MOLECULAR DISCRIMINATION OF BOVINE ORIGIN FROM PROCESSED CANNED PRODUCTS USING PCR AMPLIFICATION OF CYTOCHROME B GENE

by

NG YU-CHEN

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AW	Wash buffer
Bp	Base pair
CO	Cytochrome oxidase
Cyt b	Cytochrome b
ddH_2O	Double distilled water
dH_2O	Distilled water
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
EB	Elution buffer
g	Grams
HMW	High molecular weight
Kbp	Kilo base pair
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mL	Millilitre
mM	Millimolar
mPCR	Multiplex PCR
mtDNA	Mitochondrial DNA

ND	NADH dehydrogenase
^{o}C	Degree Celcius
PAGE	Polyacrylamide Gel Electrophoresis
PB	Phosphate buffer
PCR	Polymerase Chain Reaction
Pmol	Picomol
RAPD	Random Amplified Polymorphic DNA
RCF	Relative centrifugal force
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SSCP	Single Strand Conformation Polymorphism
ssDNA	Single strand DNA fragments
TBE	Tris Borate EDTA
TE	Tris EDTA
Tm	Melting point
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
v	Volt
µg	Microgram
µL	Microlitre

DISKRIMINASI MOLEKULAR ASAL LEMBU DARI PRODUK TIN YANG DIPROSES MENGGUNAKAN PENGUATAN PCR GEN SITOKROM B

ABSTRAK

Kaedah molekular menjadi teknik pilihan yang digunakan untuk pengenalpastian spesies haiwan daripada produk daging yang diproses. Primer khusus spesies berasaskan PCR menawarkan kelebihan dengan mensasarkan spesies tertentu menggunakan gen tertentu. Oleh itu, kajian ini bertujuan untuk mengenal pasti kandungan daging lembu daripada produk tin yang diproses di sekitar Kubang Kerian, Kelantan. Pengekstrakan DNA 12 produk bertin dan satu daging lembu mentah segar tertakluk kepada kit makanan DNeasy mericon dari Qiagen dan kauntifikasi di lakukan menggunakan spektrofotometer Nanodrop^{TM}. Penguatan PCR menggunakan primer khusus spesies dengan mensasarkan gen sitokrom b DNA mitokondria telah dikuatkan pada kawasan sasasran 120 bp. Kejayaan penguatan dilihat dalam sampel kawalan positif (daging lembu mentah) dan dua produk dalam tin. Daripada 12 produk tin yang diproses, 83.33% gagal dikesan atau tiada jalur DNA dilihat pada 120 bp. Ini mungkin dipengaruhi oleh proses pembuatan produk tin yang melibatkan suhu tinggi dan rawatan pada tekanan tinggi, seterusnya menjejaskan DNA genomik menjadi terdegradasi dan/atau mungkin jumlah DNA yang sangat rendah diperolehi daripada pengekstrakan DNA. Secara ringkasnya, penggunaan penanda DNA mitokondria khususnya gen sitokrom b telah meningkatkan kemungkinan penguatan gen yang disasarkan pada produk tin yang diproses disebabkan bilangan salinan yang tinggi.

MOLECULAR DISCRIMINATION OF BOVINE ORIGIN FROM PROCESSED CANNED PRODUCTS USING PCR AMPLIFICATION OF CYTOCHROME B GENE

ABSTRACT

The molecular method become the preferred technique used for the identification of animal species from processed meat products. PCR-based species-specific primers offer an advantage by targeting a specific species using a certain gene. Therefore, this study is aimed to identify beef meat content from processed canned products around Kubang Kerian, Kelantan. DNA extraction of 12 canned products and one fresh raw beef meat was subjected to a DNeasy mericon food kit from Qiagen and quantification was performed using a Nanodrop spectrophotometer. PCR amplification using species-specific primer by targeting a mitochondrial DNA cytochrome b gene was amplified 120 bp of the target region. Success amplification was seen in the positive control sample (raw beef meat) and two canned products. Out of 12 processed canned products, 83.33% failed to detect or no DNA band was seen at 120 bp. This could be influenced by the manufacturing process for the canned products which involves high temperature and pressure treatment, hence affecting the genomic DNA to become degraded and/or possibly a very low amount of DNA was obtained from DNA extraction. In summary, the use of mitochondrial DNA markers specifically the cytochrome b gene has increased the possibility of the amplification of the targeted gene from processed canned products due to high copy number.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Over the years, food safety scandals involving contamination and forged meat products have been commonly practised in the world of the food industry. Based on the research published by Nikolovska *et al.* (2019), foods can be contaminated with substituted meat from other species, chemical substances, illicit drug residues, additives, dyes, pathogenic microorganisms, and other pests. In 2013, the Horsemeat Scandal highlighted the vulnerabilities within the European beef supply chain as many supermarket stores in various parts of European countries reported foods advertised as containing beef were found to contain undeclared horse meat (Robson *et al.,* 2020). These reports result in mass product recalls a downturn in the beef sale and economic losses for many unrelated to the scandal.

