

MOLECULAR DETECTION OF BOVINE DNA IN PROCESSED CAT WET
FOOD PRODUCTS THROUGH PCR AMPLIFICATION USING
MITOCHONDRIAL DNA ND5 GENE

By

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Dissertation submitted in partial fulfilment of the requirements for degree of
Bachelor of Science (Honors) (Forensic Science)

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CERTIFICATE

This is to certify that the dissertation entitled “**MOLECULAR DETECTION OF BOVINE DNA IN PROCESSED CAT WET FOOD PRODUCTS THROUGH PCR AMPLIFICATION USING MITOCHONDRIAL ND5 GENE**” is the bona fide record of research work one by MS. WAN NUR AZMIERA NAZIHAH BINTI WAN AZNAN during the period from October 2025 to January 2026 under my supervision. I have read this dissertation, and in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted I partially fulfilment for the degree of Bachelor of Science (Honours) (Forensic Science).

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DECLARATION

I hereby certify that this dissertation is the result of my own investigation, except where otherwise stated and duly knowledge. I also declare that it has not been previously for concurrently submitted as whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use dissertation for teaching, research or promotional purposes.

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LIST OF ABBREVIATIONS

Bp	Base pair
ND5	NADH dehydrogenase subunit 5
°C	Degree Celsius
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
dsDNA	Double-stranded DNA
dNTPs	Deoxyribonucleoside triphosphate
g	Gram
V	Volt
Kbp	Kilo base pair
mg	Milligram
mL	Milliliter
M	Molar
mM	Micromolar
MgCl ₂	Magnesium chloride
PCR	Polymerase chain reaction
μL	Microliter
UV	Ultraviolet
RCF	Relative Centrifugal Force
T _m	Melting temperature
TE	Tris EDTA

TBE	Tris Borate EDTA
rRNA	Ribosomal ribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
BLAST	Basic Local Alignment Search Tool
EDTA	Ethylenediaminetetraacetic Acid
LOD	Limit of Detection
NADH	Nicotinamide Adenine Dinucleotide Hydride
NaOH	Sodium Hydroxide
qPCR	Real-time PCR
T _a	Annealing Temperature

**PENGESANAN MOLEKUL DNA LEMBU DALAM PRODUK MAKANAN
BASAH KUCING YANG DIPROSES MELALUI AMPLIFIKASI PCR
MENGUNAKAN GEN ND5 DNA MITOKONDRIA**

ABSTRAK

Pemalsuan makanan dan pelabelan yang tidak tepat bagi produk berasaskan haiwan menimbulkan kebimbangan yang ketara terhadap kepercayaan pengguna dan keselamatan makanan. Isu yang sama turut dilaporkan dalam makanan haiwan peliharaan, di mana spesies haiwan mungkin tidak diisytiharkan, digantikan, atau dinyatakan pada label produk tetapi sebenarnya tidak terdapat dalam produk. Produk makanan basah kucing kebanyakannya dihasilkan daripada sumber protein haiwan seperti daging lembu, ayam dan ikan. Dalam kajian ini, sebanyak 15 sampel produk makanan basah kucing yang diproses telah dianalisis untuk pengesanan DNA lembu menggunakan primer khusus spesies yang menyasarkan gen ND5 DNA mitokondria (mtDNA). Elektroforesis gel agarosa menunjukkan bahawa DNA genomik yang diekstrak daripada semua sampel yang diproses sangat terdegradasi. Walaupun demikian, amplifikasi tindak balas rantai polimerase (PCR) berjaya mengesan fragmen bersaiz 214 bp dalam tiga sampel berlabel lembu (37.5%) dan tiga sampel tanpa label lembu (42.9%), sekali gus menunjukkan kehadiran DNA lembu. Menariknya, tiga sampel positif lembu daripada produk makanan basah kucing tanpa label lembu pada asalnya dikenal pasti sebagai daging kambing, manakala dua produk lagi diklasifikasikan sebagai kuah daging tidak diketahui. Keputusan ini mungkin mencadangkan kemungkinan berlakunya pemalsuan dan penipuan dalam produk makanan basah kucing. Secara keseluruhannya, dapatan kajian ini menunjukkan

bahawa gen ND5 mtDNA merupakan penanda molekul yang sesuai untuk pengesanan DNA lembu dalam produk makanan haiwan peliharaan yang diproses. Kaedah ini tidak menunjukkan sebarang kereaktifan silang dengan tujuh spesies bukan sasaran dan berjaya mengesan DNA lembu pada kepekatan serendah 10 pg/ μ L, sekali gus menonjolkan kekhususan dan kepekaannya. Kesimpulannya, amplifikasi PCR menggunakan primer khusus spesies menyediakan pendekatan yang boleh dipercayai untuk pengesanan ketulenan makanan haiwan peliharaan dan penyiasatan forensik.

