; Matsunaga et al., 1999), the primers designed here contained 7-15 bp (23-45%) mismatches with other relevant species (Appendix B, Table 1S-5S) and very closely spaced *Tm* (61-62 °C). These ensure primers' annealing only with the DNA template of target species and not with any non-target species (Matsunaga et al., 1999). Moreover, primer annealing at a higher temperature increases specificity and eliminates nonspecific hybridization (Ali

et al., 2012a). Therefore, an optimized high annealing temperature (Table 3.3), as determined through repeated run of gradient PCR, was used for the amplification of the selected region of mitochondrial genes from raw, heat treated, admix, and commercial samples. Pairwise distance analysis of primer sequences (Tables 4.9-4.13) against 15 animal and five plant species computed using the maximum composite likelihood method (Tamura et al., 2011) revealed a minimum distance between monkey and pigeon and maximum distance between rat and tuna reflecting very high genetic distance and very little or no probability of cross-amplification of non-target species (Ali et al., 2013).

The specificity of the each pair of the designed primers was checked using species-specific simplex PCR, one by one and also against the twelve meat-providing and expensive terrestrial (beef, chicken, goat, lamb, buffalo, venison, duck, and pigeon) and aquatic species (salmon, cod, tuna, and carp) and five plant materials (wheat, tomato, garlic, onion, and pepper) and no cross-amplification was detected. When cross-species specificity was tested using optimized multiplex PCR system developed in this study, only specific target was amplified (Figure 4.4-4.6, lanes 1-5), suggesting the stringent specificity of the designed primers.

After passing through the simplex PCR assays, duplex, triplex, tetraplex and multiplex PCR systems were optimized by successively adding dog and pork; dog, pig and rat; cat, pig and rat; cat, dog, monkey and rat and finally all the five target species (cat, dog, pig, monkey and rat) as shown in Figure 4.2a. This eliminated the probability of potential primer dimer or multimers formation (Matsunaga et al., 1999; Zhang, 2013). The products of the duplex and triplex PCR were well-separated in agarose gel but agarose gel eventually failed to separate the products of the tetraplex and pentaplex PCR systems since it cannot resolute DNAs of less than 40–50 bp difference in length

(Dooley et al., 2005; Bottero & Dalmasso, 2011; Chen et al., 2014). On the other hand, complex DNA banding patterns can be analysed by microchip-based capillary electrophoresis technology, in which the gel electrophoresis step is replaced by an automated Lab-on-a-Chip micro-fluidic electrophoretic system and offers better accuracy, precision, resolution (~10 bp) with minimum consumption of samples and time (Ali et al., 2011a; Chen et al., 2014; Rashid et al., 2015). Since the differences of amplicon lengths for cat and dog, and pig and monkey, were 9 and 12 bp, respectively, I used Bio-Rad Experion Automated Electrophoresis Station which provided automated banding patterns (Figure 4.2a) along with the electropherograms (Figure 4.2b) for all of the five targets amplified in this multiplex systems.

The sensitivity of the multiplex system was checked using template DNA dilution methods (Matsunaga et al., 1999) as shown in Figure 4.3 (gel-image and electropherograms). Figure 4.3a shows that the bands for cat and pig appeared as a smear and it was difficult to detect when 0.01 ng DNA templates (lane 10) were used. These bands were so thin that they remained undetected in ectropherogram. Thus the limit of detection (LOD) for cat and pig was 0.02 ng. However, both gel-image (Lane 10 in Figure 4.3a) and electropherograms clearly detected dog, monkey and rat when 0.01 ng DNA from each species were used as template. Thus the LOD for dog, monkey and rat was 0.01 ng DNA. However, higher sensitivity might be achieved for cat and pig specific primers through simplex PCR assay.

Previously reported works were carried out on cattle, chicken, goat, sheep, deer, lamb, ostrich, pork and horse meat with amplicon-sizes between 200 and 450 bp (Matsunaga et al., 1999; Zha et al., 2011; Zhang, 2013; Kitpipit et al., 2014). The LOD of the assays documented by Matsunaga et al. (1999) and Di Pinto et al. (2005) was

0.25 ng, whereas, Dalmasso et al. (2004) 0.0025-0.025 ng. Recently, Zhang (2013) obtained enhanced sensitivity up to 0.001 ng by semi-nested multiplex PCR and shortening primers for the detection of chicken, beef, pork and mutton. However, semi-nested multiplex PCR is more time consuming, expensive and cumbersome since the PCR has to be performed first by using a pair of common primers to amplify a product which is used as a template for the multiplex PCR. Additionally, the shortened primers hardly meet identical efficiency for different templates and frequently fail to identify the exact species. Most recently, Kitpipit et al. (2014) proposed a direct multiplex PCR assay for the detection of six meat species, namely, pork, lamb, chicken, ostrich, horse, and beef with a detection limit of 7-21 fg. Parchami Nejad et al. (2014) achieved 0.05 ng of limit of detection with simplex PCR to detect chicken, donkey, camel, goat and cattle by multiplex PCR. Hou et al. (2014) obtained the same LOD (0.05 ng) for the detection of chicken, duck and goose. For deer meat detection by multiplex PCR, Zha et al. (2011) achieved 0.02-0.5 ng sensitivity and Eung Soo et al. (2011) obtained with 0.05-1 ng DNA template. Thus, the limit of detection of the current study is higher compared to previously documented works. Similarly, the variation in detection sensitivity in multiplex PCR assays is a normal phenomenon and it varies from species to species (Table 2.3). Moreover, the substitution of lower valued meats with higher priced one is mainly taking place to gain economic profits and mixing less than 1% of low priced meats are not profitable so detecting 1% adulteration is enough to prove the sensitivity and reliability of multiplex PCR assays (Hou et al., 2014; Ali et al., 2015b). But some researchers have carried out multiplex PCR to detect 0.1% adulteration (Safdar et al., 2014; Safdar & Junejo, 2015), so here we also tested the sensitivity of developed assay with 0.5 and 0.1% adulteration in the form of meat products (Figure 4.4-4.6, lanes 8-9). Additionally, previously developed

multiplex PCRs were tested only one food items (Hou et al., 2014; Parchami Nejad et al., 2014; Safdar et al., 2014; Safdar & Junejo, 2015), and here six in a total of dummy ready to eat beef and chicken meatballs, burgers and frankfurters were tested to validate the assay. Furthermore, detection of five meat species from extensive heat treated (121 °C, 45 psi, 2.5 h) samples where DNA would be extremely degraded (Ali et al., 2012a, 2013) proved the stringent reliability of the developed assay.

The primers of the previously developed multiplex PCR assays targeted relatively longer amplicons (≥200 bp) and in most of the cases, the assays were not tested under food processing conditions and complex matrices of commercial meat products. Recent studies appreciated that ≤150 bp amplicons have a higher chance of survival in degraded samples and thus offer superior sensitivity and method validity (Dooley et al., 2004; Rojas et al., 2011; Cammà et al., 2012; Ali et al., 2012a, 2013; Rahman et al., 2014). Lin and Hwang (2008) documented that highly degraded DNA could not be detected if ≥300 bp amplicons are used. Recently, Ali et al. (2015a) produced experimental evidence that short amplicon-length PCR assay is not only more stable and reliable but also more sensitive than previously published long amplicon-length PCR assays. Furthermore, earlier multiplex PCR assays included only pig and horse meats among the haram (not allowed) meat species (Matsunaga et al., 1999; Di Pindo et al., 2005; Kitpipit et al., 2014; Safdar et al., 2014), which have limited scope in halal authentication. Here I developed a multiplex PCR system targeting short-length amplicons (108-172 bp) to detect five most potential haram meat species having health risk and wider scope in food traceability studies. This assay was proven to be robust, reproducible and sensitive to detect as low as 0.01-0.02 ng DNA of the targeted species (Figure 4.3). In addition to novel target detection, the limit of detection of this assay was comparable to simplex and higher than previously

documented multiplex PCR assays for other species (Zha et al., 2010; 2011; Hou et al., 2014). Due to the higher prices and extreme popularity of beef and chicken meatballs, burgers, and frankfurters (Rohman et al., 2011; Ali et al., 2012a; Rahman et al., 2014; Rahmania et al., 2015), the substitution of beef and chicken in these meat products with lower valued meats such as pig, dog, cat, rat and/or monkey meats were suspected (Rohman et al., 2011; Rahman et al., 2014) and consequently we tested the developed assay in these meat products authentication. In addition to deliberately adulterated model meat products, five different brands of commercial beef and chicken meatballs, burgers, and frankfurters (Table 4.11) under raw and heat treated conditions were blindly tested (Figure 4.4-4.6). The positive targets were detected only in model meat products with 1%, 0.5% and 0.1% target adulteration but no contamination was found in commercial ready to eat meat products.