In Malaysia, food fraud crimes are very common due to the demand for meat production which increases every year, making it the easiest target prospect for fraudsters (Ariffin *et al.,* 2021). The majority of food crime issues involve adulteration, manipulation and mislabelling of meat products. Oon Chuah *et al.* (2016) show that out of 143 prepacked beef and poultry meat products (sausages, cold cut meats, cooked whole muscle meats, breaded products, meatballs and ground meats) that were purchased from several national and international supermarket chains in Malaysia, 112 (78.3%) samples were mislabelled, attributed by the false declaration of species and/or presence of undeclared meat species. In addition, there were numerous reports on the exchange of prohibited meat for halal such as pork is exchanged for beef, dog meat is

exchanged with mutton and also the meat of animals that are not slaughtered (Ariffin *et al.,* 2021).

Previous methods for species identification of meat were performed based on anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic, and immunological analytical methods (Wu *et al.,* 2018). However, those methods have been replaced by DNA-based methods due to limitations such as heat-labile as well as producing inaccurate results and lack of reproducibility (Karabasanavar *et al.,* 2017). DNA-based methods particularly the polymerase chain reaction (PCR) technique using species-specific primers, allow the identification of species even in complex and processed foods (Mafra *et al.,* 2008). It has been used extensively to determine the genetic relationships among closely related species, especially the bovine species origin.

The adulteration and substitution of beef have gained concern for several reasons such as possible economic loss from fraudulent substitutions or adulterations, medical requirements of individuals who might have specific allergies and religious reasons (Abdul-Hassan & Tauma, 2014). Therefore, meat species identification plays an important role in the field of quality control management in the meat industry as it helps consumers to detect the adulteration of processed meat with unwanted food ingredients (Hong *et al.,* 2017).

1.2 Objectives of Study

1.2.1 Main Objective

- To screen processed canned products using PCR amplification of bovine specific primer.

1.2.2 Specific objectives

1. To measure the quality and quantity of the extracted genomic DNA of processed canned products.
2. To perform PCR amplification of fresh raw meat beef using cytochrome b gene primers.
3. To identify the beef content in processed canned products using PCR amplification of the cytochrome b gene.

1.3 Significance of Study

In recent years, the adulteration and substitution of beef have become a concern for several reasons such as economic loss due to immoral practice, medical requirements of individuals who might have specific allergies and religious prohibitions (Abdul-Hassan & Tauma, 2014). Therefore, meat species identification plays an important role in the field of quality control management of the meat industry to detect the adulteration of processed meat with unwanted food ingredients as well as protect consumer rights.

The use of high specificity and sensitivity techniques is needed to achieve precise accuracy of detection. The designed primer also should be amplified with a smaller targeted gene to increase the success of amplification because the samples used originated from processed food, specifically canned products. Hence, this study aims to identify the authenticity of beef meat in the canned product as claimed in the list of ingredients on the canned product.

CHAPTER 2

LITERATURE REVIEW

2.1 Species Identification

The authentication of animals in meat products is significant to the contribution of fair trade as well as providing correct information to the consumers based on proper labelling as stated on the product packing. There are several options methods for species identification which can be based on proteins, lipids, volatile organic compounds, and DNA analyses (Kitpipit *et al.,* 2014).

Recently, DNA-based methods have been considered essential tools for species identification of food products (Abdul-Hassan & Tauma, 2014). Based on Murugaiah *et al.* (2015), the DNA-based technique has the advantage because it does not require a standard for each tissue since all the cells in an individual have the same DNA. Furthermore, the structure of DNA is relatively stable at high temperatures and has a conserved region within all tissues of an individual (Murugaiah *et al.,* 2015). These features allow the identification and discrimination of species to be carried out not only in raw samples but also in cooked and processed foods (Karabasanavar *et al.,* 2017).

The current development in animal identification gives more attention to the mitochondrial DNA genes, especially the cytochrome b gene (cyt b) (Mutalib *et al.,* 2012). Based on the research carried out by Chandrika *et al.* (2010), the cyt b gene sequence has been well characterized among different vertebrate groups, thus allowing the identification of genetic variation between animal species.