MOLECULAR DETECTION OF BOVINE DNA IN PROCESSED CAT WET FOOD PRODUCTS THROUGH PCR AMPLIFICATION USING MITOCHONDRIAL DNA ND5 GENE

ABSTRACT

Food adulteration and mislabelling of animal-derived products pose significant concerns for consumer trust and food safety. Similar issues are also reported in pet foods, where animal species may be undeclared, substituted, or declared on product labels but not actually present in the final product. Cat wet food products are predominantly derived from animal protein sources such as beef, chicken, and fish. In this study, 15 samples of processed cat wet food products were analysed for bovine DNA using species-specific primers targeting the mitochondrial DNA (mtDNA) ND5 gene. Agarose gel electrophoresis revealed that genomic DNA extracted from all processed samples was highly degraded. Despite this, polymerase chain reaction (PCR) amplification successfully detected a 214 bp fragment in three bovine-labelled samples (37.5%) and three samples without a bovine label (42.9%), indicating the presence of bovine DNA. Interestingly, 3 positive bovines from cat wet food products without a bovine label were initially identified as lamb meat and two products were classified as unknown meat gravy. This result may suggest the possibility of fraud in cat wet food products. Overall, these findings demonstrate that the mtDNA ND5 gene is a suitable molecular marker for bovine detection in processed pet food products. The method showed no cross-reactivity with 7 non-target species and successfully detected bovine DNA at concentrations as low as 10pg/ μ L, highlighting its specificity and sensitivity. In summary, PCR amplification using species-specific primers provides a reliable approach for pet food authentication and forensic investigations

CHAPTER 1

INTRODUCTION

1.1 Study Background

Pets like cats are considered companions because they live closely with humans and are emotionally bonded. An accumulating body of research has shown that pets can have a positive physiological impact, especially for people facing major life challenges (Kanat-Maymon *et al.*, 2015). Therefore, ensuring nutrients and maintaining good health in pets are essential responsibilities of pet owners. Food is one of the most fundamental needs that is consumed for everyday activities, and its nature play significant role in the health and productivity of living organisms (Anagaw *et al.*, 2024). Although ingredient labelling is intended to inform consumers of food product composition, concerns have emerged regarding the accuracy of such labels, particularly related to species identification and product authenticity.

Adulteration in processed cat food products has been a concern, as it not only affects food quality but also has a tremendous effect on health without our knowledge. Food adulteration refers to the reduction of food quality by substituting ingredients, adding non-authenticated substances or removing essential components from food for financial gain or other incidental reasons (Choudhary *et al.*, 2020). This may occur in various forms, including the substitution of claimed ingredients with cheaper ingredients. Such discrepancies may arise due to intentional adulteration, cross-contamination during processing, or limitations in quality control. In addition, meat is

particularly susceptible to fraudulent substitution by less valuable meat or meat coming from domestic animals (Böhme *et al.*, 2019).

Bovine is one of the common meats used as ingredients of processed cat wet food products (Darwin's, 2026). As per the Cambridge dictionary, bovine species refer to animals belonging to the cattle family, which include domestic cattle, *Bos taurus*. *Bos taurus* is typical herbivorous domestic cattle descended from Europe, north-eastern Asia, and parts of Africa (Keeton *et al.*, 2014). Malaysia's beef cattle population are dominated by the Kedah Kelantan (KK) and its crossbred. Malaysia started the crossbreeding program with the Brahman and Charolais in 1970 to increase the performance of Kedah Kelantan cattle (Islam *et al.*, 2021). In 2020, Malaysia's cattle population was reported to be 699,424, with 75% of them categorised as KK crossbreed. It is also worth noting that the other percentage, though it also has *Bos taurus* genes like Limousine and Brangus (Mohd Saufi *et al.*, 2023).

One of the primary concerns associated with ingredient mislabelling in cat food products is the risk of food allergies. Although beef is a great source of protein, some cats may be allergic to beef, resulting in symptoms like itching, vomiting or diarrhoea (Dial A Vet, 2025). When bovine-derived ingredients are present but not declared on the product label, owners may unknowingly expose cats to allergenic substances, leading to adverse health effects. Additionally, like humans, cats have appetitive preferences for specific meat types and components, which can influence their acceptance of commercial foods formulated with different animal proteins (Amir & Mona, 2013).

Therefore, verifying the authenticity of cat food and detecting adulterants are important for quality assessment and to assure consumer protection against fraudulent activities. A DNA-based molecular method is ideal for adulterant detection, especially in determining the presence of biological substances. The three main strategies commonly used in DNA-based methods are polymerase chain reaction, DNA sequencing and hybridisation (Bansal *et al.*, 2017).