The findings of this study of non-fraudulent labelling of pig, dog, rat, cat, and monkey in commercial products was against the conventional wisdom due to fraudulent labelling is quite common throughout the globe (Doosti et al., 2014; Fajardo et al., 2010). It is because of Malaysian government is dedicated to develop “Halal Hub” in local and international arenas (Talib et al., 2008) and has been strictly monitoring local markets time to time. Therefore, this study was in the line with the government policy, and I found the validity and applicability of this assay for the detection of cat, dog, pig, monkey, and rat tissues both in raw and processed commercial products. Similarly, the findings suggesting the halal sanctity of Malaysian meatballs, burgers and frankfurters.

5.1 Conclusion

Multiple targets detection in a single assay platform is a technological challenge but has potential to significantly cut analysis cost and time since the primers need to be selectively annealed with their respective targets under a single set of PCR conditions (reaction volume and cycling conditions) with high sensitivity and offers easy analysis of food components in the industry and retail outlets. Carefully designed species-specific PCR under optimized conditions is conclusive to detect and identify species, eliminating the need of restriction digestion and/or sequencing of PCR products. However, most of the documented multiplex assays have used long amplicon targets which have limited stability under extreme conditions. Moreover, previously developed multiplex PCRs only included pig and horse as non-halal species which constrains the applications of the assays in multiple haram meat detection.

Here I developed multiplex PCR system with self-designed species-specific primers targeting short-length amplicons (108-172 bp) of mitochondrial genes, whose stability were proven under extreme food processing treatments such as boiling and autoclaving. The assay successfully detected five non-halal meat species, namely, cat, dog, monkey, pig and rat with a detection limit of 0.01-0.02 ng DNA under raw, and 0.1% (w/w) meats under admixed and heat-treated samples, showing its clear appeal in halal food industry and halal regulatory bodies.

The developed assay was adapted and validated for the analysis of processed foods such as meatballs, burgers and frankfurters with 0.1% target meat adulteration. Such an assay is a first time report for multiple haram meat authentication under pure, raw and mixed states as well as processed foods. As well pentaplexing, the assay could be used in simplex, duplex, triplex, tetraplex and multiplex PCR systems based on

requirements. Thus, could be recommended for the detection of animal tissue by food control agencies or laboratories as a reliable and practical method for the determination of technically inevitable contamination and/or intentional admixtures in highly processed meat products.

In addition, the potential of the real-time procedure to detect and quantify small amounts of DNA in raw and heat treated meats may make it a useful tool for inspection programs not only in halal but also in all food industry.

5.2 Recommendations for future work

- Real-time PCR assays are automated and more sensitive. Although intercalating probes were designed, a multiplex real-time PCR assay development could not be completed due to time constraints. Future works could explore this area with much interest.
- Three commercially available and dummy food items have tested both in raw and processed states, more food products can be examine to prepare a matrix-adapted reference material for preventing false positive detection under mixed matrices in multiplex real-time PCR.
- The use of internal positive control would add assay reliability and eliminate the chances of false negative detection in species-specific simplex PCRs. However, it was avoided in multiplex PCR due to primer proximity and complexity with pig and monkey primers. Further optimization might be done using an appropriate universal endogenous control for potential species in future.

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APPENDICES

Appendix A

Related Patent and Publications

a) Patent:

Ali, M.E., **Razzak, M.A.,** Hamid, S.B.A. (2014). FaithGuard Halal Detection Kit- Patent file no. PIC/P/654/14/UM/EAQUB/768-PCR KIT.

b) Journal Papers:

Ali, M.E., **Razzak, M.A.,** Hamid, S.B.A., Rahman, M.M., Amin, M.A., Rashid, N.R.A., Asing. (2015). Multiplex PCR Assay for the Detection of Five Meat Species Forbidden in Islamic Foods. *Food Chemistry*. 177:214-224. (**ISI IF 3.33, Scopus, Quartile 1**).

Ali, M.E., **Razzak, M.A.,** & Hamid, S.B.A. (2014). Multiplex PCR in Species Authentication: Probability and Prospects—A Review. *Food Analytical Methods*. 7:1933-1949. (**ISI IF 1.96, Scopus, Quartile 2**).

Rahman, M.M., Ali, M.E., Hamid, S.B.A., Bhassu, S., Mustafa, S., Amin, M.A., **Razzak, M.A.** (2015). Lab-on-a-Chip PCR-RFLP Assay for the Detection of Canine DNA in Burger Formulations. *Food Analytical Methods*. DOI 10.1007/s12161-015-0090-1. (**Accepted ISI IF 1.96, Scopus, Quartile 2**).

c) Conference Proceedings:

Ali, M.E., **Razzak, M.A.,** Hamid, S.B.A. (2014). Development of PCR-Targets for Multiplex PCR Assay. International Conference on Food Innovation-2014. Organized by Universiti Sains Malaysia (USM), Penang, Malaysia.

Ali, M.E., **Razzak, M.A.,** Hamid, S.B.A. (2014). Multiplex Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Assay for the Detection of Five Haram Meat Species. IUFoST World Congress of Food Science and Technology and EXPO, Montreal Convention Center, Montreal, Canada.

Ali, M.E., **Razzak, M.A.,** Hamid, S.B.A. (2014). Systematic Development of Multiplex Polymerase Chain Reaction Assay for the Detection of Five Haram Meat Species. BIT's 2nd Annual World Congress of Nutrition and Health- 2014. Taiyuan, China.

Amin, M.A., Ali, M.E., Hamid, S.B.A., **Razzak, M.A.** (2014). Primer design targeting mitochondrial ND5 gene for cat meat detection in halal foods. International Conference on Food Innovation-2014. Organized by Universiti Sains Malaysia (USM), Penang, Malaysia.

Abstract and front page of published articles:

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Multiplex PCR in Species Authentication: Probability and Prospects—A Review

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Abstract Food forgery is one of the most articulated socio-economic concerns which contributed to increase people's awareness on what they eat and how and where it is produced. Consumers are anxious about the consequences of food falsification on their choices, religious rituals, health, and hard-earned fortunes. The recent scandals of horse and rat meats in Europe and China have given us a brainstorming apprehension on the detection, differentiation, and identification of meat products. To restore consumers' trust and protect wildlife in natural habitats, researchers and policy-making and policy-implementing authorities have massively monitored all steps in the production of foods and food materials. Analytical approaches based on lipids, proteins, and DNA have been proposed for the authentication of meat species under pure and complex matrices. However, protein and lipid-based methods are less effective since the target biomarkers could be modified throughout the processing treatments. On the other hand, DNA-based species identification schemes have gained wider acceptance and reliability because of the superior stability and universality of DNA in all tissues and cells. We systematically presented here major species detection schemes with special emphasis on multiplex polymerase chain reaction (PCR) of both end-point and real-time platforms. We believe this short but comprehensive review would serve as a reference guide for the developers and users of multiplex PCR and others DNA-based techniques.