2.2 Mitochondrial DNA

Mitochondria are intracellular organelles that play a crucial role in generating adenosine triphosphate (ATP) by aerobic respiration for cells to survive. For over a hundred million years ago, mitochondria originated as aerobic bacteria which form a symbiotic relationship with pre- or early eukaryotic cells. Over time, the endosymbiotic bacterium went through a massive number of evolutionary changes to become a permanent organelle (Roger *et al.,* 2017). The legacy of mitochondria is reflected in the fact that the present-day mitochondria retain their own respective bacterial-type ribosomes and DNA (mtDNA) which are distinct from the genetic material found in the cell nucleus (Gunn *et al.,* 2019).

The structure of mtDNA is a small, double-stranded, circular DNA that contains 16, 569 base pairs in length and it is made up of 2 regions, namely the coding and non-coding regions as shown in Figure 2.1. The non-coding region is also known as the displacement loop (D-loop) or control region. The D-loop is 1124 bp in length (positions 16024-576), which contains 2 hypervariable regions: HV1 and HV2. Both of these hypervariable regions demonstrate a higher mutation rate as compared to the coding region. Furthermore, the rate of mutation is approximately 5-10 times higher than that of nuclear genomic DNA (Sharma *et al.,* 2005).

The coding region of the mtDNA genome encodes for the synthesis of 37 genes which include 2 ribosomal RNA (rRNA), 22 transfer RNA (tRNA) and 13 polypeptides. The following 13 polypeptides consist of 7 subunits of Complex I (ND1-6 and ND4L), 1 subunit of Complex III (cyt b), 3 subunits of Complex IV (COI-III) and 2 subunits of Complex V (ATPase 6 and 8) (Yang *et al.,* 2014).

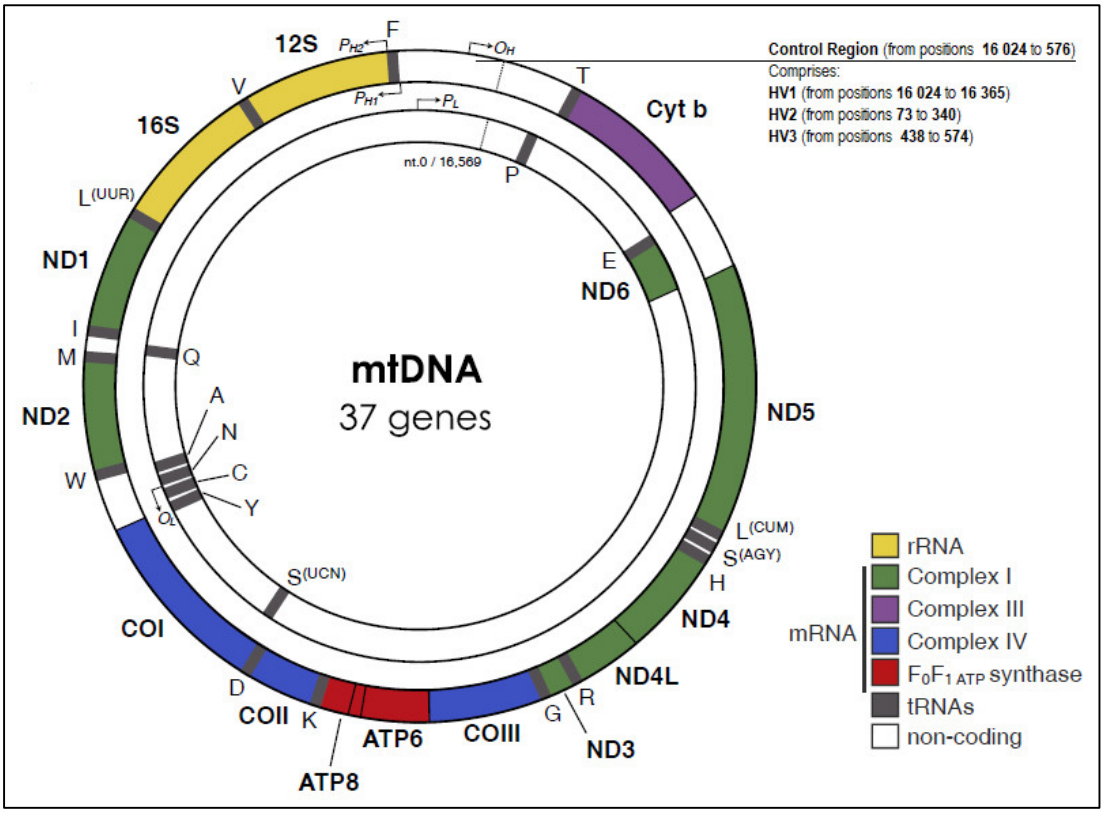

Figure 2.1: The structure of mitochondrial DNA

(Source: https://peerj.com/articles/7314/)