1.2 Problem Statement

According to Mordor Intelligence (2022), cats account for approximately 55.3% of the total pet population in Malaysia based on 2022 reported data. Between 2019 and 2022, the cat populations increased by 32.2% and are expected to reach 1.3 million by 2025 (GlobalPETS News, 2025). This rapid growth has led to increasing demand for commercial cat foods due to their wide availability and convenience. Thus, concerns regarding the authenticity and accuracy of ingredient labelling within the pet food industry have increased.

Mislabelling of processed cat wet food products includes the presence of undeclared meat used in cat wet foods, occur and has been reported previously. Cat wet food products are commonly formulated with animal protein, such as beef and chicken (Era Pet Food, 2024). However, the undisclosed presence of bovine-derived material may pose risks to pets with food allergies or intolerances (Wang *et al.*, 2024). This is an issue particularly for pet owners who rely on ingredient information due to dietary restrictions, allergies or religious considerations. Additionally, there have been reported cases where declared ingredients were absent from the final product. In this situation, usually the artificial flavours were used, as modern food and technology can

produce cultured or artificial meat in laboratories (Kashim *et al.*, 2024). Such practices not only violate labelling regulations but also raise ethical, economic and health concerns.

In this study, mitochondrial DNA (mtDNA), namely NADH dehydrogenase subunit 5 (ND5), was used as a target marker for *Bos taurus* identification in processed cat wet food products. The detection of bovine may be challenging, as the processing method involves mechanical stress, high temperature, pH variations, enzymatic activities, and fermentations that degrade DNA into short fragments (Gryson, 2010; Wang *et al.*, 2024). Therefore, polymerase chain reaction-based identification of bovine mtDNA is suitable for processed and degraded samples due to the high copy number and stability of mtDNA (Kowalczyk *et al.*, 2021).

1.3 Significance of the Study

With the rapid growth of the pet food industry, the need for molecular authentication of cat food ingredients is increasing. This study is significant as it provides molecular-based evidence on the authenticity of both bovine-labelled and non-bovine-labelled processed cat wet food products. By applying polymerase chain reaction (PCR) techniques, this study demonstrates the suitability of DNA-based methods for species identification in highly processed pet food matrices.

Additionally, this study also provides a reference method for future research in species authentication of processed cat food products by establishing a framework for DNA extraction and PCR amplification. From a forensic science perspective, this study highlights the applicability of PCR as a reliable analytical tool in investigating

potential food adulteration and assessing quality control. It also contributes to scientific and forensic research by demonstrating the suitability of mitochondrial DNA genes as a marker for species identification in situations where nuclear DNA is degraded or unreliable.

1.4 Objectives

1.4.1 Main Objectives

To identify bovine DNA in processed cat wet food products using PCR amplification of the mitochondrial DNA gene

1.4.2 Specific Objectives

- I. To extract genomic DNA from various processed cat wet food brands using the silica-based method.
- II. To amplify bovine DNA using species-specific primers of the mtDNA ND5 gene.
- III. To determine the presence of bovine DNA in processed cat wet food products through agarose gel electrophoresis.

CHAPTER 2

LITERATURE REVIEW

2.1 Detection Method of Adulteration in Food Products

Authentication of food and detection of adulterants in various food products is required not only to comply with Section 272 of the Malaysian Penal Code, but also to protect consumers from fraud and maintain public confidence in the food supply chain. Various methods have been developed to authenticate processed food products and identify adulterants, including analytical, physical, chemical and DNA-based molecular techniques (Bansal *et al.*, 2017; Böhme *et al.*, 2019; Banti, 2020). Among them, the detection of adulterants by proving the presence of foreign substances is considered the best and simplest technique for adulterant detection (Bansal *et al.*, 2017).

Detection based on the physical methods is usually conducted through microscopic examination, macroscopic visual structural analysis and physical parameters analysis such as morphology, texture and solubility. Though these visual analyses do not guarantee qualitative adulterant detection, combining physical methods with chemical profiling can offer better identification and authentication, especially for herbs and medicinal plants (Bansal *et al.*, 2017; Banti, 2020). It is worth noting that relying solely on physical appearance can be misleading in food adulterant detection, as some adulterated food may look like genuine products.