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Keywords Food forgery · Lipid · Protein- and DNA-based biomarkers · Multiplex PCR

Introduction

It is a universal desire of all that foods should be compliant with the consumers' lifestyle, culture, religion, diet, and health. The world is increasingly getting busier, and to keep up pace with its increasing work-volumes, a growing number of people are being increasingly forced to spend more time in their working places. These growing numbers of population do not have enough time to make their own meals and are being acquiesced to accept whatever they could have from a nearby restaurant or grocery. Thus, the demands and prospects of restaurant business and ready-made foods, such as burgers, pizzas, hot dogs, sandwiches, soups, cookies, candies, and creams, are at the growing spree (Ali et al. 2012c; van der Spiegel et al. 2012). However, the consumers' concern over ingredients and quality of packaged and ready-made foods are not being abated. This is because of the zoonotic threats, religious prohibition, food allergy, and fraudulent labeling of various food brands with materials of certain animal origins (Ali et al. 2012c; Dalvit et al. 2007; Nakyinsige et al. 2012a). Ecological, environmental, and wildlife protection are some of the other factors that have been added over the years (Opara and Mazaud 2001).

In 2010, beef consumption in Europe was drastically fallen because of bovine spongiform encephalopathy (BSE), avian and swine influenza, and contamination with toxic dioxin (Bottero and and Dalmasso 2011). Researchers believe that the most fatal and infectious disease, HIV/AIDS, has come to human race from an African chimpanzee meat infected with simian immunodeficiency virus (Fajardo et al. 2010). Religious rituals are also one of the prominent issues determining food avoidance, taboos, and special regulation with

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Analytical Methods

Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods

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ABSTRACT

Food falsification has direct impact on public health, religious faith, fair-trades and wildlife. For the first time, here we described a multiplex polymerase chain reaction assay for the accurate identification of five meat species forbidden in Islamic foods in a single assay platform. Five pairs of species-specific primers were designed targeting mitochondrial *ND5*, *ATPase 6*, and *cytochrome b* genes to amplify 172, 163, 141, 129 and 108 bp DNA fragments from cat, dog, pig, monkey and rat meats, respectively. All PCR products were identified in gel-images and electrochromatograms obtained from Experion Bioanalyzer. Species-specificity checking against 15 important meat and fish and 5 plant species detected no cross-species amplification. Screening of target species in model and commercial meatballs reflected its application to detect target species in process foods. The assay was tested to detect 0.01–0.02 ng DNA under raw states and 1% suspected meats in meatball formulation.

1. Introduction

Authentication of declared components in foods is an ever increasing public demand and a key priority in policy making and regulatory bodies. It helps to safeguard public health, consumers' lifestyle, food choice, religious faith, fair-trade economy and wildlife in natural habitats (Fajardo, González, Rojas, García, & Martín, 2010; Hou et al., 2014; Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Kesmen, Celebi, Güllüce, & Yetim, 2013). The turnover of halal food items has crossed USD 661 billion (Ali et al., 2012) and has been expanding rapidly even among the non-Muslim consumers because of its perceived quality attributes and significantly reduced risk to be a carrier of zoonotic diseases (Gregory, 2008; Mohamad, El Sheikha, Mustafa, & Mokhtar, 2013). Perceiving the huge opportunities of the halal food markets, even the European food industries have been investing in the production of halal foods (van der Spiegel et al., 2012). For the Muslims, choices of foods depend on the "Halal" (means allowed) attributes enshrined by the Qur'an and holy prophet Mohammed (peace be upon him) and Islamic fiqah (opinions of a team of Islamic scholars) (Nakyinsige, Man, & Sazili, 2012). It is more hygienic than the conventional foods since it must meet both the religious and health requirements. Consequently, the prices of halal foods are significantly higher than the conventional ones and thus it offers a significant scope of adulteration (illegal mixing of low-priced items).

The Islamic law prohibits Muslims from eating pork and ingredients derived from animals having canine teeth or fangs such as dog, cat, monkey and rat. However, in certain countries such as Vietnam, Switzerland, Tahiti, Mexico, South Korea, Taiwan and some parts of the United States, these animals have been consumed for ages (Ali et al., 2013). Additionally, these animals could be obtained without any offered prices and hence there is a significant chance of mixing them in halal foods (Rahman et al., 2014). The recent horse meat scandal in Europe and pig and rat meat scandal in China (Ali, Razzak, & Hamid, 2014) have put Muslim consumers in red alert in determining the presence of prohibited ingredients in marketed foods. Moreover, some of the species such as rat, cat, dog and monkey are potential carrier of several zoonotic diseases such as plague, ringworm, hydrophobia (rabies), and herpes virus (*Herpevirus simiae*), simian virus 40 (SV40) (Conly & Johnston, 2008; Fajardo et al., 2010) and thus they are not safe and hygienic for public consumption.

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Lab-on-a-Chip PCR-RFLP Assay for the Detection of Canine DNA in Burger Formulations

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Abstract Canine species detection in foods is important in the perspectives of health, religions, and fare-trade food business. This study describes a very short-amplicon length Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) assay with lab-on-a-chip detection platform for the authentication of canine DNA in processed foods. A 100-bp fragment of canine mitochondrial Cytochrome b (cytb) gene was selected and amplified using a pair of canine-specific primers. The amplified PCR products were validated by RFLP analysis using lab-on-a-chip microfluidic bioanalyzer kit. Both gel-image and electropherograms authenticated the canine-specific PCR products before (100 bp) and after restriction digestion (51, 30, and 19 bp). The assay successfully detected 0.0001-ng canine DNA under pure state and 0.01 % (*w*/*w*) canine meat spiked in chicken and beef burger formulations. Screening of eight commercial burgers across Malaysia did not reveal any canine adulteration. We believe the assay would find potential applications in food industries, Halal food regulatory bodies and animal right protection authorities across the globe.

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Introduction

It is a long-term envision of human civilization that food will be compliant with health, religions, culture, and age (Ali et al. 2014). While elders are concerned of healthy foods, younger often run after taste, appearance, and availability (Nam et al. 2010). In contrast to the consumer ages, religions often play a key role in controlling the consumption, preparation, processing, and purchasing of foods (Bonne and Verbeke 2008; Nam et al. 2010). Most religions have food taboos, for example, pork is not allowed to be consumed for the followers of Islam and Judaism (Bonne and Verbeke 2008). In Islam, meats of the ritually slaughtered animals with split hoof such as sheep, cattle, buffalo, and goat are allowed, but those of the carnivores with sharp teeth such as dog and cat are forbidden to be consumed (Khattak et al. 2011).

The term "Halal" is an Arabic word which defines the permitted things for the Muslims by the Islamic law drawn from the divine book of Quran and the compilation of Prophet Muhammad (Hadith). The "Halal" status of processed foods cannot be verified by consumers using organoleptic test or even after consumption since the processing treatments significantly modify the organophysical biomarkers, making the physical identification extremely difficult (Bonne and Verbeke 2008). Therefore, "Halal" logo on food products are trusted by the consumers, and it authenticates the halal status of the food and its ingredients. Due to huge demand (US$700 billion annually) and higher price of Halal foods, fraudulent labelling of halal brands are frequently taking place (Ali et al.

Appendix B

Table 1S. The mismatch comparison of the pig specific forward and reverse primers against commercially important species.