For over the past 40 decades, the adoption of mtDNA as a genetic marker has been widely applied in phylogenetic studies (Moritz *et al.,* 1987). There are several reasons the mtDNA genes are more preferred to be used such as the nature of mtDNA is haploid and monoclonal (maternal) (Amorim *et al.,* 2019). Hence, assist in the interpretation of DNA sequencing results for a better representation of closely related species variation data. Secondly, the evolution of mtDNA is neutral as it is involved in basic metabolic function (respiration) and very rarely involved in adaptive processes (Galtier *et al.,* 2009). Finally, the rate of evolution of mtDNA is assumed to be clock-like and its divergence of nucleotide sequencing should approximately reflect divergence times (Moritz *et al.,* 1987).

Among the various availability of mtDNA markers used for species identification, the cytochrome b gene is the most suitable gene for phylogenies analysis concerning its protein structure and function (Esposti *et al.,* 1993).

2.2.1 MtDNA Cytochrome b Gene

The mitochondrial cytochrome b (cyt b) gene is widely used in numerous studies of phylogenetics as well as in forensic investigations of species identification (Parson *et al.,* 2000). The nucleotide sequence of cyt b gene provides phylogenetic information of animals which extends from the intraspecific level to the intergeneric level. Kocher *et al.* (1989) reported that the phylogenetic relationships among animal species were successfully identified using the primer of cyt b gene based on the homologous segments of more than 100 animal species, including mammals, amphibians, reptiles, birds, fish, insects, and spiders. Furthermore, Ni'mah *et al.* (2016)

presented that pork contamination in both fresh and cooked beef can be detected up to 1% of the contamination using Duplex-PCR of mtDNA cytb gene.

The identification of species in processed foods especially in canned products becomes more challenging due to the nature of the final product. The exposure to a higher temperature during heat treatment of the processed food can degrade the DNA (Ni'mah *et al.,* 2016). Therefore, reducing the sensitivity of the molecular analysis. Nevertheless, a study by Cutarelli *et al.* (2018) has successfully identified species of 60 canned seafood products using cyt b gene. This study showed that the quality of the mtDNA was not affected despite the product was preserved in different conditions such as salt-curing, cooking, and packaging in oil (Cutarelli *et al.,* 2018).

As reported by Pesole *et al.* (1999) the cyt b gene of mammals mtDNA genome is one of the slowest-evolving genes in terms of non-synonymous mutation. This suggests that the data obtained in the present study reinforce the previous findings on the applicability of cyt b gene for species diagnosis, thus making this gene ideal for species identification (Silva-Neto *et al.,* 2016).

2.2.2 Other MtDNA Markers

Species identification can also be performed using other mtDNA markers such 12S rRNA, 16S rRNA, ND (1-6 genes), cytochrome c oxidase subunit I (COI), ATPase and control region (De Mandal *et al.,* 2014). In a study reported by Karabasanavar *et al.* (2017), beef specific PCR assay that was developed by targeting a conserved region of the mitochondrial D-loop to detect raw beef materials, cooked as well as adulterated meat samples (beef meat mixed with different animal species).

Results showed that positive amplification was reported in all samples indicate that PCR based mtDNA gene is sensitive and rapid (Karabasanavar *et al.,* 2017).

Similarly, Yang *et al.* (2014) demonstrated that by using the mitochondrial 12S rRNA and 16S rRNA genes as molecular markers, the nucleotide variations among interspecies and intraspecies were able to be identified in birds, insects, fish, amphibians, and mammals including human beings. The use of the universal primers from 12S and 16S rRNA was able to identify a wide range of animals based on a conserved region (Yang *et al.,* 2014). While cytochrome oxidase I (COI) gene which is the protein-coding gene was frequently found to be used as molecular marker for evolutionary studies (De Mandal *et al.,* 2014). COI gene is the largest gene among the three mitochondrial genes encoding cytochrome oxidase subunit and shows high rate of nucleotide substitution which is useful to discriminate ambiguous or cryptic species (De Mandal *et al.,* 2014). TABLE 2.1 displayed each mtDNA gene with its respective characteristics (Arif *et al.,* 2009).

Table 2.1: Characteristics of each of mtDNA gene (Arif et al., 2009).