Meanwhile, identification techniques relying on chemical and biochemical can be divided into 4 main categories, such as chromatograph-based, spectroscopy-based,

immunology-based and electrophoresis-based. Besides that, high-performance liquid chromatography (HPLC) and gas chromatography (GC) are also common techniques used but are limited to volatile compounds only. Although these methods proved to be more accurate and sensitive compared to physical methods, however it requires an in-depth understanding of the machinery and are very costly (Banti, 2020). Methods such as enzyme-linked immunosorbent assay (ELISA) have also been widely used in meat adulteration due to their high sensitivity and specificity. A study shows 0.01% and 0.1% of pork adulteration are detected for direct and competitive formats, respectively (Mandli *et al.*, 2018). ELISA is an immunological technique that involves an enzyme to detect the presence of an antibody or an antigen in a sample (Asensio *et al.*, 2008). This method relies on a stable antigen for species detection, making it unsuitable for routine analysis of food since the biological activities of most proteins are lost after animal death, and some foods are highly processed (Chi *et al.*, 2014).

Another method of detection is DNA-based molecular tools, which is ideal when the adulterant to be detected is a biological substance. Presence of variation in species-specific regions, like mitochondrial, enables differentiation for adulterant detection (Bansal *et al.*, 2017). Compared to ELISA, polymerase chain reaction has higher specificity, sensitivity and molecular stability, making DNA a perfect target molecule for food authenticity purposes. This allows detection of mislabelling and food fraud at trace levels and processed food (Böhme *et al.*, 2019).

2.2 Mitochondrial DNA (mtDNA)

For successful PCR amplification, the primer must anneal to a complementary target region within the DNA template. Various types of DNA have been used as target

regions, including nuclear DNA and mitochondrial DNA. Both nuclear and mitochondrial genomes have been widely used in forensic DNA for PCR and profiling (McDonald *et al.*, 2024). Nuclear DNA (nDNA) encodes most genes essential for cellular function and protein synthesis (Sidler, 2016). It is inherited from both parents, whereas mtDNA is exclusively from the mother (Ahmad, 2019).

Mitochondrial DNA is the circular chromosome found inside the cellular organelles called mitochondria, located in the cytoplasm. Mitochondria are the site of the cell's energy production (National Human Genome Research Institute, 2026). Owing of high copy number, mtDNA has strong inter-species specificity and high intra-species conservation, making it often selected for sensitive qualitative assays (Baptista *et al.*, 2021). Due to a lack of protective histones, mtDNA is vulnerable to mutations (Vadakedath *et al.*, 2023). The presence of hundreds to thousands of mitochondrial genomes per cell enables mtDNA analysis when standard methods markers fail. Additionally, when traditional sources like tissues and bodily fluids of nDNA are not available, mtDNA is an alternative way (McDonald *et al.*, 2024).

In forensic investigations, mtDNA analysis has long been used for challenging forensic samples because of its ubiquitous presence in biological material, even in cases where nDNA is absent or highly degraded (Pajnič, 2025; Court, 2021). The high copy number per cell and circular structure of mtDNA enhance its stability compared to nDNA (Pajnič, 2025). Both nuclear DNA and mitochondrial DNA can be used for PCR-based authentication. However, mtDNA is generally preferred because it is present in high copy numbers, has a highly conserved sequence within a species, and shows greater resistance to degradation, making it suitable even for small or

compromised samples (Baptista *et al.*, 2021). In contrast, nDNA can be less reliable for short DNA fragments (<150 bp), as miniSTRs may suffer from allele imbalances and dropouts (Bose *et al.*, 2018). Therefore, when DNA is highly degraded or limited, mtDNA serves as the alternative marker for detection and species identification.

2.3 Polymerase Chain Reaction (PCR)

Food authentication using DNA-based methods, particularly PCR is gaining ground due to advantages notably in the areas of food adulteration in plant and animal-based food (Kumar, 2022). Polymerase chain reaction (PCR) has been universally used in basic science and biomedical research. It is a laboratory technique to amplify specific DNA segments for various laboratory and clinical applications (Ghannam & Varacallo, 2023). PCR has been applied in the food industry as a technique for the detection and identification of animal and plant species due to its high sensitivity and specificity. The exponential amplification of the target DNA molecule using a thermal cycler can generate thousands to millions of copies, thus allowing detection of target species at very low concentrations (Böhme *et al.*, 2019).

One of the first PCR types is the conventional PCR, a well-established method with advantages including simple operation, lower processing time and ease of use. Main components of PCR are the DNA template, primers, free nucleotide bases (dNTPs), and DNA polymerase enzymes (Ghannam & Varacallo, 2023). It utilises specific primers that were designed based on the genetic sequence of the target DNA, enabling target DNA to be amplified for detection, analysis and determination of food components (Zhao *et al.*, 2025). Now, advances in gene detection technology have been developed, including real-time PCR, multiplex PCR, restriction fragment length

polymorphisms (RFLPs) and droplet digital PCR (ddPCR). PCR methods are suitable for use in highly processed food. According to Ulca *et al.* (2013), the PCR kit used correctly identified the animal species for both raw and cooked meats (Ulca *et al.*, 2013). In another study, PCR was used to identify bovine in ovine or caprine milk and cheese (Baptista *et al.*, 2021).