Forward	C	C	A	T	C	C	C	A	A	T	T	A	T	A	A	T	A	T	C	C	A	A	C	T	C	Number of Mismatch	Reverse	A	C	A	C	A	C	A	G	G	C	C	A	A	G	A	A	A	T	A	A	T	C	A	Number of Mismatch
Pig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	Pig	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Dog	.	T	T	.	G	.	.	C	.	.	C	.	.	T	.	G	T	.	A	.	T	C	T	A	.	13	Dog	C	T	T	.	T	.	G	.	A	.	.	T	G	.	C	.	.	.	G	.	.	.	.	10
Cat	T	.	C	.	G	.	.	C	.	.	C	.	.	T	C	.	.	A	.	T	G	T	.	A	.	8	Cat	C	T	C	.	T	A	.	.	.	A	.	.	C	.	.	.	G	C	.	.	.	T	.	10
Rat	.	A	.	C	A	.	.	.	.	.	C	.	.	T	.	.	T	.	.	T	.	T	G	A	.	10	Rat	T	T	.	.	T	A	C	A	A	T	A	C	.	.	.	.	T	A	T	.	.	A	.	14
Monkey	.	.	C	.	T	.	.	.	.	.	.	T	.	T	G	C	C	A	.	.	C	T	.	A	T	12	Monkey	C	.	T	.	C	T	.	A	A	.	G	.	.	T	.	.	.	C	.	.	.	C	.	.	10
Cow	.	T	.	.	A	.	.	C	.	A	.	G	.	.	T	.	.	A	T	A	.	G	.	.	T	11	Cow	C	A	.	G	T	.	.	C	.	.	.	.	G	.	.	.	C	.	.	.	.	T	.	8
Buffalo	.	A	T	.	A	.	.	C	.	.	C	.	.	.	.	.	.	A	.	A	.	G	.	C	T	10	Buffalo	C	.	.	T	.	G	.	.	.	A	G	.	.	A	C	.	T	.	.	G	.	.	.	8
Sheep	.	.	.	.	A	.	.	C	.	.	C	G	C	.	G	C	.	A	T	.	.	.	T	.	T	11	Sheep	C	T	.	.	G	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	6
Deer	.	T	.	.	.	.	.	C	.	.	C	.	.	.	.	C	T	A	.	A	.	G	T	.	.	9	Deer	T	.	C	T	.	.	T	.	A	.	.	.	G	.	.	.	.	.	.	.	.	T	.	7
Chicken	.	.	C	C	T	A	T	T	.	.	C	C	.	T	C	C	C	C	.	.	C	T	.	C	T	17	Chicken	C	.	.	.	T	.	.	.	.	G	G	C	.	.	.	.	.	G	C	.	.	T	G	9
Duck	.	T	C	C	A	A	T	C	.	.	C	C	.	C	C	C	.	C	T	.	C	T	.	.	T	17	Duck	C	T	.	.	T	.	C	.	.	A	.	T	.	.	.	.	T	C	C	.	.	T	.	10
Salmon	A	T	C	C	T	.	T	T	.	.	.	.	C	T	.	C	C	C	T	T	.	.	.	C	.	15	Salmon	.	G	.	.	C	A	.	.	.	G	A	C	C	.	.	.	.	C	.	.	.	T	G	10
Tuna	A	.	C	C	T	G	T	G	T	.	C	.	C	.	.	C	C	C	T	.	T	C	.	C	.	17	Tuna	.	A	.	.	G	A	G	.	.	G	G	C	C	.	.	.	G	C	C	.	.	T	.	12
Cod	A	.	C	C	A	.	T	T	T	.	A	G	.	.	.	C	C	.	T	T	.	C	A	.	.	15	Cod	.	A	G	.	T	.	T	.	.	T	A	T	.	.	.	G	G	C	T	G	.	A	.	13
Horse	.	.	C	.	.	.	.	.	.	.	C	.	.	.	T	.	.	G	.	A	.	T	.	.	T	7	Horse	T	.	G	.	T	.	T	.	.	A	.	.	.	C	.	.	.	C	.	.	.	T	.	8
Donkey	.	T	C	.	A	.	.	.	.	.	C	.	.	.	T	C	.	.	.	A	.	T	.	C	T	10	Donkey	T	.	.	G	T	.	T	.	C	A	.	.	T	.	G	.	.	C	.	.	.	T	.	10
Goat	.	.	G	C	A	.	.	C	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	C	T	8	Goat	T	T	C	T	.	.	T	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	7
Yak	.	A	G	.	A	.	.	C	.	.	.	.	.	.	.	C	.	A	.	A	.	G	.	C	T	10	Yak	T	T	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	T	.	6
Shad	A	T	C	C	A	A	T	T	.	.	.	.	C	.	.	C	C	A	T	T	G	.	.	C	.	16	Shad	G	G	.	T	C	A	.	.	.	A	G	T	.	.	.	.	.	C	C	.	.	.	.	10
Pigeon	T	A	C	C	.	A	T	C	C	.	A	C	.	G	C	C	.	C	T	.	.	T	A	.	.	17	Pigeon	G	T	.	.	C	T	.	.	.	.	.	C	.	.	.	.	.	G	C	.	.	.	.	7
Carp	A	.	C	C	.	.	T	.	C	.	C	.	C	C	.	C	.	.	.	T	A	.	.	.	C	.	12	Carp	G	A	.	.	C	T	.	A	A	A	A	C	.	.	.	.	G	G	C	.	.	T	.	13
Turtle	T	A	C	C	T	T	T	.	.	.	A	.	.	.	C	C	T	A	T	A	T	.	.	C	.	16	Turtle	T	T	.	T	C	T	.	.	A	.	A	T	T	.	.	.	T	C	.	.	.	.	.	11

Table 2S. The mismatch comparison of the dog specific forward and reverse primers against commercially important species.

Forward	T	G	G	C	T	C	T	A	G	C	C	G	T	T	C	G	A	T	T	A	Number of Mismatch	Reverse	T	C	C	T	A	G	A	A	T	T	T	G	C	T	G	T	T	G	C	C	T	T	Number of Mismatch
Dog	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	Dog	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Cat	.	A	.	T	.	.	.	C	.	.	A	.	.	A	.	G	.	C	.	T	8	Cat	G	.	T	.	T	.	C	.	.	G	.	.	.	C	.	.	A	.	.	.	-	.	7
Rat	.	A	.	.	A	.	.	T	.	.	A	.	.	A	.	A	.	C	.	G	8	Rat	.	A	.	.	T	.	.	G	.	.	C	.	.	.	.	.	A	.	.	T	.	.	6
Pig	.	A	.	.	C	.	.	.	A	.	.	.	.	A	.	.	.	C	.	G	6	Pig	.	T	.	C	T	.	T	.	.	A	.	.	.	A	.	.	A	.	.	T	C	.	9
Monkey	.	A	.	.	C	.	.	T	.	.	A	.	.	A	G	.	.	C	.	T	8	Monkey	.	T	G	.	.	C	T	.	A	.	.	.	.	A	.	.	A	.	.	.	.	.	7
Cow	G	A	.	.	C	.	.	C	.	.	.	A	.	G	A	.	G	.	.	C	9	Cow	.	T	.	G	.	.	.	G	.	.	.	.	.	A	.	.	A	.	.	T	A	.	7
Buffalo	.	A	.	.	G	.	.	C	.	.	T	.	.	A	T	.	-	C	.	.	7	Buffalo	.	T	.	A	.	.	C	G	.	.	.	.	.	A	.	.	G	.	.	T	A	.	8
Sheep	.	A	.	.	C	.	.	T	.	.	T	.	.	A	G	.	.	C	.	G	8	Sheep	.	T	.	A	C	.	.	C	.	.	C	.	.	A	.	.	G	.	.	T	A	.	9
Deer	.	C	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	C	.	C	5	Deer	.	T	.	G	T	.	.	G	.	.	.	.	.	A	.	.	A	.	.	.	A	.	7
Chicken	.	A	.	.	C	.	.	.	.	G	A	.	.	A	.	.	C	C	.	.	7	Chicken	G	.	T	.	C	.	.	.	G	.	G	.	.	A	.	.	A	.	.	.	A	.	8
Duck	.	A	.	.	A	.	.	C	.	G	A	.	.	C	.	.	C	C	.	C	9	Duck	.	A	.	G	.	.	.	.	G	.	A	.	.	A	.	.	G	.	.	.	A	.	7
Salmon	.	C	.	.	C	.	.	T	.	G	T	.	.	A	.	.	.	C	.	C	8	Salmon	.	T	G	.	T	.	.	.	A	.	.	.	.	C	.	.	C	.	.	.	A	.	7
Tuna	.	A	.	.	A	.	.	T	.	G	A	.	.	G	.	.	G	.	.	.	7	Tuna	.	T	.	.	C	.	G	.	A	.	C	.	.	.	.	.	C	.	.	A	A	.	6
Cod	.	C	.	.	.	T	.	.	.	G	.	.	.	.	.	.	G	C	.	T	6	Cod	.	A	.	.	C	.	.	.	G	.	A	.	.	.	.	.	.	.	.	.	A	.	5
Horse	.	A	.	.	C	.	.	T	.	G	.	.	.	G	.	.	G	C	.	T	8	Horse	C	.	A	.	C	.	T	.	.	.	C	.	.	A	.	.	A	.	.	T	A	.	9
Donkey	.	A	.	.	C	.	.	C	.	.	.	A	.	A	.	.	G	C	.	.	7	Donkey	A	.	G	.	C	.	T	.	.	.	C	.	.	A	.	.	A	.	.	.	A	.	8
Goat	.	A	.	.	C	.	.	C	.	.	.	.	.	A	.	.	.	C	.	G	6	Goat	.	T	.	.	C	.	.	.	.	.	C	.	.	A	.	.	A	.	.	T	A	.	7
Yak	.	A	.	.	C	.	.	C	.	.	.	.	.	G	.	.	G	C	.	C	7	Yak	.	T	.	A	.	.	.	C	.	.	.	.	.	A	.	.	A	.	.	T	A	.	7
Shad	.	A	.	.	G	T	.	.	.	G	A	.	.	C	.	.	G	C	.	.	8	Shad	.	A	.	.	C	.	.	.	G	.	.	.	.	C	.	.	A	.	.	.	A	.	6
Pigeon	.	A	.	.	C	.	.	.	.	G	A	.	.	C	.	.	T	C	.	C	8	Pigeon	.	A	.	.	.	.	.	G	G	.	A	.	.	A	.	.	A	.	.	.	A	.	7
Carp	.	A	.	.	C	.	.	G	.	G	A	.	.	C	.	.	.	C	.	.	7	Carp	.	A	.	.	.	.	.	.	G	.	.	.	.	A	.	.	A	.	.	A	A	.	6
Turtle	.	A	.	.	C	T	.	.	.	G	T	.	.	A	.	.	.	C	.	T	8	Turtle	.	T	.	.	.	.	.	.	C	.	A	.	.	A	.	.	G	.	.	.	A	.	6

