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Development of Species-Specific Primers Targeting Mitochondrial *Cyt b* Gene for Porcine DNA Detection in Halal Authentication via Polymerase Chain Reaction (PCR)

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Abstract

Background: *The detection of porcine DNA is critical to ensuring adherence to halal standards, particularly in food and pharmaceutical products. This study aimed to design and validate species-specific primers targeting the mitochondrial Cyt b gene of Sus scrofa for porcine DNA identification. Using in silico tools such as NCBI, Primer3Plus, SnapGene, Mega and NetPrimer, four primer pairs were designed and assessed for specificity and efficiency.* **Methodology:** *Laboratory validation involved PCR amplification and bi-directional Sanger sequencing.* **Findings:** *The findings demonstrated that primers 2F/2R and 3F/3R successfully amplified the target DNA, producing amplicons of 514 bp and 456 bp, respectively. The primers exhibited high specificity, with no amplification observed in non-target DNA samples, including chicken and beef. Sanger sequencing confirmed that the amplified products corresponded to the Cyt b gene sequence of Sus scrofa with 100 % similarity, as validated through BLAST analysis. This study presents an accurate and dependable molecular method for detecting porcine DNA, with valuable applications in halal authentication and molecular diagnostics.* **Contribution:** *The developed primers offer an effective tool for accurately identifying porcine-derived components, addressing the critical demand for species-specific DNA detection to support halal compliance.*

Keywords: *Cyt b gene; Halal compliance; Porcine DNA; PCR; Species-specific primer*

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INTRODUCTION

The necessity of halal certification in the food industry has become increasingly important to ensure compliance with Islamic dietary laws by eliminating prohibited substances. With growing concerns among Muslim consumers about food authenticity and the prevalence of counterfeiting, the demand for rigorous verification processes for animal-derived products has steadily increased (Kua et al., 2022). The increasing complexity of food supply chains and the variety of products, including those containing animal ingredients, present significant challenges in confirming their halal status. Furthermore, limited public knowledge about food composition and manufacturing processes has amplified the call for reliable halal certification methods (Yakub, 2021).

Indonesia, as the country with the largest Muslim population globally, estimated at 237.55 million people according to the Royal Islamic Strategic Studies Centre (RISSC) in 2023—has experienced a surge in the adoption of halal lifestyles (Widayat et al., 2019). This demographic context emphasizes the critical need for comprehensive halal regulations. The enactment of the 2014 Halal Product Assurance (JPH) Act established a legal framework for halal certification in Indonesia, mandating the labeling of halal-certified products and prohibiting items deemed non-halal by Majelis Ulama Indonesia (MUI) (Ariny & Nurhasanah, 2020; Sari, 2017). Within this regulatory framework, ensuring the absence of porcine derivatives is a pivotal requirement for halal compliance.

To address the need for reliable detection of porcine DNA contamination, advanced molecular techniques have proven indispensable. Among these, Polymerase Chain Reaction (PCR) has emerged as the method of choice due to its ability to amplify specific DNA regions with high precision. PCR offers a robust approach for detecting even minimal traces of porcine DNA in food products (Chilmi et al., 2021; Muflihah et al., 2023; Rachmawati et al., 2018; Budiarto, 2015). The success of PCR, however, is critically dependent on the design of primers, which are short sequences of DNA essential for targeting the desired amplification region. The application of in silico bioinformatics tools enables the efficient and accurate design of primers, ensuring the specificity and effectiveness of PCR-based assays (Yustinadewi et al., 2018; Fakih et al., 2021).

The mitochondrial Cytochrome b (*Cyt b*) gene, a key component of the electron transport chain, is a highly conserved genetic marker extensively used in species identification and phylogenetic studies (Chilmi et al., 2021; Widayanti et al., 2004). Its high degree of conservation makes it an ideal target for developing species-specific primers for the detection of porcine DNA. Despite the proven utility of PCR and primer design in halal authentication, the inconsistent application of these methodologies remains a significant challenge.

This study addresses these gaps by designing primers specific to the *Cyt b* gene for porcine DNA detection. These primers are intended to serve as reliable molecular markers, providing a consistent and efficient foundation for biomolecular approaches to halal certification.

METHOD

The study was carried out at the Microbiology Laboratory of Pharmacy and the Microbial Biotechnology Laboratory (UPA) at Universitas Singaperbangsa Karawang, West Java, between March and November 2024.

Samples and Reagents

This study employed porcine, beef, and chicken meat samples, each weighing 200 mg. The reagents employed included the Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan), primers (IDT, USA), PCR master mix (Bioline, USA), nuclease-free water (Himedia, India), TE buffer (Himedia, India), agarose gel 1 % (Vivantis, Malaysia), and GelRed dye (Biotium, California).

Analysis of the *Cyt b* gene with the NCBI

The nucleotide sequence of the *Cyt b* gene was obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). To ensure species-specific detection of porcine DNA, *Sus scrofa* was selected as the target organism. By selecting the appropriate gene category from the database's dropdown menu and entering the query term "*Cyt b Sus scrofa*," the relevant sequence was identified. The sequence associated with GenBank accession number KJ476218.1 was selected based on its high quality, complete annotation, and prior validation in molecular authentication studies. The sequence was then downloaded in FASTA format for subsequent bioinformatics analysis.

Primer Design

The development of primers targeting the *Cyt b* gene of *Sus scrofa* is a crucial aspect of achieving precise and specific detection of porcine DNA. The process involves adherence to stringent criteria to ensure the primers are efficient and selective in amplifying the target gene segment. To create primer candidates, the *Cyt b* gene sequences in FASTA format were analyzed using Primer3Plus software, available at https://www.bioinformatics.nl/cgi-bin/primer3plus/primers3plus.cgi. This software evaluates key parameters, including primer length, GC content, melting temperature (Tm), and amplicon size, to propose suitable primer options. The final selection of primer pairs was guided by optimal design principles to guarantee specificity to the target gene, compatibility with PCR conditions, and prevention of cross-reactivity with non-target species (Untergasser et al., 2007).The mitochondrial DNA, *Cyt b* gene sequence reference for *Sus scrofa* used is GenBank accession number: KJ476218 (Kang et al., 2018).

Primer Quality Analysis

A thorough evaluation was performed to determine the quality and appropriateness of the primers selected for amplifying the *Cyt b* gene of *Sus scrofa*. This assessment was crucial to ensure the primers met essential criteria for specificity, efficiency, and alignment with the required PCR conditions, which are vital for precise and dependable DNA amplification.

