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Primer Design of Porcine DNA using Mitochondrial DNA (*Cyt b* Gene) for Halal Authentication using Polymerase Chain Reaction (PCR)

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Abstract

The Muslim community has difficulties in determining the halal status of animal-derived items and their derivatives due to their extensive prevalence. Islamic customers are increasingly becoming more selective and are demanding halal certification for culinary goods. Developing a way to identify porcine DNA in goods is of utmost importance. The *Cyt b* gene is being used to evaluate swine DNA samples by using primer candidates. The process of generating primers utilizing bioinformatics tools, such as NCBI, MegaXI, Primer3Plus, SnapGene Viewer, and Net Primer websites, is utilized to assess and analyze the effectiveness of laboratory research. The study concluded that the primers effectively amplified pig DNA, but they were unsuccessful in amplifying chicken or beef DNA. After conducting in silico experiments, a total of 7 possible primers were generated. The most advantageous pair of primers was identified, which includes the forward primer 5'-AACATCCGAAATCACACCC-3' and the reverse primer 5'-AGAATGATATTTGTCCTCAGGG-3'. The efficacy of these primers was evaluated in a controlled laboratory environment. Results from the laboratory experiments demonstrate that these particular primers have the ability to amplify the *Cyt b* gene from the *Sus scrofa* species at a temperature of 58°C, producing a DNA fragment that is 415 base pairs long. DNA sequencing is essential to verify that the amplified DNA band matches to the *Sus scrofa Cyt b* gene.

Keywords: PCR, Porcine, Primer, *Cyt b*, Halal

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1 Introduction

Halal certification is presently crucial in the food sector to guarantee that food is devoid of any ingredients that are forbidden under Islamic law. Muslim customers have expressed worry on the problem of food diversity and counterfeiting [1]. The distribution of different products in the market, including both finished goods and raw materials sourced from animals or animal-derived products, poses challenges for Muslims in verifying the authenticity of these products. Furthermore, the general public lacks comprehensive knowledge about the composition and manufacturing processes of food products. Given these circumstances, the demand for halal certification is growing significantly as Muslim customers become more discerning and want assurance regarding the authenticity of the food items they eat [2].

Based to the 2023 assessment by the Royal Islamic Strategic Studies Centre (RISSC), Indonesia has the highest Muslim population, with 237.55 million people. The substantial Muslim population in Indonesia has significantly impacted the adoption of the halal lifestyle, which serves as the foundation for product choices [3]. The removal of items is a paramount concern for a nation where the majority of its population follows the Islamic faith. Currently, producers still have the option to comply with the legal obligation [4]. The JPH Act, enacted in 2014, serves as the legal framework for safeguarding the public's choice of halal products in Indonesia. According to Article 25 of the JPH Act (Garantie of Halal goods), the governing body responsible for ensuring the authenticity of halal goods mandates that businesses who have earned a halal certificate must affix a halal label on items that have been officially certified. As per the legislative laws, the invitation states that all items deemed unlawful by the fatwa MUI (Majelis Ulama Indonesia) must display the legitimate logo [5].

A suitable test parameter for a product is the absence of swine contamination. Ensuring the authenticity of food goods becomes an essential concern. Verification of the presence of porcine DNA in a food product can only be achieved using molecular techniques [1]. Rachmawati et al. [6] state that molecular technology can accurately detect even trace levels of porcine components in a food sample by identifying the nucleotide base sequence. PCR is the prevailing molecular method used for detecting swine contamination in food preparation. PCR, short for Polymerase Chain Reaction, is the predominant technique employed to replicate specific regions of DNA [7],[8],[9]. The success of primer designs in PCR is significantly impacted by the features of the primers utilized. Prudent primer design is essential for producing precise primers that accurately match the amplification goal. Acquiring primer substances that match the requirements of being excellent primaries may be achieved by in silico methods, which involve designing primaries using computer programs [10],[11].

Cytb is a gene located in mitochondrial organelles that has a role in electron transport [9]. The *Cyt b* sequence is a highly conserved region, making it a popular choice for investigations aiming to identify and determine the phylogenetics of different species [12].