The principle of the polymerase chain reaction consists of three major phases: denaturation, annealing, and extension. During denaturation, DNA is heated to 95°C to disrupt the hydrogen bonds between complementary base pairs of double-stranded molecules. The annealing phase cooled the denatured DNA to the optimal annealing temperature of the primer, allowing primers to bind to single-stranded DNA by pairing their 3' ends to the template strand. In the final phase, DNA polymerase synthesises new strands in the 5' to 3' direction, generating sequences complementary to the template strands. These 3 phases are repeated for 30 - 40 cycles, amplifying multiple copies of the target DNA within the tube (Khehra *et al.*, 2025).

In conventional PCR analysis, amplified DNA products from each sample are subsequently separated by agarose gel electrophoresis according to their amplicon size. The presence of the target DNA is then visualized using a DNA-binding dye under a UV transilluminator (Baptista *et al.*, 2021). Although advanced PCR like qPCR offers higher accuracy and sensitivity, conventional PCR remains a practical choice as it is more accessible, cost-effective and suitable for routine analysis.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

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

Table 3.1: List of Chemicals and Reagents

Chemicals/ Reagent	Company/ Supplier
Food lysis buffer	Qiagen
Proteinase K	Qiagen
Binding buffer	Qiagen
Elution Buffer	Qiagen
Washing Buffer	Qiagen
Chloroform	Merck KGaA, Germany
Tris Base	Vivantis
Boric Acid	Merck KGaA, Germany
EDTA Disodium Salt Dihydrate	Sigma-Aldrich, USA
Ethidium Bromide	Sigma-Aldrich, USA
Orange G Loading Dye	Sigma-Aldrich, USA
1 Kbp DNA Ladder	GeneDireX
100 bp DNA Ladder	Vivantis
Taq Buffer	Vivantis
MgCl ₂	Invitrogen, Brazil

dNTPs	Promega
Taq Polymerase	Vivantis
Absolute Ethanol	HMBG
Primers	Integrated DNA Technologies
Agarose Powder	Matrioux

3.1.2 Consumables Materials

All consumables utilised in this study are listed in Table 3.2.

Table 3.2: List of Consumables Materials

Consumables Materials	Brand
p10 μ L, p200 μ L and p1000 μ L pipette tips	Kirgen
2.0mL and 1.5mL microcentrifuge tubes	Teraslab, SDN BHD
0.2mL PCR tubes	Teraslab, SDN BHD
Parafilm	Teraslab, SDN BHD
Nitrile gloves	GLOF
50mL Falcon tubes	Teraslab SDN BHD
Kimwipes	Kimtech Science
Aluminum foil	-
Masking tape	Apollo M502

3.1.3 Instrument and Equipment

All instruments and equipment utilised in this study are listed in Table 3.3.

Table 3.3: List of Instruments and Equipment

Instrument/ Equipment	Brand	Model
Pipette	Gilson/ Eppendorf	P10, P20, P100, P200 and P100
Analytical Balance	Sartorius	BSA 224S-CW
Shaking Water Bath	Memmert	WNB14L4
Hot Air Oven Sterilizer	Memmert	UE600
Vortex Mixer	ERLA	EVM-6000
Protector Laboratory Fume Hood	Labconco	-
Hot plate and Magnetic Stirrer	ERLA	-
Microcentrifuge (Refrigerated)	Hettich Universal	Micro 22R
Microwave Oven	Elba	Emo-1706
DNA Electrophoresis System	Owl	B1 and B2
Power Pack	Bio Rad	Power Pac 3000V
UV Transilluminator	Uvitec, Cambridge	BTS-26.MS
Nanodrop Microvolume Spectrophotometer	Denovix	DS-11
Thermal Cycler PCR System	Veriti	Thermal Cycler, 96-well Fast

3.1.4 Buffer and Other Solutions Preparations

In this study, several buffers and solutions were prepared. This included 0.5 M EDTA solution (pH 8.0), 10X TBE buffer and 0.5X TBE buffer. In addition, 70% ethanol was also prepared for routine laboratory use. Lastly, Orang G loading dye was also prepared according to standard protocols.