Table 3S. The mismatch comparison of the rat specific forward and reverse primers against commercially important species.

Forward	A	T	C	A	T	C	A	G	A	A	C	G	C	C	T	T	A	T	T	A	G	C	Number of Mismatch	Reverse	T	A	C	A	C	C	A	A	A	A	G	G	A	C	G	A	A	C	C	T	Number of Mismatch
Rat	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	Rat	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Dog	T	.	.	.	C	.	T	A	.	C	.	.	A	.	.	A	.	G	.	.	A	T	10	Dog	C	.	A	T	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	6
Cat	T	.	.	.	C	.	T	A	.	C	.	.	A	.	.	A	.	.	.	.	A	T	9	Cat	.	.	A	T	.	A	T	.	C	.	.	T	.	.	A	.	.	.	.	.	7
Pig	.	A	.	.	C	.	C	A	.	.	.	.	A	.	.	C	.	.	.	.	A	T	8	Pig	C	.	A	C	.	A	.	.	.	.	.	.	C	.	A	.	.	.	.	.	6
Monkey	.	A	.	C	.	.	T	A	.	.	.	A	.	.	C	C	.	.	.	.	A	T	9	Monkey	C	.	A	C	G	.	T	.	.	G	.	.	.	.	A	.	.	.	.	.	7
Cow	.	A	.	.	.	.	.	A	.	C	.	.	A	.	.	A	G	.	A	.	G	.	8	Cow	C	.	A	T	T	.	T	.	.	.	.	.	.	.	A	.	.	.	A	.	7
Buffalo	.	A	.	T	C	.	.	A	.	T	.	.	A	.	.	A	.	.	A	.	.	T	9	Buffalo	C	.	A	T	A	.	C	.	.	G	.	.	.	.	A	.	.	.	A	.	8
Sheep	C	A	.	.	.	.	.	A	.	C	.	.	A	.	.	A	G	.	C	.	A	.	9	Sheep	.	.	A	T	A	.	C	.	.	.	.	.	.	.	A	G	.	.	A	.	7
Deer	.	A	.	.	.	.	.	A	.	T	.	.	T	.	.	A	G	.	A	.	A	T	9	Deer	C	.	A	T	G	.	C	.	.	.	.	.	.	.	A	.	.	.	A	.	7
Chicken	.	.	.	.	C	.	.	.	.	C	.	.	A	T	G	G	.	.	C	.	A	.	8	Chicken	A	.	A	C	A	A	G	G	C	.	.	.	T	.	A	C	.	A	A	.	13
Duck	.	.	.	C	C	.	.	.	.	C	.	.	A	T	G	A	.	.	C	.	A	.	9	Duck	A	.	A	C	A	A	.	.	.	C	.	.	C	.	A	C	.	A	A	.	11
Salmon	.	A	.	C	C	.	.	T	C	C	.	.	A	T	G	A	C	.	A	.	A	.	13	Salmon	C	.	A	T	T	T	.	G	G	.	.	.	C	.	A	T	.	A	A	.	12
Tuna	.	A	.	.	C	.	.	A	C	C	.	.	A	T	G	A	C	.	A	.	A	.	12	Tuna	.	.	A	T	.	T	G	C	C	C	.	.	C	.	A	C	.	A	A	.	12
Cod	T	A	.	.	C	.	T	A	C	C	.	.	A	T	G	A	C	.	A	.	.	.	13	Cod	A	.	A	T	G	T	G	G	G	.	.	.	.	.	A	C	.	A	A	.	12
Horse	C	.	.	.	C	.	C	A	.	C	.	.	A	.	.	A	.	.	C	.	A	.	9	Horse	.	.	A	C	A	G	C	.	.	.	.	.	.	.	A	.	.	.	.	.	6
Donkey	C	.	.	.	.	.	C	A	.	C	.	.	A	.	.	A	.	.	.	.	A	.	7	Donkey	.	.	A	C	A	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	6
Goat	C	.	.	.	.	.	.	A	.	C	.	.	A	.	.	A	.	.	.	.	A	.	6	Goat	.	.	A	C	A	.	C	.	.	.	T	.	.	.	A	.	.	.	A	.	7
Yak	.	A	.	.	.	.	.	.	.	T	.	.	A	T	.	A	G	.	G	.	A	T	9	Yak	C	.	A	C	.	.	C	.	.	.	.	.	.	.	A	.	.	.	A	.	6
Shad	C	A	.	C	C	.	T	A	C	.	.	.	A	T	G	A	C	.	A	.	A	T	15	Shad	C	.	A	C	.	.	.	G	G	.	.	.	.	.	A	T	.	A	A	.	9
Pigeon	.	.	.	C	C	.	T	A	.	C	.	.	T	T	G	A	.	.	.	.	C	T	11	Pigeon	A	.	A	C	A	A	.	.	.	C	.	.	C	.	A	C	.	A	A	.	11
Carp	.	A	.	C	C	.	.	C	C	T	.	.	A	T	G	A	.	.	.	.	A	T	12	Carp	G	.	A	T	G	T	.	G	G	.	.	.	.	.	A	T	.	A	A	.	11
Turtle	.	A	.	C	C	.	.	A	.	C	.	.	A	T	G	A	C	.	A	.	C	T	13	Turtle	.	.	A	C	A	A	.	.	C	.	.	.	.	.	A	T	.	A	A	.	9

Table 4S. The mismatch comparison of the cat specific forward and reverse primers against commercially important species.