Currently, there is a lack of uniformity in the application of primer and PCR technology approaches for halal authentication. The

objective of this work is to identify potential primers for DNA analysis of the presence of porcine in the *Cyt b* gene. These primers may then be employed as a molecular marker for regular biomolecular-based halal certification testing.

2 Methods

2.1 In Silico Studies

2.1.1 Searches of the Cyt b gene using the NCBI

Cyt b gene site sequence nucleotides were found by searching the National Center for Biotechnology Information (NCBI) database at http://www.ncbi.nlm.nih.gov. The gene category might be chosen from the accessible menu box. Then to begin the search, put *Cyt b Sus scrofa* into the text box. The acquired *Cyt b Sus scrofa* s sequences are next transformed to the FASTA format [13].

2.1.2 The primer design

In order to generate a primer candidate, the format of the FAST gene is processed using the Primer3Plus software, which can be found at https://www.bioinformatics.nl/cgi-bin/primer3plus/primers3plus.cgi. After obtaining the primer candidate, the selection of the primaries is done based on the principles of optimum primer conditions [14].

2.1.3 Analysis primer quality

Analysis is utilized to evaluate the quality of the selected primer. To begin the analysis, enter the primer sequences for both the forward (F) and reverse (R) strands. Next, select the "Analyze" option to get its results. The Net Primer utility may be accessed at the URL https://www.premierbiosoft.com/netprimer/ [15].

2.1.4 Assessment of Primer Candidate

Implantation using SnapGene Viewer The primer candidate retrieved from the primer3plus web server comprises a pair of forward and reverse primers. The primer pairs are subsequently assessed utilizing the SnapGene Viewer web server, accessible through the URL https://www.snapgene.com/snapgene-viewer/, to analyze their capacity to bind to the DNA template [16].

2.2 Wet Lab Experimental

2.2.1 Sampling preparation and DNA extraction

The primer forward and reverse experimental evaluation of the design results in silico was carried out using the meat matrix of porcine, beef, and chicken. The entire sampling process from the market has been closely monitored and is in line with the standard sample process procedures. Genome DNA extraction is carried out by heating method according to the standard protocol Genomic DNA Mini Kit (Tissue) Geneaid. The result of the DNA extract of the sample is stored at a temperature of 20°C.

2.2.2 DNA amplification using PCR

The amplification of the target gene in porcine DNA is achieved by conventional PCR, utilizing primer F and primer R designed findings obtained from in silico analysis. The thawing process is conducted as a preliminary step to ensure that all relevant steps are prepared. Running involves the execution of three repetitions for every action. The K+ used is the artificially created DNA derived from the pig genome. Genescript Biotech is located in Nanjing, China. A negative control was performed using nuclease-free water obtained from ThermoScientific, a company based in the United States. The total volume of the reaction is 20µL, consisting of 10µL of master mix (Tiangen, USA), 4µL of extracted DNA templates, 2µL of primer F/R with a final concentration of 400nM each, and 4µL of nuclease-free water. The company ThermoScientific is based in the United States. The methodology for PCR amplification involves utilizing the Tiangen Master Mix, which includes a dye. The amplification process began with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds. This was followed by a precipitation step at a temperature range of 55-60°C for 30 seconds, an extension step at 72°C for 60 seconds, and a final extension in 72°C for 5 minutes. PCR products are separated using electrophoresis on an agarose gel and then visualized using gel documentation with UV light.

3 Results and Discussions

3.1 Cyt b Gen Search with NCBI Website

Based on the results of the NCBI database search, one sequence of the *Sus scrofa Cyt b* gene was obtained. The FASTA format of the *Cyt b* gene of *Sus scrofa* is shown in Figure 1.