The analysis involved entering the forward (F) and reverse (R) primer sequences into the NetPrimer software, available at https://www.premierbiosoft.com/netprimer/. This tool examines key characteristics such as melting temperature (Tm), GC content, secondary structures (including hairpins and primer dimers), and overall primer stability. These parameters are pivotal in optimizing PCR functionality while minimizing the risk of non-specific binding and amplification errors.

Upon initiating the analysis, the software provided a comprehensive report detailing the properties of each primer, identifying both their strengths and any potential areas for improvement. The evaluation confirmed that the primers complied with optimal design standards, including well-matched Tm values between the forward and reverse primers, appropriate GC content for stable hybridization, and the absence of significant secondary structures that could interfere with the amplification process. By verifying these parameters, the study ensured the selected primers were ready for laboratory validation and practical application.

Assessment of Primer Candidate

The primer candidates designed using Primer3Plus, including forward and reverse sequences, were further evaluated with the SnapGene Viewer tool. This analysis was conducted to confirm the primers' binding efficiency and alignment accuracy with the *Cyt b* gene sequence of *Sus scrofa.* SnapGene Viewer, accessible at https://www.snapgene.com/snapgene-viewer/, offers a visual and interactive environment to simulate the interaction between primers and the target DNA. The forward and reverse primers were entered into the software and mapped onto the target sequence to validate their alignment and ensure compatibility. The evaluation focused on confirming that the primers annealed precisely to their designated binding regions on the template without mismatches that could affect amplification effectiveness (Rahma et al., 2025).

Additionally, the tool facilitated the determination of the expected amplicon size, adding another layer of verification to the primer design process. Through the simulation of primer-template interactions, SnapGene Viewer validated the selected primer pair's suitability for amplifying the targeted region of the *Cyt b* gene. This assessment confirmed that the primers were adequately prepared for subsequent laboratory testing and experimental use (Clontech, 2016).

Sample Preparation and Genomics DNA Extraction

The forward and reverse primers designed through in silico methods were experimentally validated using a meat matrix consisting of porcine, beef, and chicken samples. The sampling process was carefully supervised to ensure adherence to established standard operating procedures for proper collection and handling (Nuraeni et al., 2023).

DNA Extraction and Evaluation

Genomic DNA was extracted following the standard procedure outlined in the Genomic DNA Mini Kit (Tissue) protocol (Geneaid, China). The extracted DNA's concentration and purity were evaluated using a Nanodrop EPOCH2 spectrophotometer (BioTek, USA). Duplicate readings were performed to enhance measurement reliability, utilizing the elution buffer provided in the Genomic DNA Mini Kit (Tissue) as the blank for calibration. This method ensured accurate quantification and a thorough assessment of DNA quality for further experimental applications (Rahma et al., 2025).

PCR and Sequencing

The target gene in porcine DNA was amplified through conventional PCR and bi-directional Sanger sequencing using forward (F) and reverse (R) primers designed via in silico analysis. The PCR reaction was optimized to a total volume of 20 µL, comprising 10 µL of master mix (Tiangen, USA), 4 µL of extracted DNA template (100 ng/µL), 2 µL of forward and reverse primers (final concentration of 400 nM each), and nuclease-free water to complete the volume. The PCR conditions included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 55–63 °C for 30 seconds, and extension at 72 °C for 60 seconds. A final extension was conducted at 72 °C for 5 minutes. To ensure the reliability of the results, a no-template control was included, and all reactions were carried out in triplicate. Amplifications were performed on a T100 PCR System (Bio-Rad, USA).

Post-amplification analysis involved electrophoresis of the PCR products on a 1 % agarose gel (Vivantis, Malaysia) at 70 volts for 45 minutes, using the Mupid-exU system (Japan). DNA bands were visualized under UV light using a transilluminator (Accuris-Benchmark Scientific, USA). Products showing single, distinct bands of the expected size were purified and subjected to Sanger sequencing via capillary electrophoresis (Macrogen, Korea). The sequence data obtained were examined using SnapGene Viewer software to identify partial Cyt b gene and confirm the accuracy and specificity of the amplified gene region. This protocol ensured the precision of the primers and the validity of the amplification process.

Data Analysis

Sequencing results were examined to verify that the designed primers and genomic DNA selectively amplified the intended target sequence from porcine meat, producing amplicons approximately 400 – 550 bp in length. SnapGene software was utilized for analyzing cycle sequencing outcomes, including base-calling and sequence trimming. Sequencing quality was assessed through peak quality metrics, and segments with peak scores below 20 were removed from both ends of the electropherogram to enhance accuracy (Koh et al., 2021).

After trimming, a consensus sequence was generated. The sequence data were assembled using BioEdit software, which provided tools for sequence alignment and

editing. Base-calling was meticulously reviewed to ensure the integrity and reliability of the final sequence results (Logan et al., 2014). This systematic approach confirmed the specificity of the amplified region and demonstrated the effectiveness of the designed primers.

RESULT AND DISCUSSION

Analysis of the *Cyt b* gene with the NCBI

The design and computational evaluation of primers are essential to ensure their accuracy and efficiency in detecting and amplifying specific target DNA sequences. This process is pivotal for the success of PCR-based experiments, as primers must bind precisely to the target region while avoiding unintended amplification of non-target sequences (Kumar & Chordia, 2015).

The primer design process began with retrieving the reference gene sequence for the *Cyt b* gene of *Sus scrofa* from the NCBI database. This step is critical to ensure that the primers are specifically designed for the target gene. High-quality reference sequences are essential as they serve as the foundation for subsequent in silico analyses (Goldfarb et al., 2025). The NCBI database search yielded a single sequence of the *Sus scrofa Cyt b* gene. The retrieved sequence was formatted into FASTA, a standardized text-based representation of nucleotide sequences that facilitates compatibility with bioinformatics tools for primer design and quality assessment. The FASTA format of the *Cyt b* gene sequence for *Sus scrofa* is shown in Figure 1.