3.1.4.1 0.5M Ethylenediaminetetraacetic acid (EDTA) Solution (pH 8.0)

To prepare a 0.5 M EDTA solution, 93 g of ethylenediaminetetraacetic acid (EDTA) powder was placed into a 500 mL autoclave-safe Schott bottle. Next, 400 mL of deionised water was added to a beaker and stirred with a magnetic stir bar until the EDTA dissolved. The pH of the solution was adjusted to 8.0 using NaOH pellets. The solution was then poured into a graduated cylinder and made up to a final volume of 500 mL with deionised water. After that, the solution was transferred into the Schott bottle, autoclaved, and stored at room temperature.

3.1.4.2 10X Tris-Borate EDTA (TBE) Buffer

The 10X TBE buffer was prepared by weighing out 108g of Tris base and 55g of boric acid. Next, 200mL of deionised water was added to the 1 L beaker containing Tris-base and boric acid and was stirred using a magnetic bar until fully dissolved. Then, 40 mL of 0.5 M EDTA solution (pH 8.0) and 100 mL of deionised water were added. The solution then transferred to a 1L volumetric flask and deionised water was added until the calibration mark. The solution was homogenised by inverting the flask several times and transferred into a Schott bottle for autoclaving. The autoclaved solution was stored at room temperature.

3.1.4.3 0.5X Tris-borate EDTA (TBE) Buffer

Preparation of 0.5X TBE buffer was done by mixing 50 mL of 10X TBE buffer with 950 mL of deionised water. The solution was homogenised by inverting the flask several times, then transferred into a Schott bottle, autoclaved and stored at room temperature.

3.1.4.4 70% Ethanol

A total of 700mL of absolute ethanol was measured using a 1 L measuring cylinder and transferred to a 1 L volumetric flask. Deionised water was then added until the final volume reached the calibration mark. The mixture was gently mixed by repeatedly inverting the flask before being transferred into a spray bottle and kept at room temperature.

3.1.4.5 Orange G Loading Dye

A total of 0.125g of Orange G powder and 20 g of sucrose were dissolved in 30 mL of deionised water. Deionised water was then added to bring the final volume to 50 mL. The solution was subsequently aliquoted into 1.5 mL microcentrifuge tubes and stored at -20°C .

3.2 Sample Collection

For this study, raw beef meat (*Bos taurus*) was used as the positive control. Several other commonly consumed meat species, namely buffalo (*Bubalus bubalis*), goat (*Capra hircus*), quail (*Coturnix japonica*), duck (*Anas platyrhynchos*), chicken (*Gallus gallus domestica*), fish (*Oreochromis mossambicus*) and pig (*Sus scrofa*) were

used as non-target species. All samples were purchased from wet markets in Kota Bharu and were kept at -20 °C until DNA extraction.

A total of 15 processed cat wet foods were bought from various pet shops around Kota Bharu. There are three types of wet food packaging used, such as cans, pouches and sticks. Out of 15 cat wet food samples, 7 were labelled as containing beef, and 6 samples did not have information about the meats used. Two products lacked a clear ingredient list but were advertised as beef-flavoured and fish-flavoured. Details of all processed wet cat food samples are provided in Appendix A and Appendix B.

3.3 DNA Extraction

The QIAgen DNeasy Mericon Food Kit was used for the DNA extraction from raw meat and processed wet food samples. Approximately 100 mg of homogenised raw meats were placed into a 2 mL microcentrifuge tube and labelled accordingly. DNA extraction of the processed cat wet food samples required 200 mg of homogenised cat wet food and placed into a 2 mL microcentrifuge. Cat wet food samples were labelled as S1 to S15.

Subsequently, 1000 µL of food lysis buffer and 20 µL of Proteinase K solution were added to each tube. Each tube was vortexed briefly to ensure complete distribution and moistening of the sample. All raw meat samples were then incubated for 2 hours in a water bath at 56°C with constant shaking, while processed cat wet food samples were incubated overnight under the same setting conditions.

Following the incubation, all tubes were centrifuged for 5 minutes at $2500 \times g$. The clear supernatant was carefully transferred into new tubes without disturbing the precipitated inhibitors at the bottom. Then, 500 μL of chloroform was added to each new tube, followed by brief mixing. The tubes were then centrifuged again at $14,000 \times g$ for 15 minutes.

After that, 250 μL of the aqueous upper layer from the previous step was drawn into a new 2 mL microcentrifuge tube. Then, 1000 μL of buffer PB was added to the tube and mixed. Next, 600 μL of the mixture was pipetted into the QIAquick spin column that placed in a 2 mL collection tube. All samples were then centrifuged at $17900 \times g$ for 1 minute and the flow-through was discarded. This step was repeated for the remaining mixture. Then, 500 μL of buffer AW2 was added to the QIAquick spin column, centrifuged at $17900 \times g$ for 1 minute and the flow-through was discarded. To dry the membrane, the spin column was centrifuged again at $17900 \times g$ for 5 minutes.