Forward	G	G	A	A	T	A	A	T	G	T	T	T	C	G	A	C	C	A	C	T	A	A	G	C	Number of Mismatch	Reverse	A	T	C	C	T	A	A	T	A	C	C	C	A	T	C	T	C	A	G	G	C	A	Number of Mismatch
Cat	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	Cat	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Dog	A	.	C	C	.	G	.	.	A	.	.	C	.	.	G	.	.	C	G	.	T	.	T	.	11	Dog	T	G	A	.	.	G	.	.	.	.	.	A	.	C	.	G	T	T	A	.	.	.	10
Rat	A	.	T	C	.	.	.	C	A	.	.	C	.	.	C	.	.	.	A	.	C	.	C	.	10	Rat	.	.	T	T	.	C	.	.	.	.	.	A	.	.	.	.	.	C	.	.	A	.	6
Pig	A	.	C	.	.	.	.	.	A	.	.	.	.	.	.	G	.	.	G	.	.	C	.	T	7	Pig	G	.	A	T	.	G	.	.	.	.	.	A	.	.	.	A	.	T	A	.	.	.	8
Monkey	A	.	T	.	.	.	.	.	A	.	.	C	.	.	C	.	.	.	.	.	C	.	T	.	7	Monkey	.	C	.	A	.	.	.	.	.	.	.	A	C	.	G	G	.	C	T	C	.	C	10
Cow	A	.	C	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	5	Cow	G	.	G	.	.	.	.	.	.	.	.	A	.	C	G	G	.	C	.	.	.	.	7
Buffalo	A	.	T	.	.	G	.	.	.	.	.	C	.	.	G	.	.	.	T	.	C	.	.	.	7	Buffalo	G	.	G	.	.	.	.	.	.	.	.	A	.	C	G	G	.	C	A	.	.	.	8
Sheep	A	.	C	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	A	.	C	.	.	T	7	Sheep	G	.	.	A	.	.	.	.	.	.	.	A	G	.	A	G	.	T	A	.	.	.	8
Deer	A	.	C	.	.	G	.	.	A	.	.	C	.	.	.	.	.	.	T	.	C	.	.	.	7	Deer	G	.	.	.	.	T	.	.	.	.	.	G	.	.	.	A	.	C	A	.	.	.	6
Chicken	A	C	.	.	.	.	.	C	C	.	.	C	.	.	.	.	.	.	.	.	C	T	C	.	8	Chicken	.	.	.	.	.	C	T	.	C	.	.	.	.	C	A	A	T	C	.	.	A	.	9
Duck	A	C	.	.	.	.	.	C	A	.	.	C	.	.	G	.	.	G	.	.	C	T	C	.	10	Duck	T	.	.	.	.	T	T	.	C	.	.	T	G	C	.	G	T	.	A	.	.	G	11
Salmon	.	.	.	C	.	G	.	C	C	.	.	.	.	.	C	.	.	.	.	.	C	.	C	.	7	Salmon	G	.	.	.	.	T	G	C	C	.	.	.	C	.	G	G	.	T	.	.	.	T	10
Tuna	A	C	T	C	.	.	.	C	A	.	.	C	.	.	.	.	.	.	G	.	T	T	C	.	11	Tuna	G	.	T	T	.	C	T	.	C	.	.	A	C	.	T	G	.	.	.	.	.	T	11
Cod	.	.	T	T	.	.	.	C	A	.	.	C	.	.	C	.	.	T	.	.	T	.	C	.	9	Cod	G	.	T	T	.	.	T	.	C	.	.	.	C	.	T	G	.	.	.	.	A	.	9
Horse	A	.	C	.	.	.	.	.	A	.	.	C	.	.	G	.	.	T	.	.	C	.	.	.	7	Horse	.	.	T	T	.	T	.	.	.	.	.	A	C	.	.	G	.	.	A	.	.	.	7
Donkey	A	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	T	.	.	T	6	Donkey	.	.	.	T	.	C	.	.	.	.	.	A	C	.	.	G	.	.	A	.	.	.	6
Goat	A	.	C	.	.	.	.	.	A	.	.	C	.	.	C	.	.	.	A	.	C	.	.	.	7	Goat	G	.	A	A	.	.	.	.	.	.	.	A	G	C	A	G	.	T	A	.	.	.	10
Yak	A	.	C	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	5	Yak	G	.	A	.	.	.	.	.	.	.	.	A	.	C	A	G	.	C	.	.	.	.	7
Shad	.	.	.	T	.	.	.	C	C	.	.	C	.	.	G	.	.	C	.	.	C	.	C	A	9	Shad	.	.	.	.	.	.	G	C	.	.	.	T	C	.	A	G	.	.	.	.	G	T	8
Pigeon	A	C	.	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	C	T	C	T	9	Pigeon	G	.	.	.	.	C	T	.	C	.	.	T	G	C	T	A	.	C	.	C	A	G	13
Carp	.	.	G	C	.	.	.	C	A	.	.	C	.	.	C	.	.	C	A	.	C	.	C	.	10	Carp	.	.	T	T	.	T	.	.	G	.	.	A	C	.	A	G	.	.	.	.	A	T	10
Turtle	A	C	.	G	C	T	T	C	A	.	.	C	.	.	.	.	.	.	T	.	.	.	C	.	11	Turtle	.	C	A	.	.	T	.	.	.	.	.	.	C	.	T	A	.	.	.	.	A	C	8

Table 5S. The mismatch comparison of the monkey specific forward and reverse primers against commercially important species.

Forward	T	G	A	G	A	C	C	T	C	C	A	A	C	A	A	A	T	A	C	T	A	G	C	Number of Mismatch	Reverse	C	T	G	A	C	T	A	C	C	T	T	C	T	G	C	C	A	T	A	G	A	G	Number of Mismatch
Monkey	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	Monkey	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Pig	.	.	.	.	.	T	.	.	T	.	.	T	.	.	G	.	.	C	T	.	T	A	T	9	Pig	A	.	A	.	T	.	G	.	.	C	.	.	A	.	.	A	.	.	.	.	.	.	A	8
Dog	.	.	.	.	.	.	.	.	T	.	.	G	.	.	C	.	.	C	T	.	C	A	T	8	Dog	G	.	A	.	G	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	6
Cat	.	.	G	.	.	.	T	.	T	.	.	.	.	.	.	.	G	C	T	.	T	A	T	9	Cat	G	.	A	T	.	.	G	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	A	7
Rat	.	.	G	.	.	A	.	.	T	.	.	.	.	.	.	.	.	T	T	.	C	T	T	8	Rat	A	.	A	.	.	A	C	.	.	A	.	.	C	.	.	A	.	.	.	.	.	.	A	8
Cow	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	T	.	C	A	T	6	Cow	G	A	.	T	.	.	T	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	A	7
Buffalo	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	C	A	T	5	Buffalo	A	.	C	T	.	.	C	.	.	C	.	.	.	.	.	A	.	.	G	.	.	A	8
Sheep	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	T	.	T	A	T	6	Sheep	A	.	C	.	.	A	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	5
Deer	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	.	.	T	T	.	T	.	.	5	Deer	A	.	T	.	.	C	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	A	6
Chicken	.	.	.	.	.	A	A	.	T	.	.	.	.	.	.	.	.	C	A	C	C	C	A	9	Chicken	A	G	.	G	.	.	T	.	.	A	.	.	.	.	.	A	.	.	.	.	.	A	7
Duck	.	.	.	.	.	A	A	.	T	.	.	.	.	.	.	G	C	C	G	.	.	C	A	9	Duck	A	C	T	.	T	.	.	.	.	.	G	.	A	.	.	.	.	.	G	.	.	A	8
Salmon	.	.	.	.	.	A	A	.	T	.	.	.	.	.	.	.	.	.	T	.	T	.	T	6	Salmon	A	.	A	.	A	.	T	.	.	.	.	.	A	.	.	G	.	.	.	.	.	A	7
Tuna	.	.	.	.	.	A	A	.	A	.	.	.	.	.	.	.	.	.	T	.	C	.	T	6	Tuna	A	.	A	.	G	.	C	.	.	C	.	.	.	.	.	T	.	.	G	.	.	.	7
Cod	.	.	G	.	.	T	A	.	T	.	.	.	.	.	.	.	.	.	T	.	T	A	T	8	Cod	A	.	A	.	.	.	G	.	.	A	G	.	C	.	.	A	.	.	.	.	.	A	8
Horse	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	C	.	C	T	.	C	A	T	8	Horse	A	.	C	C	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	6
Donkey	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	C	.	C	T	.	C	A	T	8	Donkey	A	.	C	.	.	.	T	.	.	C	.	.	G	.	.	.	.	.	.	.	.	A	6
Goat	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	T	.	T	A	T	7	Goat	A	.	C	.	.	.	G	.	.	C	.	.	.	.	.	A	.	.	.	.	.	A	6
Yak	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	C	T	.	C	A	T	7	Yak	A	.	.	T	.	C	T	.	.	-	.	.	.	.	.	A	.	.	.	.	.	A	6
Shad	.	.	C	.	.	A	A	.	A	.	.	T	.	.	G	.	.	C	T	.	T	T	.	10	Shad	G	.	A	.	.	.	G	.	.	.	.	.	C	.	.	A	.	.	G	.	.	A	7
Pigeon	.	.	.	.	.	A	A	.	.	.	.	C	.	.	T	.	C	.	A	C	C	T	.	9	Pigeon	T	G	.	C	.	A	G	.	.	G	G	.	.	.	.	.	.	.	.	.	.	A	8
Carp	.	.	.	.	.	A	A	.	T	.	.	.	.	.	T	.	.	C	T	.	T	.	A	8	Carp	A	C	.	.	G	.	.	T	.	G	.	.	C	.	.	.	.	.	G	.	.	.	7
Turtle	.	.	.	.	.	G	A	.	.	.	.	.	.	.	G	.	.	C	T	.	T	A	.	7	Turtle	A	G	.	A	.	.	C	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	6