Types of Molecular Markers	**Characteristics**
Mitochondrial DNA	Inherited from the mother (maternal lineage); rare exceptions do exist.
	Degrade slower than nuclear DNA. It can be used in degraded or old samples.
	Evolves about 10–fold faster than nuclear DNA; no proofreading activity.
12S rRNA	Highly conserved; used for high-category levels: phyla and subphyla.
16S rRNA	Usually used in mid–category differentiation such as families.
Protein-coding Genes	Used in low categories such as families, genera, and species.
Control Region	Used for identification of species and subspecies.

2.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is the most well-developed molecular technique due to simple, rapid, highly sensitive and specific tool for detecting constituents of animal origin in foods (Mafra *et al.*, 2008; Fajardo *et al.*, 2010). It is an enzymatic process in which a specific target region of the DNA genome is replicated repeatedly to produce multiple copies of a particular sequence (Figure 2.2). The PCR process involves heating and cooling of samples in a precise thermal cycling pattern for over approximately 30 cycles. Each cycle of synthesis of DNA fragment consists of 3 steps being carried out at 3 different temperatures respectively: 1) denaturation step where the DNA strands are separated at 94°C; 2) At annealing step the primers bind to DNA template at 60°C; and 3) elongation step where the DNA polymerase extends the primers by copying the target region using the deoxynucleotide triphosphate building blocks at 72°C (Spychaj *et al.*, 2009). The amplification of DNA fragments (also known as amplicons) followed by fragment size verification using agarose gel electrophoresis, is the simplest PCR strategy applied to identify and evaluate the presence of animal species in meat products (Fajardo *et al.*, 2010).

PCR-based species determination method is proven to be more suitable in the analysis of processed food products due to the greater stability as well as the DNA structure is more stable compared to protein structure (Calvo *et al.*, 2002; Dalmasso *et al.*, 2004). During food production, proteins are easily degraded under extreme temperature and pH treatments, thus rendering the method of using protein analysis unreliable for species identification purposes.

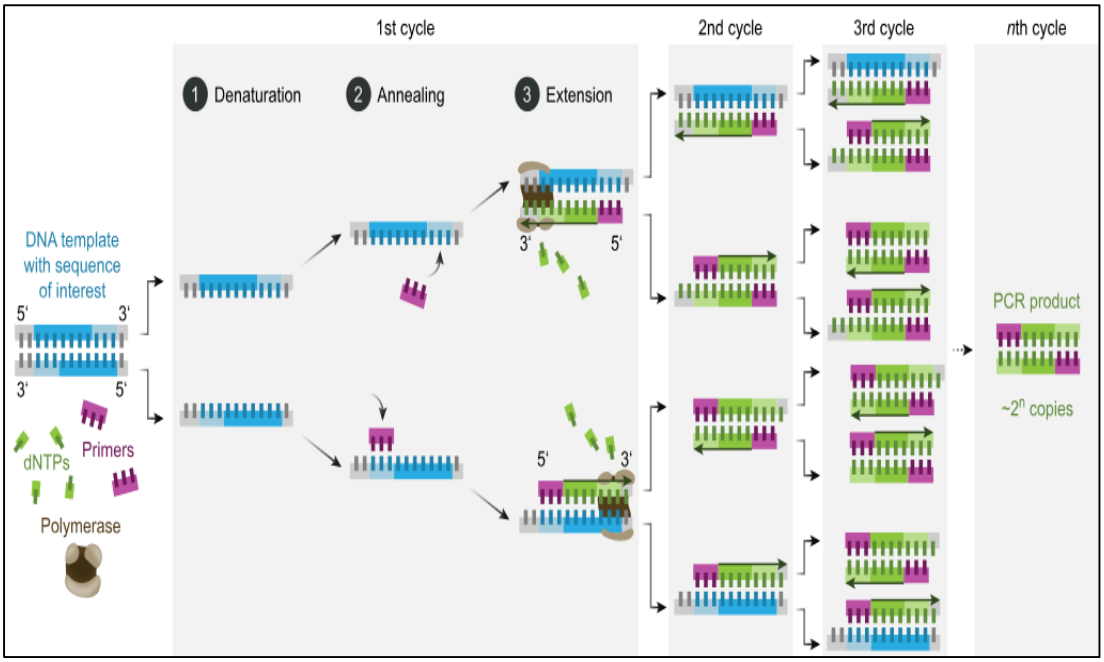

Figure 2.2: The diagram shows the process involves during PCR Amplification.

(Source: https://en.wikipedia.org/wiki/Polymerase_chain_reaction)

Previous studies have shown that PCR-based methods can distinguish different species of closely related animals in the processed food individually or in a complex mixture (Lockley *et al.* 2000; Spychaj *et al.*, 2009).