The analysis revealed that the *Sus scrofa Cyt b* gene has a sequence length of 16,613 base pairs (bp) and is part of the mitochondrial DNA, with accession number NC_000845.1. The *Cyt b* gene encodes a protein involved in mitochondrial electron transport and is widely recognized as a DNA marker for uncovering the evolutionary history of animals (Jagielski et al., 2018). Given its significance, this gene serves as an ideal target for designing species-specific primers.

```
>NC_000845.1:15342-16481 Sus scrofa mitochondrion, complete genome
ATGACCAACATCCGAAAATCACACCCACTAATAAAAATTATCAACAACGCATTCATTGACCTCCCAGCCC
CCTCAAACATCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAAC
AGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATTTGT
CGAGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGCATCCATATTCTTTATTTGCC
TATTCATCCACGTAGGCCGAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGAGT
AGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTC
TGAGGAGCTACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAA
TCTGAGGGGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTCCACTTTATCCTGCCATT
CATCATTACCGCCCTCGCAGCCGTACATCTCCTATTCCTGCACGAAACCGGATCCAACAACCCTACCGGA
ATCTCATCAGACATAGACAAAATTCCATTTCACCCATACTACACTATTAAAGACATTCTAGGAGCCTTAT
TTATAATACTAATCCTACTAATCCTTGTACTATTCTCACCAGACCTACTAGGAGACCCAGACAACTACAC
CCCAGCAAACCCACTAAACACCCCACCCCATATTAAACCAGAATGATATTTCTTATTCGCCTACGCTATT
CTACGTTCAATTCCTAATAAACTAGGTGGAGTGTTGGCCCTAGTAGCCTCCATCCTAATCCTAATTTTAA
TGCCCATACTACACACATCCAAACAACGAAGCATAATATTTCGACCACTAAGTCAATGCCTATTCTGAAT
ACTAGTAGCAGACCTCATTACACTAACATGAATTGGAGGACAACCCGTAGAACACCCGTTCATCATCATC
GGCCAACTAGCCTCCATCTTATACTTCCTAATCATTCTAGTATTGATACCAATCACTAGCATCATCGAAA
ACAACCTATTAAAATGAAGA
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Figure 1. FASTA Format of the *Cyt b* Gene Sequence for *Sus scrofa*

To utilize the sequence in primer design, the *Cyt b* gene retrieved from the NCBI database was converted into FASTA format, which simplifies sequence

representation by omitting numbering and focusing solely on the nucleotide sequence (Sihotang et al., 2021). This format enables seamless integration with tools such as Primer3Plus and SnapGene for designing and validating primers (Clontech, 2016). These primers are then evaluated to ensure specificity to the *Sus scrofa Cyt b* gene and compatibility with PCR-based applications.

Primer Design

The design process resulted in four pairs of candidate primers, each consisting of forward and reverse sequences tailored to amplify specific regions of the *Cyt b* gene. The binding sites of these primers on the reference sequence are shown in Figure 2, with detailed locations and resulting amplicon sizes for each primer pair. For the 2F/2R primer pair, the forward primer anneals at positions 15,406–15,425, and the reverse primer binds at positions 15,900–15,919, producing an amplicon of 514 base pairs (bp). The 3F/3R pair has a forward primer binding at positions 15,428–15,449 and a reverse primer at positions 15,863–15,883, yielding an amplicon of 456 bp. Similarly, the 4F/4R pair generates a 458-bp amplicon, with the forward primer annealing at positions 15,857–15,876 and the reverse primer at positions 16,293–16,314. Lastly, the 5F/5R pair produces a 415-bp amplicon, with the forward primer annealing at positions 15,348–15,367 and the reverse primer binding at positions 15,741–15,762.

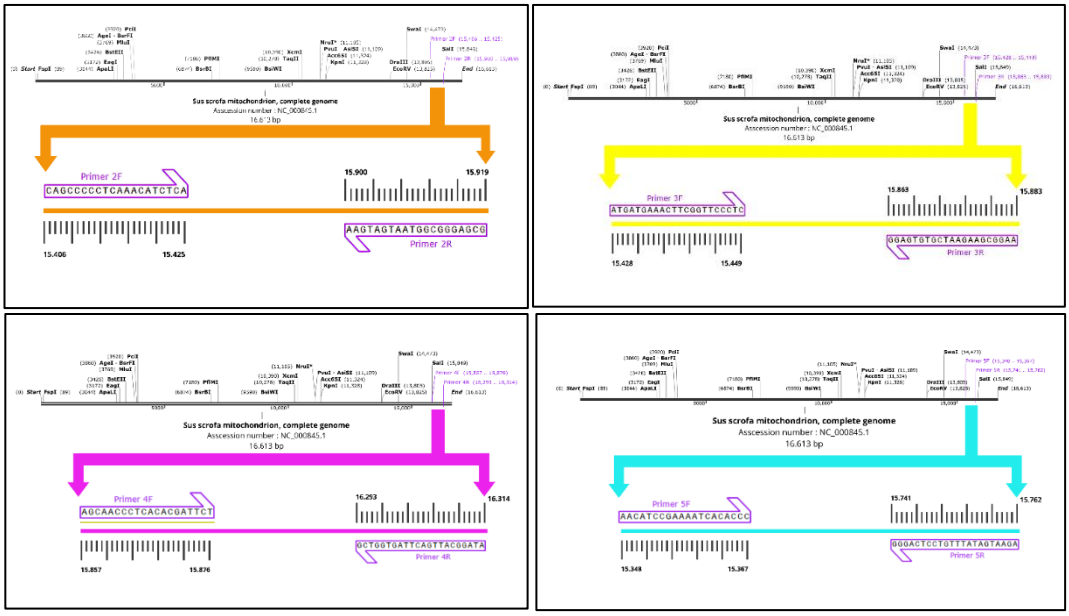


Figure 2. Mapping of Forward and Reverse Primer Annealing Sites. (A) Primer pair 2F and 2R produces a 514 bp amplicon, (B) Primer pair 3F and 3R produces a 456 bp amplicon, (C) Primer pair 4F and 4R produces a 458 bp amplicon, (D) Primer pair 5F and 5R produces a 415 bp amplicon on the *Sus scrofa* mitochondrial genome.

The sequences of the primers are detailed in Table 1. All forward primers are designed to be no longer than 25 bases, as are the reverse primers, ensuring optimal performance during PCR. The resulting amplicon lengths, ranging from 400 to 500 bp,

fall within the ideal range for efficient amplification and downstream applications. This range is critical for ensuring the primers align with the target sequence with high specificity while avoiding non-specific amplification (Nguyen et al., 2023). The primer design ensures that forward primers synthesize DNA in the 5' to 3' direction, whereas reverse primers synthesize DNA in the complementary 3' to 5' direction (Messe et al., 2020). This directional synthesis is fundamental for the accurate replication of the target DNA sequence. The high specificity and alignment precision of these primers underscore their suitability for detecting the mitochondrial *Cyt b* gene in *Sus scrofa*. These results provide a solid foundation for subsequent experimental validations.