Appendix C

Table 6S. Pairwise distances of the pig specific 141 bp ND5 site against tested species.

	Pig	Dog	Cat	Rat	Monkey	Cow	Buffalo	Sheep	Deer	Chicken	Duck	Salmon	Tuna	Cod	Horse	Donkey	Goat	Yak	Shad	Pigeon	Carp	Turtle
Pig	0.00																					
Dog	0.63																					
Cat	0.63	0.50																				
Rat	1.08	1.00	1.04																			
Monkey	0.84	0.70	0.64	1.20																		
Cow	0.57	0.40	0.42	0.83	0.66																	
Buffalo	0.60	0.40	0.39	0.91	0.55	0.20																
Sheep	0.66	0.45	0.44	0.86	0.62	0.25	0.20															
Deer	0.65	0.47	0.49	0.87	0.70	0.17	0.24	0.26														
Chicken	1.15	0.86	0.81	1.12	0.80	0.82	0.69	0.74	0.90													
Duck	1.00	0.88	0.91	1.06	0.71	0.80	0.81	0.74	0.95	0.42												
Salmon	1.06	1.03	0.91	1.26	1.08	1.05	1.07	1.04	1.03	0.77	1.16											
Tuna	1.01	1.25	1.19	1.24	1.21	1.39	1.38	1.15	1.42	1.07	1.08	0.47										
Cod	1.06	1.11	1.08	1.14	1.20	1.22	1.27	1.21	1.20	1.21	1.03	0.71	0.73									
Horse	0.59	0.41	0.32	1.01	0.59	0.30	0.33	0.41	0.35	0.73	0.76	0.95	1.11	1.19								
Donkey	0.63	0.44	0.37	0.97	0.57	0.36	0.29	0.40	0.39	0.70	0.77	1.03	1.13	1.39	0.12							
Goat	0.60	0.45	0.45	0.86	0.65	0.29	0.22	0.25	0.33	0.87	0.74	1.18	1.15	1.24	0.37	0.36						
Yak	0.64	0.45	0.46	0.80	0.69	0.10	0.22	0.30	0.20	0.83	0.85	1.12	1.42	1.24	0.32	0.33	0.22					
Shad	1.09	1.13	1.16	1.46	1.12	1.00	1.04	1.09	1.16	0.99	1.08	0.39	0.49	0.76	1.08	1.07	0.90	1.06				
Pigeon	1.18	1.08	1.07	1.30	0.94	0.97	0.99	1.03	1.05	0.52	0.46	0.87	1.00	1.00	0.96	1.10	1.01	1.01	1.01			
Carp	1.04	1.12	0.89	0.96	1.28	1.14	1.10	0.92	1.18	0.96	1.22	0.64	0.56	0.86	0.91	0.90	0.97	1.07	0.55	0.93		
Turtle	1.14	1.11	1.02	1.14	0.81	0.81	0.70	0.87	0.75	0.89	0.87	0.81	0.98	1.09	0.91	0.85	0.79	0.77	0.76	0.80	0.92	0.00

Table 7S. Pairwise distances of the dog specific 163 bp ATPase 6 site against tested species.

	Dog	Cat	Rat	Pig	Monkey	Cow	Buffalo	Sheep	Deer	Chicken	Duck	Salmon	Tuna	Cod	Horse	Donkey	Goat	Yak	Shad	Pigeon	Carp	Turtle
Dog	0.00																					
Cat	0.52																					
Rat	0.53	0.48																				
Pig	0.50	0.44	0.30																			
Monkey	0.71	0.40	0.45	0.47																		
Cow	0.52	0.49	0.44	0.54	0.39																	
Buffalo	0.50	0.34	0.31	0.26	0.44	0.14																
Sheep	0.55	0.30	0.29	0.26	0.42	0.18	0.22															
Deer	0.48	0.32	0.31	0.27	0.44	0.18	0.19	0.22														
Chicken	0.87	0.57	0.48	0.55	0.52	0.50	0.53	0.54	0.54													
Duck	0.85	0.59	0.52	0.55	0.58	0.50	0.50	0.47	0.50	0.28												
Salmon	0.69	0.49	0.41	0.48	0.52	0.49	0.45	0.44	0.42	0.39	0.34											
Tuna	0.79	0.70	0.56	0.67	0.60	0.48	0.54	0.56	0.60	0.33	0.42	0.32										
Cod	0.76	0.58	0.48	0.58	0.68	0.61	0.60	0.54	0.46	0.46	0.40	0.33	0.42									
Horse	0.51	0.30	0.32	0.28	0.39	0.22	0.27	0.26	0.30	0.45	0.51	0.49	0.55	0.59								
Donkey	0.50	0.32	0.29	0.32	0.39	0.26	0.30	0.25	0.27	0.48	0.53	0.51	0.55	0.51	0.13							
Goat	0.64	0.28	0.28	0.23	0.41	0.16	0.21	0.12	0.23	0.50	0.47	0.43	0.61	0.56	0.23	0.25						
Yak	0.55	0.29	0.29	0.25	0.42	0.07	0.18	0.22	0.20	0.51	0.56	0.49	0.53	0.59	0.22	0.26	0.14					
Shad	0.77	0.62	0.55	0.58	0.58	0.52	0.56	0.58	0.46	0.35	0.42	0.30	0.33	0.32	0.53	0.49	0.61	0.57				
Pigeon	0.83	0.67	0.58	0.55	0.62	0.52	0.55	0.55	0.53	0.21	0.24	0.36	0.42	0.50	0.50	0.50	0.53	0.57	0.35			
Carp	0.63	0.58	0.49	0.58	0.62	0.49	0.49	0.57	0.51	0.33	0.34	0.27	0.27	0.39	0.55	0.60	0.59	0.54	0.24	0.35		
Turtle	0.64	0.50	0.58	0.49	0.46	0.52	0.54	0.51	0.49	0.36	0.40	0.41	0.43	0.40	0.54	0.52	0.58	0.59	0.37	0.43	0.35	0.00

Table 8S. Pairwise distances of the rat specific 108 bp ATPase 6 site against tested species.