Kitpipti *et al.*, (2014) has applied PCR to detect adulterations (such as meat substitution or trace contaminations) and misbranding from a variety of meats and food products, including highly degraded or processed food samples. There are various methods based on the PCR technique can be used for species identification and food authentication include PCR with species-specific primers, PCR-single strand conformation polymorphism (PCR-SSCP), multiplex PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-random amplified polymorphic DNA (PCR-RAPD) and real-time PCR (Spychaj *et al.*, 2009).

2.3.1 Randomly Amplified Polymorphic DNA (PCR-RAPD)

The concept of random amplified polymorphic DNA technique involves the amplification of multiple random segments of genomic DNA using a single primer of arbitrary nucleotide sequence, followed by the separation of amplicons based on their sizes using gel electrophoresis (Arslan *et al.*, 2005). The DNA bands are then compared on the gel to identify the samples. PCR-RAPD technique has been used for species identification of-animals, plants, and microorganisms (Arslan *et al.*, 2005; Koveza *et al.*, 2005; Fajardo *et al.*, 2010). Wu *et al.* (2006) and El-Jaafari *et al.* (2008) were able to identify different species belonging to the family of *Cervidae* such as sika deer, sambar deer, tufted deer, black muntjac and Reeve's muntjac by using PCR-RAPD.

The main advantage of the PCR-RAPD method does not require prior information on the examined DNA sequence to reveal species-specific patterns

(Williams *et al.*, 1990). This method is also cheap and easy to perform as it avoids more complex analytical steps such as DNA sequencing, restriction, or hybridisation. However, there are A few drawbacks to this method: 1) the PCR amplifications have to be developed under strictly controlled conditions since the temperature, number of cycles or concentration of PCR components can affect THE RAPD results; 2) due to the non-specific nature of PCR reaction, THE RAPD technology is not suitable to identify THE target species organisms in mixed meats containing multiple species; 3) mismatch between the primer and the DNA template may affect the PCR products, thus interpretation of result becoming difficult (Wu *et al.*, 2006; Fajardo *et al.*, 2010).

2.3.2 Restriction Length Fragment Polymorphism (PCR-RFLP)

The PCR restriction fragment length polymorphism (PCR-RFLP) method is based on the amplification of the selected DNA fragment followed by digestion using an appropriate restriction enzyme which allows species differentiation within closely related species (Spychaj *et al.*, 2009). The PCR-RFLP method has been thoroughly applied for the identification of species in meat products (Girish *et al.*, 2005; Maeda *et al.*, 2006). In an experiment conducted by Girish *et al.* (2007), various types of poultry including chicken, duck, turkey, guinea fowl and quail were prepared in 3 different conditions (raw, heated, and autoclaved) and analysed using PCR-RFLP based on the mitochondrial 12S rRNA gene. All the poultry species were able to be identified from raw meats as well as in heated products. However, the autoclaved meat showed barely visible band on the gel (Girish *et al.*, 2007).

Carrera *et al.* (1999) reported that the PCR-RFLP technique has been used to identify a wide range of fish species by targeting the molecular markers of 16S rRNA

and COII genes. As stated by Pfeiffer *et al. (*2004), the PCR-RFLP technique does indeed present the advantages of being simple, cheap, and especially adaptable for large-scale studies. On the contrary, this technique might not be applicable in the analysis of meats which are subjected to thermal DNA degradation.

2.3.3 Single-Strand Conformation Polymorphism (PCR-SSCP)

The single-strand conformation polymorphism (SSCP) of DNA is an electrophoretic technique THAT IS widely exploited for the detection of mutations and polymorphisms in DNA (Peters *et al.,* 2000). Initially, double-stranded DNA fragments (dsDNA) are amplified with the aid of the PCR technique whereby the fragments undergo denaturation (most commonly due to thermal conditions) in the presence of denaturation agent such as formamide or sodium hydroxide (Spychaj *et al.,* 2009). The single-stranded DNA fragments (ssDNA) are then subjected to polyacrylamide gel electrophoresis (PAGE). Under non-denaturing conditions, the ssDNA will fold itself into a unique characteristic of a 3-dimensional secondary structure based on nucleotide sequences, temperature, and ionic strength (Schwieger *et al.,* 1998). Depending on the conditions applied for PCR, denaturation, and electrophoresis, respectively, multiple ssDNA conformation states can occur. However, with the presence of reference samples subjected to PCR-SSCP under similar conditions as the analysed samples, species identification is possible (Rehbein *et al.,* 1997).