Table 1. Oligonucleotide Primers

No.	Primer Pair	Oligonucleotide Sequence (5'→3')	Base Length	Product Length (bp)
1	2F	CAGCCCCCTCAAACATCTCA	20	514
	2R	GCGAGGGCGGTAATGATGAA	20	
2	3F	ATGATGAAACTTCGGTTCCCTC	22	456
	3R	AAGGCGAAGAATCGTGTGAGG	21	
3	4R	AGCAACCCTCACACGATTCT	20	458
	4F	ATAGGCATTGACTTAGTGGTCG	22	
4	5F	AACATCCGAAAATCACACCC	20	415
	5R	AGAATGATATTTGTCCTCAGGG	22	

Primer Quality Analysis

Evaluating primer quality in molecular biology research requires analyzing several critical parameters that directly influence the specificity and performance of PCR reactions (Rodríguez et al., 2015). These parameters include primer length, GC content, melting temperature (Tm), Tm difference between primer pairs, annealing temperature (Ta), Ta variation, and the likelihood of secondary structure formation such as hairpins, self-dimers, and cross-dimers (Borah, 2015; Hung & Weng, 2016). The requirements for these parameters, as applied in this study, are outlined in Table 2.

Primer length is an important factor in ensuring the specificity and efficiency of amplification. The ideal length of primers typically falls within the range of 15 to 30 nucleotides, as shorter primers may reduce specificity, while excessively long primers may decrease PCR efficiency (Borah, 2015; Hung & Weng, 2016; Rodríguez et al., 2015). The four potential primers evaluated in this study ranged from 20 to 23 nucleotides, which aligns with the recommended range, ensuring effective annealing during amplification (Asif et al., 2021).

Table 2. Primer Quality Analysis Using online-based NetPrimer website, Including Primer Length, GC%, Melting Temperature (*Tm*), *Tm* Difference, and Secondary Structure Analysis.

Parameter	**Acceptable Value**	**Primer 2 (5'→3')**		**Primer 3 (5'→3')**		**Primer 4 (5'→3')**		**Primer 5 (5'→3')**	
		2F	**2R**	**3F**	**3R**	**4F**	**4R**	**5F**	**5R**
Sequences	-	CAGCCCCCTCAAACATCTCA	GCGAGGGCGGTAATGATGAA	ATGATGAAACTTCGGTTCCCTC	AAGGCGAAGAATCGTGTGAGG	AGCAACCCTCACACGATTCT	ATAGGCATTGACTTAGTGGTCG	AACATCCGAAAATCACACCC	AGAATGATATTTGTCCTCAGGG
Primer length (bp)	18-24	20	20	22	21	20	22	20	22
Rating (%)	80–100	100	100	100	99	100	92	100	89
Temperature Melting (Tm) °C	50-65	60.57	63.19	59.81	61.70	56.84	56.57	57.03	55.38
GC Content (%)	40-60	55.00	55.00	45.45	52.38	50.00	45.45	45.00	40.91
GC Clamp	≤ 3 G or C	1	1	3	2	1	2	3	3
Self-Dimer (ΔG) (kcal/mol)	ΔG ≥ -6	-	-	-	-	-	3.92	-	5.67
Hairpin (ΔG) (kcal/mol)	ΔG ≥ -2 kcal/mol	-	-	-	-0.69	-	-	-	-0.33
Crossdimer	ΔG ≥ -6 kcal/mol	-9.31		-8.73		-6.19		-5.36	

The melting temperature (Tm), defined as the temperature at which half of the DNA double strands dissociate, is a crucial parameter influencing the annealing temperature in PCR procedures. Optimal Tm values for primers typically range from 55 °C to 65 °C. Exceeding this range may hinder amplification due to reduced annealing efficiency, while lower Tm values may result in non-specific binding (Green & Sambrook, 2020). The Tm values for the primers analyzed in this study ranged from 55.38 °C to 63.19 °C, with a Tm difference of no more than 3 °C between primer pairs, ensuring compatibility and minimizing the risk of failed amplification (Asif et al., 2021; Sari, 2018).

The GC content of primers, representing the proportion of guanine and cytosine bases, significantly affects the melting temperature and overall stability of primer-template binding. An effective primer typically contains a GC content of 40 % to 60 % (Green & Sambrook, 2020; Saraswati et al., 2019). All primers designed in this study adhered to this range, exhibiting GC contents between 40 % and 55 %. Low GC content could hinder PCR efficiency by reducing the stability of primer binding (Pradnyaniti & Yowani, 2013; Zhao, 2014).