Finally, the QIAquick spin column was transferred to a 1.5mL microcentrifuge tube and 100 μL of buffer EB was pipetted directly onto the membrane. All samples were incubated for 1 minute at room temperature and centrifuged at $17900 \times g$ for another 1 minute to ensure all DNA was eluted.

3.4 Agarose Gel Electrophoresis of Genomic DNA

Firstly, the casting tray was prepared by sealing both ends with masking tape and the comb was placed. After that, the agarose gel solution was prepared by weighing 1g of agarose powder and transferring it into a 250 mL Schott bottle. Then,

100 mL of 0.5X TBE buffer was added and the mixture was gently swirled to disperse the powder. The bottle was closed and heated in a microwave oven for 2 minutes at medium-low temperature until the agarose was completely dissolved.

After heating, the solution was allowed to cool until warm to the touch. Then, 2 μ L of ethidium bromide was added and the solution was gently swirled to ensure even mixing. The molten agarose was poured into the casting tray and left to solidify for 1 hour. Once the gel had fully hardened, the comb was removed and the tray was placed into an electrophoresis buffer tank. The remaining 0.5X TBE buffer was poured into the tank until it covered the gel.

Next, 2 μ L of 1 Kbp DNA ladder was pipetted into the first well to serve as a molecular size marker. A parafilm was prepared and 2 μ L of orange G loading dye was placed onto its surface. For sample loading, 5 μ L of the extracted DNA was mixed with the orange G loading dye by pipetting up and down. Then, the mixture was carefully loaded into the designated well.

After all samples had been loaded, the electrophoresis tank cover with electrodes was placed onto the tank and the electrodes were connected to the appropriate terminals of the power pack. The run was set at 90 V for 1 hour. Upon completion of the run, the agarose gel was visualised using a UV transilluminator, and all results were recorded.

3.5 DNA Quantification

All extracted raw meats and processed cat wet food samples were quantified using a Nanodrop Microvolume Spectrophotometer. The instrument was switched on, and a user account was created. Prior to measurement, the sampling surface of the spectrophotometer was cleaned thoroughly. Then, 2 μ L of distilled water was pipetted onto the sampling surface, the sampling arm was lowered, and the water was allowed to sit for approximately 15 seconds. The arm was then lifted, and the surface was wiped clean using a Kimwipe tissue.

For DNA quantification, the dsDNA measurement mode was chosen as the samples being identified were double-stranded DNA. The same elution buffer from DNA extraction was used as a blank for quantification. For extracted DNA samples, 1 μ L of each extracted sample was loaded onto the sampling surface and measured. After each measurement, the sampling surface was wiped using the Kimwipe tissue.

Once all samples had been quantified, the sampling surface was cleaned again. The data was sent via email. The results were recorded and graphs for each sample were plotted.

3.6 Oligonucleotide Primers

In this study, two primer sets were used for PCR amplifications, namely the 18S ribosomal RNA (rRNA) and mtDNA NADH dehydrogenase subunit 5 (ND5) primers. The 18S rRNA primer served as an internal control to confirm the presence of DNA in the samples. This primer was adapted from a previously published study from Fajardo et al. (2008). The primer sequence of 18S rRNA for forward orientation

was: 5'- TCTGCCCTATCAACTTTCGATGG -3' and reverse: 5'- TAATTTGCGCGCCTGCTG -3'. Amplification of the internal control primer generated a 140 bp amplicon. of the 18S rRNA gene.

Meanwhile, detection of bovine DNA from cat wet foods was carried out using a species-specific primer targeting the mtDNA ND5 gene. This primer was newly designed based on the *Bos taurus* mitochondrial reference genome (NC_006853) and had been validated by a previous student. However, before being used in this study, the mtDNA ND5 primers were evaluated in silico using NCBI Primer-BLAST and BioEdit to confirm the specificity, melting temperature and expected amplicon size. The primer sequence of mtDNA ND5 for the forward and reverse strands were 5'- ATTCCTCATTACTATGCTCATCCT-3' and 5'- ATTGAGATTTGTTAGGAACCATGCT-3', respectively. The amplified product of 214 bp was generated from this amplification.

Both primers were verified using the BioEdit Sequence Alignment Editor software to confirm the primer binding sites. Besides that, all sequences from non-target species were also checked to ensure the specificity of the mtDNA ND5 primer. The ClustalW DNA sequencing was performed to identify the primer binding site against the non-target species and the target species.

3.7 Polymerase Chain Reaction (PCR) Optimisation

PCR optimisation was performed to determine the optimum annealing temperature for the primers used in the PCR amplification process. There are a few criteria that must be taken into consideration, some of which are the annealing

temperature, the number of cycles, and the concentration of reagents used in the PCR mixture. In this study, the annealing temperature was optimised starting from 56°C, 58°C, 60°C, 62°C and 64°C using the Veriti™ 96-Well Thermal Cycler (Applied Biosystem).