The main limitation of the PCR-SSCP method is due to the high rate of reannealing of DNA strands after an initial denaturation during electrophoresis (Selvakumar *et al.,* 1997). Furthermore, excessive temperatures could reduce the effectiveness of conformer production, which in turn might cause the obtained results

in gel difficult to interpret. Nevertheless, based on the research led by Rehbein *et al.* (1997), the PCR-SSCP method was applied to identify closely related fish species in raw meat, thermally treated tinned fish as well as fish products which were not made up of muscle tissue i.e., caviar.

2.3.4 Real-Time PCR

Earlier study on PCR was mainly focus on the qualitative detection of DNA, where amplicons were separated by visual detection using gel electrophoresis method (Ulca *et al.,* 2013). Recently, more work has progressed towards using real-time PCR to achieve a rapid and highly sensitive quantification of DNA (Rodríguez *et al.,* 2005; Ulca *et al.,* 2013). The quantity of amplicons in each PCR reaction cycle is monitored in real time by measuring the intensity of signals derived from DNA-binding dyes or fluorescently labelled sequence-specific primers or probes. The increase in fluorescence signals is directly proportional to the quantity of amplicons produced in the reaction (Rocha et al., 2015).

The main advantage of real-time PCR is that the continuous measuring of fluorescence during each PCR cycle enables the skipping of gel preparation and electrophoresis steps that are typically required after executing the PCR reaction (Spychaj *et al.,* 2009). The other main advantage is that since the samples containing the reaction mixture were sealed during the analysis, real-time PCR significantly reduces the likelihood of contamination (Rodríguez *et al.,* 2005). Based on Oh *et al.* (2020), due to its high specificity and sensitivity, the real-time PCR was able to detect very small amounts of target DNA in complex processed foods.

Real-time PCR technique has been widely used for the identification of meat species in processed food products (Chen *et al.,* 2020). According to Chisholm *et al.* (2005), the real-time PCR was applied to detect trace quantities of donkey and horse meats in commercial food products. Rodríguez *et al.* (2005) was able to detect pork meat at the level of 0.5% in a beef and pork mixture subjected to sterilisation. In the analysis of qualitative and quantitative detection of mealworm DNA in processed food products, Natonek-Wiśniewska *et al.* (2022) demonstrated that out of the examined 13 processed foods consisting of dried mealworm larvae, dried crickets, freeze-dried yellow mealworms, crustaceans and dipterans, yellow mealworm species was successfully identified in 7 products by using species-specific real-time PCR assay.

Due to the diversity of various meat species in the composition of processed food products as well as the different manufacture processes subjected to processed foods, the accuracy of real-time PCR analysis could be affected. However, Chen *et al.* (2020) presented that the lowest detectable level of bovine DNA in different types of beef meat products with varying matrix and composition was 0.025 ng at a 95% confidence level. Thus, highlighting the specificity and sensitivity of real-time PCR assay in the identification and quantification of bovine ingredient in commercial meat products (Chen *et al.,* 2020).

A real-time PCR assay is a highly sensitive, rapid, and specific method to measure the extracted DNA of target species in processed food complexes (Oh *et al.,* 2020). Nevertheless, several parameters such as good design primers and high purity levels of extracted DNA are important to ensure the results produced in the analysis of real-time PCR are more reliable and accurate (Rocha *et al.,* 2015).

2.3.5 Multiplex PCR (mPCR)

Multiplex polymerase chain reaction (mPCR) assay offers the possibility of identifying several target regions on a single test platform, hence reducing the amount of time and money spent on analytical procedures (Ali *et al.,* 2014). Targeting two or more genes at the same time with a shorter amplicon length in a mPCR assay would be more reliable and trustworthy than targeting a single gene. In this method, the detection of an alternative target can compensate for the absence of the original target if it is lost due to the states of decomposition (Hossain *et al.,* 2017).

With the use of mPCR, numerous targets are amplified simultaneously, which enables the identification of a large number of species in a relatively short amount of time. Study findings from Abdul-Hassan & Tauma (2014) has showed that the multiplex PCR of the cyt-b gene was effective in identifying meat from cattle, sheep, goats, buffalo, chickens, pigs, and horses using a species-specific primers. Ali *et al.* (2015) has created a multiplex PCR assay from mtDNA ND5, ATPase 6, and cyt b genes for the detection of five types of meats belonging to cat, dog, pig, monkey, and rat.

However, it is important to note that the multiplex PCR should be constructed with careful consideration by choosing a suitable gene to be amplified, the relative sizes of the fragments, the dynamics of the primers, and the optimization of the PCR process to accommodate multiple fragments (Abdul-Hassan & Tauma, 2014). In mPCR analysis, any presence of a significant mismatch in the primer binding site might impaired the effectiveness of a PCR or will cause failed amplification.