	Rat	Dog	Cat	Pig	Monkey	Cow	Buffalo	Sheep	Deer	Chicken	Duck	Salmon	Tuna	Cod	Horse	Donkey	Goat	Yak	Shad	Pigeon	Carp	Turtle
Rat	0.00																					
Dog	0.67																					
Cat	0.67	0.58																				
Pig	0.66	0.54	0.44																			
Monkey	0.68	0.57	0.47	0.42																		
Cow	0.67	0.54	0.34	0.46	0.53																	
Buffalo	0.69	0.51	0.55	0.56	0.49	0.14																
Sheep	0.74	0.51	0.51	0.58	0.61	0.18	0.16															
Deer	0.69	0.50	0.50	0.54	0.44	0.16	0.13	0.18														
Chicken	0.88	0.65	0.65	0.55	0.76	0.63	0.61	0.55	0.70													
Duck	0.94	0.60	0.60	0.60	0.62	0.65	0.60	0.54	0.67	0.18												
Salmon	1.08	0.84	0.84	0.73	1.09	0.65	0.70	0.73	0.70	0.46	0.50											
Tuna	1.12	0.95	0.95	0.97	1.22	0.77	0.87	0.79	0.84	0.55	0.59	0.35										
Cod	1.13	0.86	0.86	1.02	1.12	0.74	0.90	0.90	0.81	0.67	0.78	0.32	0.39									
Horse	0.77	0.61	0.21	0.24	0.50	0.35	0.32	0.29	0.35	0.53	0.56	0.89	1.16	0.91								
Donkey	0.75	0.60	0.20	0.24	0.45	0.31	0.33	0.29	0.35	0.49	0.52	0.89	1.20	0.93	0.07							
Goat	0.78	0.58	0.28	0.31	0.51	0.26	0.22	0.15	0.23	0.51	0.54	0.76	0.98	0.82	0.19	0.17						
Yak	0.72	0.58	0.38	0.39	0.55	0.12	0.20	0.25	0.20	0.59	0.62	0.68	0.87	0.82	0.37	0.35	0.26					
Shad	0.93	0.89	0.89	0.82	0.84	0.82	0.81	0.87	0.76	0.68	0.61	0.28	0.50	0.36	0.98	1.01	0.96	0.79				
Pigeon	0.97	0.58	0.58	0.65	0.63	0.66	0.54	0.64	0.62	0.27	0.17	0.54	0.61	0.73	0.66	0.58	0.54	0.64	0.61			
Carp	0.93	0.79	0.79	0.62	0.81	0.80	0.66	0.72	0.64	0.44	0.45	0.21	0.49	0.39	0.80	0.77	0.71	0.71	0.33	0.49		
Turtle	0.98	0.73	0.73	0.71	0.83	0.62	0.60	0.67	0.64	0.42	0.43	0.36	0.55	0.55	0.71	0.71	0.58	0.60	0.41	0.36	0.39	0.00

Table 9S. Pairwise distances of the cat specific 172 bp cyt b site against tested species.

	Cat	Dog	Rat	Pig	Monkey	Cow	Buffalo	Sheep	Deer	Chicken	Duck	Salmon	Tuna	Cod	Horse	Donkey	Goat	Yak	Shad	Pigeon	Carp	Turtle
Cat	0.00																					
Dog	0.54																					
Rat	0.55	0.41																				
Pig	0.43	0.31	0.32																			
Monkey	0.58	0.39	0.43	0.37																		
Cow	0.60	0.31	0.37	0.22	0.33																	
Buffalo	0.67	0.34	0.32	0.26	0.38	0.13																
Sheep	0.73	0.31	0.33	0.22	0.35	0.21	0.21															
Deer	0.64	0.29	0.33	0.23	0.37	0.18	0.19	0.20														
Chicken	0.56	0.36	0.31	0.38	0.42	0.40	0.40	0.44	0.42													
Duck	0.57	0.37	0.39	0.41	0.48	0.40	0.39	0.43	0.47	0.26												
Salmon	0.82	0.55	0.47	0.46	0.48	0.41	0.44	0.44	0.43	0.41	0.41											
Tuna	0.85	0.54	0.38	0.47	0.49	0.44	0.51	0.51	0.47	0.43	0.49	0.38										
Cod	0.88	0.44	0.39	0.45	0.47	0.39	0.44	0.43	0.45	0.45	0.42	0.33	0.29									
Horse	0.75	0.32	0.40	0.28	0.40	0.27	0.31	0.31	0.27	0.49	0.44	0.52	0.39	0.42								
Donkey	0.79	0.33	0.40	0.25	0.41	0.28	0.32	0.29	0.28	0.45	0.47	0.50	0.41	0.40	0.10							
Goat	0.72	0.30	0.36	0.23	0.36	0.19	0.17	0.09	0.20	0.47	0.47	0.45	0.48	0.40	0.29	0.33						
Yak	0.53	0.32	0.34	0.22	0.37	0.10	0.11	0.16	0.20	0.35	0.38	0.37	0.43	0.40	0.29	0.29	0.15					
Shad	0.86	0.42	0.42	0.45	0.49	0.46	0.49	0.49	0.47	0.39	0.46	0.31	0.32	0.34	0.39	0.37	0.52	0.39				
Pigeon	0.59	0.43	0.34	0.36	0.36	0.42	0.43	0.42	0.44	0.18	0.22	0.43	0.41	0.43	0.49	0.45	0.45	0.42	0.40			
Carp	0.61	0.43	0.30	0.38	0.42	0.36	0.45	0.38	0.40	0.39	0.45	0.30	0.26	0.27	0.36	0.37	0.37	0.37	0.21	0.42		
Turtle	0.94	0.53	0.50	0.45	0.60	0.46	0.52	0.48	0.42	0.50	0.57	0.57	0.55	0.53	0.53	0.56	0.48	0.46	0.53	0.57	0.50	0.00

Table 10S. Pairwise distances of the monkey-specific 129 bp ND5 site against tested species.

	Monkey	Pig	Dog	Cat	Rat	Cow	Buffalo	Sheep	Deer	Chicken	Duck	Salmon	Tuna	Cod	Horse	Donkey	Goat	Yak	Shad	Pigeon	Carp	Turtle
Monkey	0.00																					
Pig	0.61																					
Dog	0.64	0.55																				
Cat	0.66	0.59	0.53																			
Rat	0.66	0.54	0.52	0.63																		
Cow	0.72	0.54	0.47	0.57	0.34																	
Buffalo	0.79	0.58	0.58	0.33	0.24	0.13																
Sheep	0.73	0.57	0.53	0.36	0.32	0.16	0.14															
Deer	0.68	0.62	0.54	0.34	0.35	0.21	0.19	0.19														
Chicken	0.80	0.57	0.52	0.40	0.40	0.43	0.34	0.40	0.40													
Duck	0.81	0.43	0.42	0.41	0.44	0.51	0.35	0.39	0.45	0.18												
Salmon	0.92	0.36	0.34	0.36	0.33	0.38	0.36	0.29	0.32	0.27	0.35											
Tuna	0.97	0.45	0.34	0.37	0.34	0.38	0.33	0.33	0.40	0.33	0.40	0.25										
Cod	0.91	0.40	0.44	0.38	0.43	0.44	0.44	0.38	0.50	0.41	0.49	0.30	0.33									
Horse	0.66	0.33	0.30	0.35	0.29	0.30	0.23	0.28	0.34	0.34	0.39	0.34	0.31	0.45								
Donkey	0.64	0.32	0.34	0.34	0.32	0.29	0.27	0.31	0.35	0.43	0.47	0.39	0.32	0.42	0.12							
Goat	0.74	0.27	0.27	0.27	0.30	0.20	0.13	0.15	0.19	0.39	0.43	0.33	0.39	0.37	0.32	0.31						
Yak	0.71	0.30	0.37	0.38	0.32	0.09	0.12	0.14	0.21	0.37	0.45	0.38	0.38	0.42	0.28	0.25	0.20					
Shad	0.82	0.38	0.46	0.40	0.37	0.39	0.36	0.36	0.46	0.37	0.36	0.28	0.32	0.34	0.42	0.38	0.36	0.39				
Pigeon	0.75	0.41	0.36	0.40	0.38	0.44	0.33	0.41	0.36	0.20	0.27	0.37	0.36	0.43	0.36	0.43	0.40	0.43	0.41			
Carp	0.95	0.41	0.34	0.35	0.34	0.34	0.27	0.30	0.30	0.34	0.37	0.27	0.22	0.37	0.36	0.44	0.35	0.38	0.29	0.35		
Turtle	0.89	0.31	0.32	0.34	0.49	0.40	0.41	0.37	0.41	0.29	0.35	0.37	0.33	0.38	0.32	0.32	0.39	0.41	0.37	0.29	0.35	0.00