Secondary structures, such as hairpins, self-dimers, and cross-dimers, can negatively impact the binding efficiency of primers by interfering with their ability to anneal to the target sequence. Hairpins are formed when complementary bases within a single primer strand pair with each other, creating a loop structure. Acceptable hairpin formation is defined by a free energy (ΔG) value greater than -3 kcal/mol, as higher negative ΔG values indicate more stable structures that may disrupt amplification (Chen et al., 2017; Nazarenko, 2002; Sasmito et al., 2014). The analysis revealed that reverse primers 3 and 5 exhibited hairpin ΔG values of -0.69 and -0.33, respectively, which are within acceptable limits.

Self-dimers form when primers bind to identical primer molecules, while cross-dimers occur between forward and reverse primers. Both structures are evaluated based on their ΔG values, with tolerable values being greater than -6 kcal/mol to avoid interference with amplification. The primer pairs evaluated in this study demonstrated acceptable self-dimer ΔG values exceeding -6 kcal/mol, indicating low stability of secondary structures. However, cross-dimers involving reverse primers 2, 3, 4, and 5 exhibited ΔG values more negative than -6 kcal/mol, suggesting potential interference with primer binding at these sites (Sasmito et al., 2014; Zhao, 2014).

The overall quality ratings for the primers were satisfactory, with primer pairs 2F/2R and 3F/3R achieving ratings close to 100% for both forward and reverse sequences. These superior ratings underscore their strong binding specificity and efficiency, making them ideal candidates for wet-laboratory validation. While some primers may require optimization to address secondary structure issues, the selected primers are expected to perform reliably in downstream experimental applications.

In summary, the primer design process demonstrated high potential for achieving efficient and specific PCR amplification. Adhering to established criteria for primer length, Tm, GC content, and secondary structure formation, this study identified two primer pairs with robust performance characteristics. These primers offer a reliable molecular tool for applications in DNA detection, including food authentication and other diagnostic contexts, where precise species identification is critical (Green & Sambrook, 2020; Rodríguez et al., 2015).

Primer Specificity Analysis

The specificity of the designed primers was analyzed to ensure accurate amplification of the target sequence, thereby reducing the likelihood of non-specific amplification that could compromise the validity and reliability of the research findings (Sabina & Leamon, 2015). This analysis employed the Primer-BLAST tool on the NCBI platform (Sihotang et al., 2021), which revealed that all primer pairs successfully bound to 480 sequences listed in the NCBI database as of December 2024, as shown in Table 3. In addition to binding specifically to *Sus scrofa*, the primers exhibited potential recognition of five additional species within the Suidae family. This characteristic highlights their relevance for halal authentication, as all these species are restricted in food and pharmaceutical products (Rahma et al., 2025).

Table 3. Result of *Cyt b* gene in *Sus scrofa* primer pair sequences alignment (100 Blast top sequence)

Primer	Species	Ordo	Family	Total
2F	*Sus scrofa*	Even-toed ungulates & whales	Suidae	50
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	5
	Sus scrofa riukiuanus	Even-toed ungulates & whales	Suidae	2
	Sus scrofa cristatus	Even-toed ungulates & whales	Suidae	4
2R	*Sus scrofa*	Even-toed ungulates & whales	Suidae	81
	Sus scrofa vittatus	Even-toed ungulates & whales	Suidae	1
	Sus scrofa critatus	Even-toed ungulates & whales	Suidae	6
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	9
	Sus scrofa riukiuanus	Even-toed ungulates & whales	Suidae	4
3F	*Sus scrofa*	Even-toed ungulates & whales	Suidae	92
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	5
	Sus scrofa riukiuanus	Even-toed ungulates & whales	Suidae	1
	Sus scrofa cristatus	Even-toed ungulates & whales	Suidae	1
3R	*Sus scrofa*	Even-toed ungulates & whales	Suidae	27
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	5
	Sus scrofa cristatus	Even-toed ungulates & whales	Suidae	3
4F	*Sus scrofa*	Even-toed ungulates & whales	Suidae	51
	Sus scrofa cristatus	Even-toed ungulates & whales	Suidae	3
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	5
4R	*Sus barbatus*	Even-toed ungulates & whales	Suidae	2
	Sus scrofa	Even-toed ungulates & whales	Suidae	78
	Sus scrofa cristatus	Even-toed ungulates & whales	Suidae	4
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	7