For the 18S rRNA primer, the Master Mix consisted of 13.4µL deionized water, 2.0µL of 10× Taq buffer, 0.2µL of 10µM forward primer and reverse primer each, 0.6µL of 50mM MgCl₂, 0.4µL of 10mM dNTPs and 0.2µL of 5U/ µL Taq polymerase for each sample. Detailed PCR parameters were displayed in Table 3.4. For the mtDNA ND5 primers, one reaction mixture of PCR master mix consisted of 13.0 µL deionised water, 2.0µL of 10× Taq buffer, 0.2 µL of 10µM forward primer and reverse primer each, 1.0 µL of 50mM MgCl₂, 0.4 µL of 10mM dNTPs and 0.2 µL of 5U/ µL Taq polymerase for each sample. The DNA template for one reaction was 3µL for each PCR amplification. A total volume for each PCR reaction mixture for both 18S rRNA and mtDNA ND5 primers was 20 µL.

Table 3.4: PCR parameters used in PCR Optimisation.

Step	Temperature (°C)	Time (s)	No. of Cycles
1	95	180	1
2	95	30	35
	56,58,60,62 and 64	30	
	72	30	
3	72	300	1
4	4	Hold	-

3.8 Specificity and Sensitivity Testing

In this study, the optimised annealing temperature from PCR optimisation was used for specificity and sensitivity tests. Primers 18S rRNA and mtDNA ND5 were cross-checked against the template of bovine, the target species and non-target species to verify their species specificity. The sensitivity of the assays was tested through the dilution method, where the extracted DNA of the target species of bovine were serially diluted from the highest to the lowest concentrations (1ng/ μ L, 100pg/ μ L, 10pg/ μ L and 1pg/ μ L). The limit of detection (LOD) was determined based on the band visibility seen on the agarose gel, as shown by the lowest DNA template.

3.9 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a nucleic acid amplification technique involving denaturation, annealing, extension, and amplification of short DNA or RNA segments. This technique was involved in multiple stages in this study, including annealing temperature optimization, specificity testing and sensitivity testing. Ultimately, this technique was used to determine the presence of cow DNA in processed cat wet food samples by targeting the mitochondrial ND5 gene.

Prior to PCR setup, all reactions were prepared in a designated closed room to minimize contamination. The workbench and pipettes were first sanitized with 70% ethanol and exposed to UV light for 30 minutes. The process started by preparing the Master Mix (MM). For the 18S rRNA primer, the Master Mix consisted of 13.4 μ L deionized water, 2.0 μ L of 10 $\times$ Taq buffer, 0.2 μ L of 10 μ M forward primer and reverse primer each, 0.6 μ L of 50mM MgCl₂, 0.4 μ L of 10mM dNTPs and 0.2 μ L of 5U/ μ L Taq polymerase for each sample.

Meanwhile, the master mix formulation for the mtDNA ND5 primer was prepared in a separate session to minimise errors during pipetting and to avoid cross-contamination. One reaction mixture of PCR master mix consisted of 13.0 μL deionised water, 2.0 μL of 10 $\times$ Taq buffer, 0.2 μL of 10 μM forward primer and reverse primer each, 1.0 μL of 50mM MgCl_2 , 0.4 μL of 10mM dNTPs and 0.2 μL of 5U/ μL Taq polymerase for each sample. The DNA template for one reaction was 3 μL for each PCR amplification. A total volume for each PCR reaction mixture for both 18S rRNA and mtDNA ND5 primers was 20 μL . PCR parameters for both primers were shown in Table 3.5.

Table 3.5: PCR Amplification Conditions for 18S rRNA and ND5 Primers

Primer	Stage 1	Stage 2 $\times$ 35 cycle			Stage 3 $\times$ 1 cycle	
	$\times$ 1 cycle	Denaturation	Annealing	Extension	Final	Hold
18S rRNA	95.0	95.0	60.0	72.0	72.0	4.0
	3 minutes	30 seconds	30 seconds	30 seconds	5 minutes	∞
ND5	95.0	95.0	56.0	72.0	72.0	4.0
	3 minutes	30 seconds	30 seconds	30 seconds	5 minutes	∞

3.10 Agarose Gel Electrophoresis of Amplified PCR Products

The amplified PCR products were subjected to agarose gel electrophoresis to confirm the presence of bovine DNA. A 2% agarose gel solution was prepared by weighing 2g of agarose powder and transferring it into 250 mL Schott bottle. Then,