2.3.6 Singleplex PCR

Currently, the PCR based species-specific primers or also known as singleplex is a novel method that has capability to identify various meat species, including raw meats as well as meats that undergone thermal treatments. The knowledge of the nucleotide sequence of a particular gene serves as a foundation for species identification (Spychaj *et al.*, 2009).

In this method, the incorporation of control samples during amplification is compulsory to eliminate any possibility of false positive or negative findings. Because of its simplicity, dependability, and capability to identify and discriminate target DNA, species-specific PCR prove to be an extremely useful tool for the identification of species (Karabasanavar *et al.*, 2017). Based on the experiment conducted by Hird *et al.* (2005), the singleplex PCR makes it feasible to differentiate between different types of meat that come from closely related animals with nucleotide sequences that have a high degree of similarity, such as chickens and turkeys. According to Hossain *et al.* (2017), species-specific PCR assays targeting two mitochondrial genes of cyt b and ND5 were designed due to its features of higher degree of divergence and availability of sufficient conserved regions within the species but adequate polymorphism among closely related species. As a result, the screening of meatball products purchased from Malaysia demonstrated that all beef processed products were bovine positive, however 35% of the composition were found to be totally replaced by other meat species (Hossain *et al.*, 2017).

In view of the advantages of the singleplex PCR method, this study was inspired to develop a bovine-specific PCR assay while targeting the conserved cyt b gene of

mtDNA for the identification of mislabelling in processed beef products in Kelantan, Malaysia.

CHAPTER 3

METHODOLOGY

3.1 MATERIALS

3.1.1 Chemicals and Reagents

All reagents, chemicals and consumables used in this study are listed in Table 3.1.

3.1.2 Instruments and Apparatus

All instruments and apparatus used in this study are listed in Table 3.2

3.1.3 Buffer and Other Solution Preparations

3.1.3.1 Proteinase K (20 mg/mL)

Approximately 20 mg of Proteinase K was dissolved in 1 mL of sterile deionized water (ddH_2O). The solution was then stored at -20°C.

3.1.3.2 10X Tris Borate EDTA (TBE) Buffer

TBE buffer stock solution was prepared by dissolving 108 g of Tris base, 55 g Boric acid and 9.3 g Na_2EDTA in 800 mL of distilled water using a magnetic stirrer. The solution was made up to 1 L by adding ddH_2O. The solution then was autoclaved and stored at room temperature.

3.1.3.3 0.5X Tris Borate EDTA (TBE) Buffer

The working solution buffer for electrophoresis was prepared by diluting 50 mL of 10X TBE Buffer in 950 mL of ddH_2O.

3.1.3.4 70% Ethanol

The solution was prepared by mixing 30 mL of ddH_2O with 70 mL of absolute ethanol and was stored at ambient temperature.

Table 3.1: All reagents, chemicals, and consumables used in this study

Reagents / Chemicals	**Company / Supplier**
Fresh beef meat (1 kg)	Wet market Kota Bharu
Absolute Ethanol	HmBG Chemicals, Malaysia
Deionised distilled water (ddH_2O)	USM, Malaysia
Chloroform	Merck Darmstadt, Germany
Proteinase K powder	Bioline, USA
Food Lysis Buffer	Qiagen, Germany
Buffer PB	Qiagen, Germany
Buffer AW2, concentrate	Qiagen, Germany
Buffer EB	Qiagen, Germany
Cyt-b primer (Forward and reverse)	Integrated DNA Technologies (IDT), USA
Taq polymerase	Fermentas, USA
Taq buffer	Fermentas, USA
Magnesium Chloride ($MgCl_2$)	Fermentas, USA
dNTP mix	Thermo Scientific, USA
Agarose gel powder	Amresco VWR Life Science, USA
Ethidium bromide	Sigma Aldrich, USA
Orange G. dye	Sigma Aldrich, USA
100 bp DNA ladder	Vivantis, USA
1kbp DNA ladder	Vivantis, USA
Boric Acid powder	Merck Darmstadt, Germany
Tris base powder	Promega, USA
EDTA powder	1st BASE, Singapore

Table 3.1, continued

Consumables	**Brand / Manufacturer**
Micropipette tips (10 μL, 200 μL, 1000 μL)	Axygen Scientific, USA
Microcentrifuge tubes (1.5 mL, 2 mL)	Bio-Rev, Singapore
QIAquick® Spin Columns (2 mL)	Qiagen, Germany
Parafilm	Bemis, Malaysia
PCR tubes (0.2 mL)	Corning Life Science, China