	Sus scrofa riukiuanus	Even-toed ungulates & whales	Suidae	2
5F	*Sus scrofa*	Even-toed ungulates & whales	Suidae	26
	Sus scrofa domesticus	Even-toed ungulates & whales	Suidae	4
5R	*Sus scrofa*	Even-toed ungulates & whales	Suidae	2
Total				480

Table 4. Results of PCR Product Sequence Alignment (100 Blast top sequence)

Primer Name	Amplicon sequence (5'-3')	Number of Target Spesies Recognized		
		Sus scrofa	other spesies	total
2F/2R	CAGCCCCCTCAAACATCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAA TCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTTACAC ACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGCATCCATATTC TTTATTTGCCTATTCATCCACGTAGGCCGAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGA AACATTGGAGTAGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGG ACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAG ACCTCGTAGAATGAATCTGAGGGGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTC CACTTTATCCTGCCATTCATCATTACCGCCCTCGC	100 *Query Cover = 100% %Ident. = 100% E-value = 0,0*	0	100
3F/3R	ATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGCCTGTTCTTAGC AATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATTTGTCGAGACGTAAATTA CGGATGAGTTATTCGCTATCTACATGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGT AGGCCGAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGAGTAGTCCTACTAT TTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGAGGAGCT ACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGG GGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTT	100 *Query Cover = 100% %Ident. = 100% E-value = 0*	0	100
4F/4R	AGCAACCCTCACACGATTCTTCGCCTTCCACTTTATCCTGCCATTCATCATTACCGCCCTCGCAGCCGT ACATCTCCTATTCCTGCACGAAACCGGATCCAACAACCCTACCGGAATCTCATCAGACATAGACAAAA TTCCATTTCACCCATACTACACTATTAAAGACATTCTAGGAGCCTTATTTATAATACTAATCCTACTAAT CCTTGTACTATTCTCACCAGACCTACTAGGAGACCCAGACAACTACACCCCAGCAAACCCACTAAACA CCCCACCCCATATTAAACCAGAATGATATTTCTTATTCGCCTACGCTATTCTACGTTCAATTCCTAATAA ACTAGGTGGAGTGTTGGCCCTAGTAGCCTCCATCCTAATCCTAATTTTAATGCCCATACTACACACATC CAAACAACGAAGCATAATATTTCGACCACTAAGTCAATGCCTAT	100 *Query Cover = 100% %Ident. = 99,74% E-value = 0*	0	100
5F/5R	AACATCCGAAAATCACACCCACTAATAAAAATTATCAACAACGCATTCATTGACCTCCCAGCCCCCTCA AACATCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGC CTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATTTGTCGA GACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGCATCCATATTCTTTATTTGCCTA TTCATCCACGTAGGCCGAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGAGT AGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATT CT	100 *Query Cover = 100% %Ident. = 100% E-value = 0*	0	100

The nucleotide alignment of PCR product amplicons, conducted using Nucleotide-BLAST, is summarized in Table 4. The analysis demonstrated that the nucleotide sequences of the amplicons shared exclusive similarity with *Sus scrofa*, achieving Query Cover and Percent Identity values of 100 %, along with an Expectation Value (E-value) of 0. While this underscores the primers' high specificity for *Sus scrofa*, they also exhibited detection of non-target species. Notably, primer pairs 2F/2R and 3F/3R amplified sequences in distantly related Suidae species, indicating the need for careful selection and optimization of primers during further wet-laboratory validation.

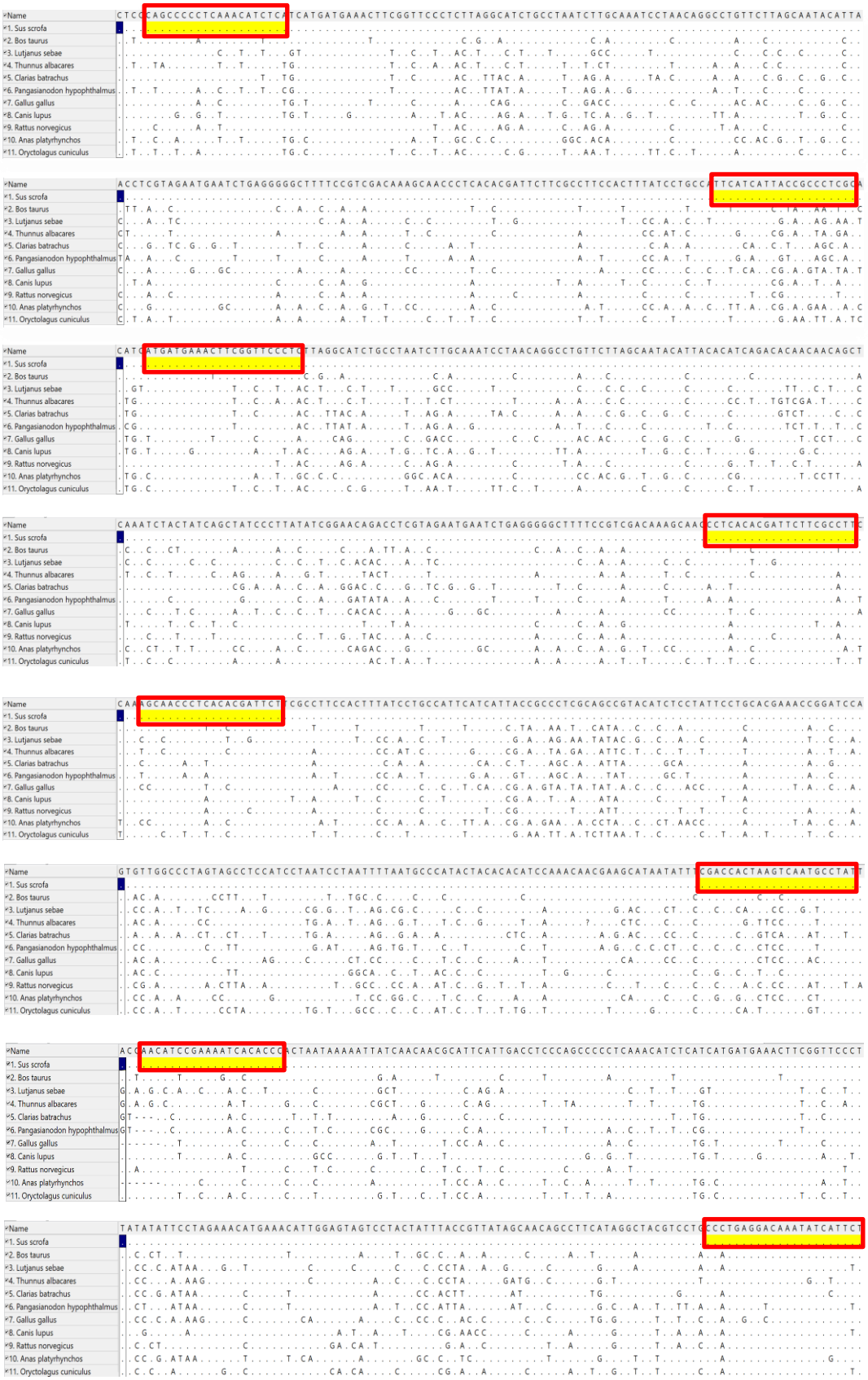
The Nucleotide-BLAST analysis queried extensive databases hosted on the NCBI GenBank, with alignment criteria requiring 100% Query Cover and Percent Identity, and an E-value threshold of ≤0.01 (Sihotang et al., 2021). This evaluation affirmed the primers' specificity for the *Sus scrofa* target while acknowledging their cross-reactivity with other Suidae species, an aspect that requires consideration in subsequent laboratory tests.

To strengthen the specificity evaluation, multiple sequence alignment (MSA) was performed using MEGA XI software (Sabina & Leamon, 2015). Target and non-target species included in the analysis are detailed in Table 5, and the MSA results are illustrated in Figure 3. The results showed that primer pairs 2F/2R, 3F/3R, 4F/4R and 5F/5R demonstrated high specificity, with no amplification observed in non-target species such as *Rattus norvegicus* (rat), *Oryctolagus cuniculus* (rabbit), *Bos taurus* (cow), *Clarias batrachus* (catfish), *Pangasianodon hypophthalmus* (pangasius), *Gallus gallus* (chicken), *Anas platyrhynchos* (duck), and *Canis lupus familiaris* (wolf). These findings align with earlier research by Ali et al., (2015); Shuhaimi & Sam-on, (2022), supporting the primers' strong specificity for *Sus scrofa*.

Table 5. Categories of Samples Used for In-silico Primer Specificity Evaluation (Target and Non-Target Species)

Assecion Number	Common Name	Scientific Name
NC_000845.1	Pig	*Sus scrofa*
NC_006853.1	Beef	*Bos taurus*
NC_012737.1	Red Snapper	*Lutjanus sebae*
NC_014061.1	Tuna	*Thunnus albacares*
NC_023923.1	Catfish	*Clarias batrachus*
NC_021752.1	Patin Fish	*Pangasianodon hypophthalmus*
NC_053523.1	Chicken	*Gallus gallus*
NC_009684.1	Duck	*Anas platyrhynchos*
NC_008092.1	Wolf	*Canis lupus*
NC_001665.2	Rat	*Rattus norvegicus*
NC_001913.1	Rabbit	*Oryctolagus cuniculus*

Figure 3. Results of Specificity Analysis for Forward and Reverse Primers Using Multiple Sequence Alignment (MSA) in MEGA11 (Target and Non-Target Species)



Ensuring this level of specificity is vital for halal authentication, given the frequent adulteration of food products with pork and its derivatives (Zia et al., 2020). Accurate detection of such materials is critical for compliance with halal standards. These results confirm that the in silico primer design successfully produced primers that are both efficient and highly specific for detecting *Sus scrofa* DNA. Validation through Primer-BLAST, Nucleotide-BLAST, and MSA establishes the primers' reliability for future experimental applications (Sabina & Leamon, 2015).

Genomic DNA Extraction

Genomic DNA was isolated from meat samples using the Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan), selected for its efficiency and ease of use compared to traditional methods like chloroform and phenol extraction (Kusec et al., 2015). DNA extraction was performed on porcine samples as the target DNA source, while beef and chicken samples served as non-target controls. The extracted DNA was analyzed for purity and separated from other substances using electrophoresis to confirm its quality.

During electrophoresis, the extracted DNA was mixed with a loading dye to increase density, ensuring it settled at the bottom of the gel wells. The dye also facilitated accurate placement of DNA within the wells and served as a visual guide for monitoring DNA migration through the gel matrix (Sundari & Priadi, 2020). To improve DNA yield, the protocol was modified by heating the elution buffer to 60 °C for 2 minutes before elution. Electrophoresis results showed red-highlighted genomic DNA bands that corresponded with expected patterns based on reference sequences from the NCBI genome database Figure 4.

Figure 4. Gel Electrophoresis Visualizations of Genomic DNA. M = 3kb DNA Marker; 1–7 = Pork Samples; 9–11 = Chicken and Beef Samples.

High-quality genomic DNA is defined by specific criteria: fragments exceeding 1000 bp indicate the absence of degradation; distinct and well-defined bands at the top of the electrophoresis gel signify intact large DNA fragments; non-smearing bands confirm the absence of contamination; and a sufficiently high DNA concentration ensures an adequate quantity of genetic material for subsequent analyses (Levin et al., 2018). The presence of clear DNA bands on the gel confirms the successful extraction and integrity of the isolated genomic material. DNA quality and concentration were assessed, with the results detailed in Table 6. The DNA concentration obtained ranged from 200 to 700 ng/µL. Purity ratios ranged from

1.8 to 2.0, aligning with the standards established by Sambrook et al., (1989); Kirby, (1990). The results indicate the effectiveness of the extraction process in yielding high-quality DNA appropriate for subsequent molecular applications.

Table 6. Concentration and Purity of Extracted Genomic DNA

No.	Samples ID	DNA Conc (ng.µl-1)	DNA Purity (260/280)
1	Pork1	566.45	1.999
2	Pork2	656.23	1.959
3	Pork3	435.35	2.012
4	Pork4	534.21	1.987
5	Pork5	323.66	1.896
6	Pork6	647.45	1.984
7	Pork7	446.33	1.986
8	Chicken1	786.44	2.011
9	Chicken2	865.21	1.911
10	Beef	233.76	1.897

The successful extraction and verification of genomic DNA from target and non-target samples confirm the reliability of the methodology employed (Kirby, 1990; Sambrook et al., 1989). Maintaining the purity and concentration of DNA is essential for achieving consistent and accurate results in subsequent analyses, particularly in distinguishing porcine DNA from non-target species. These outcomes lay a robust groundwork for the application of PCR-based techniques in halal authentication.

PCR Amplification and Sequencing Analysis of Selected Primers

To evaluate the performance of the selected primers (2F/2R and 3F/3R) in a wet laboratory environment, PCR amplification followed by bi-directional Sanger sequencing was performed. The amplification of target and non-target DNA samples was carried out using the touchdown PCR method, recognized for its ability to enhance specificity and minimize non-specific amplification (Malau et al., 2023). Porcine DNA served as the target sample, while chicken and beef DNA were used as non-target controls (Malau et al., 2023).

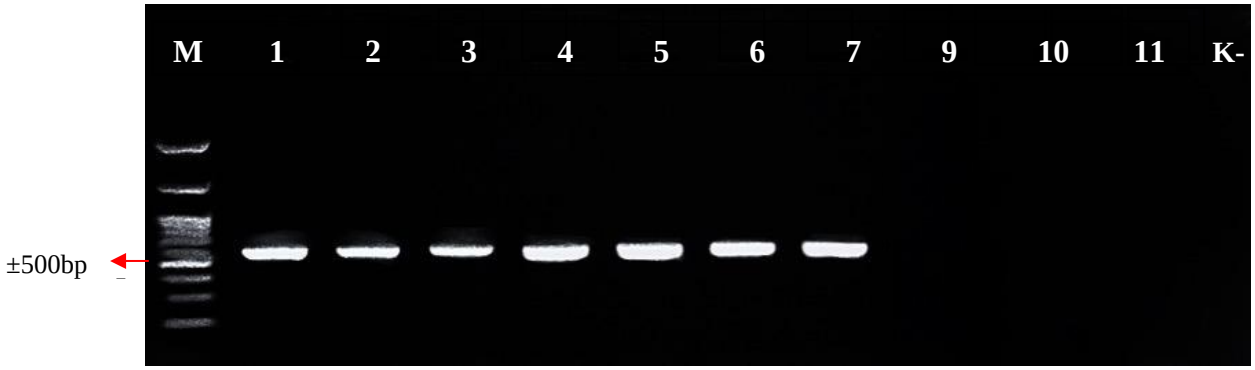


Figure 5. (A) PCR amplification of Meat samples using 2F and 2R Primer, amplicon length = ± 514 bp. M =DNA marker 3 kb; 1–7 = Pork Samples; 9–11 = Chicken and Beef Samples ; K- = negative control.

The results showed that both primers effectively amplified porcine DNA, generating products of 514 bp and 456 bp for primers 2F/2R and 3F/3R, respectively. These results aligned with the predictions from earlier in silico analyses, validating the accuracy of the primer design. Importantly, no amplification was detected in the non-target DNA samples (chicken and beef), highlighting the primers' high specificity for porcine DNA (Rahma et al., 2025). Figures 5 and 6 depict the DNA amplification outcomes, demonstrating the precision and dependability of the primers under laboratory conditions.

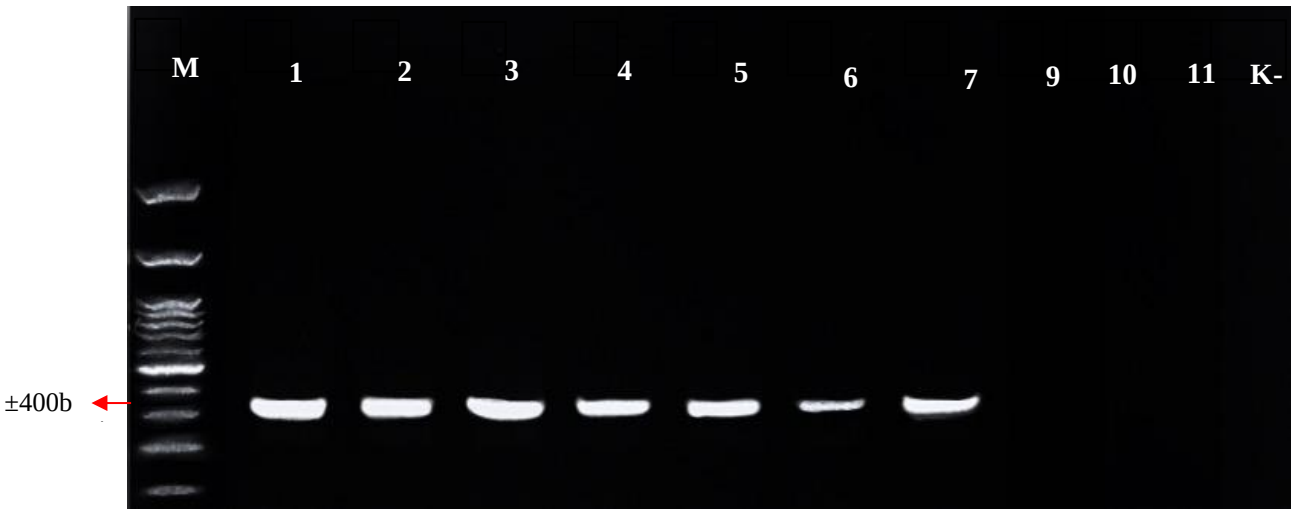


Figure 6. PCR amplification of Meat samples using 3F and 3R Primer, amplicon length = ± 456 bp. M =DNA marker 3 kb; 1–7 = Pork Samples; 9–11 = Chicken and Beef Samples ; K- = negative control.

The PCR products, observed as single bands, were subjected to Sanger sequencing to confirm base-calling accuracy. The sequencing analysis revealed complete alignment with the *Cyt b* gene sequence of *Sus scrofa*. Additionally, online BLAST analysis confirmed 100% similarity between the amplified sequences and the partial *Cyt b* gene of *Sus scrofa*, corresponding precisely with the expected target region. These findings indicate that primers 2F/2R and 3F/3R exhibited strong performance, delivering precise base-calling with no discrepancies. Figure 7 provides detailed sequencing results and alignment.

The experimental validation confirms that the primers are highly specific and efficient for porcine DNA detection, showing no cross-reactivity with non-target species. The consistency between in silico predictions and experimental outcomes underscores the robustness of the primer design process. These findings highlight the potential of these primers for applications in halal authentication and other molecular diagnostic systems requiring precise species-specific DNA identification.

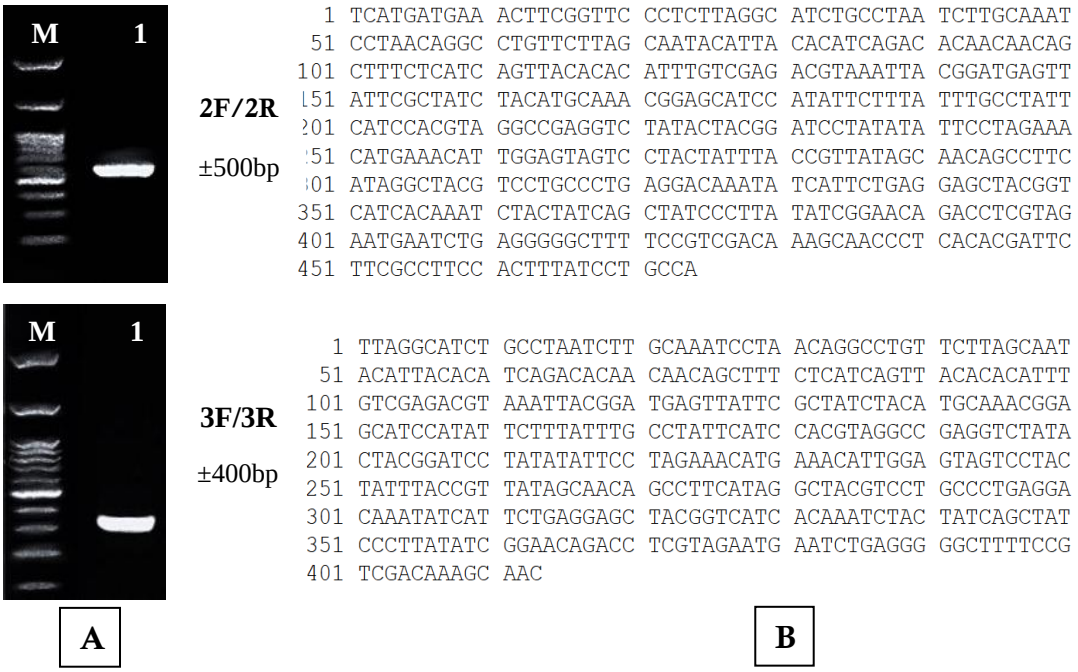


Figure 7. PCR Amplification and Sequencing Results. (A) PCR amplification of pork samples using 2F/2R and 3F/3R primers, producing amplicons of ±514 bp and ±456 bp, respectively. M = 3 kb DNA Marker. (B) Representative sequencing results of PCR products amplified with 2F/2R and 3F/3R primers.

CONCLUSION

This study successfully developed and validated species-specific primers targeting the mitochondrial *Cyt b* gene of *Sus scrofa* for porcine DNA detection. The use of in silico tools such as NCBI, Primer3Plus, SnapGene, Mega and NetPrimer ensured the precision and specificity of the primers, which were further confirmed through comprehensive laboratory validation. The selected primer pairs, 2F/2R and 3F/3R, consistently amplified target DNA, yielding amplicon sizes of 514 bp and 456 bp, respectively, while demonstrating no cross-reactivity with non-target species such as chicken and beef. Sanger sequencing verified that the amplified products matched the partial *Cyt b* gene sequence of *Sus scrofa* with 100% accuracy, as confirmed by BLAST analysis. The primers' robust performance and high specificity underscore their potential for use in halal authentication systems and other molecular diagnostic applications requiring precise species identification. This study contributes a reliable molecular tool for addressing the critical need for porcine DNA detection in compliance with halal standards.

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