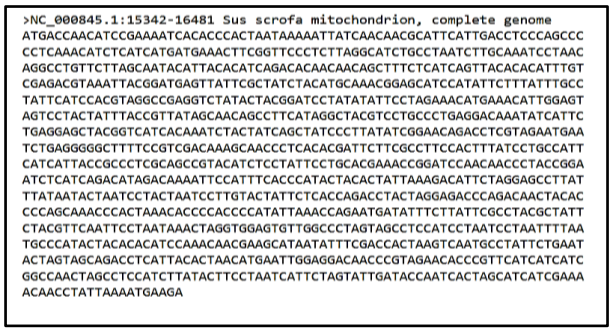

Figure 1. Sequence Gene *Cyt b Sus scrofa*

The *Cyt b* gene in *Sus scrofa* has a sequence length of 1140 bp (base pair) with accession no.NC_000845.1 and this gene is part of mitochondrial DNA. The *Cyt b* gene encodes a protein and is known as a DNA marker in revealing the evolutionary history of animals [17]. *Cyt b* gene sequences obtained from the NCBI database must be converted to FASTA format in order to be used as a template in primer design using Primer3Plus software. FASTA format is a text-based format to show nucleotide sequences without numbering [18].

3.2 Primer Design

Primer design serves as a limitation in the amplification of DNA segments. Successful primer design is accomplished using computational methods based on bioinformatics. Primers in Polymerase Chain Reaction (PCR) procedures serve the purpose of binding with template DNA and identifying the specific area to be amplified. They also provide the attachment site for DNA polymerase, which amplifies the target gene. Primer design is the initial stage in determining the optimal primer sequence for DNA amplification, specifically for in vitro sample analysis utilizing the Polymerase Chain Reaction (PCR) technique [11].

The primer design process yielded a total of 7 pairs of candidate primers (both forward and reverse). These pairs are reported in Table 1. Primer3Plus program consistently provides a suggested set of forward and reverse primers for each primer design outcome. Forward primers are designed to synthesize DNA in the 5' to 3' direction, whereas reverse primers are designed to synthesize DNA in the 3' to 5' direction [19]. These primers will thereafter function as a hindrance to the target DNA fragment that will be amplified. Each of these potential primers is assigned specific names to facilitate their identification.

3.3 Primer Quality Analysis using NetPrimer

When designing primers, it is important to take into account many factors like the length of the primer, the melting temperature (TM), the fraction of GC bases, and ΔTm [18]. The ideal length of primers typically falls within the range of 15 to 30 nucleotides [20]. Reducing the length of primers can result in decreased specificity, while increasing their length can improve the alignment with the target sequence. However, this may lead to a fall in the efficiency of PCR amplification. The study's findings are presented in Table 1. The 7 potential primers have base lengths ranging from 20 to 23, which aligns with the desired parameters for primer base length.

The second aspect pertains to the melting temperature (Tm). The melting temperature (Tm) is the temperature at which half of the DNA double strands have dissociated, and the ideal Tm is within the range of 55°C to 65°C. The melting temperature (Tm) influences the choice of annealing temperature in the polymerase chain reaction (PCR) procedure. Should the temperature exceed 65°C, the amplification process will be impeded as a result of the reduced annealing temperature. On the other hand, if the melting temperature (Tm) is low, it will lead to non-specific findings since the primers would bind to other locations [21]. The data presented in Table 1 demonstrates that all candidate primers satisfy the specified criteria. All seven primer combinations exhibit a Tm difference of no more than 5°C. When the disparity in Tm value between the two primers is more than 5°C, it will hinder the amplification process, and in some cases, prevent any amplification from occurring [22].

Table 1. Primer Candidates' Primer Properties Determination

No	Primer Pair	Sequence Primer	Product Lenght	Base Lenght	%GC (%)	Tm (°C)	ΔTm
1	Forward 1	5'GCATTCATTGACCTCCCAGC3'	986	20	55	60,1	2,94
	Reverse 1	5'TGTTCTACGGGTTGTCCTCC3'		20	55	57,16	
2	Forward 2	5'CAGCCCCCTCAAACATCTCA3'	514	20	55	60,57	2,62
	Reverse 2	5'GCGAGGGCGGTAATGATGAA3'		20	55	63,19	
3	Forward 3	5'ATGATGAAACTTCGGTTCCCTC3'	456	22	45	59,81	1,89
	Reverse 3	5'AAGGCGAAGAATCGTGTGAGG3'		21	53	61,7	
4	Forward 4	5'AGCAACCCTCACACGATTCT3'	458	20	50	56,84	0,27
	Reverse 4	5'ATAGGCATTGACTTAGTGGTCG3'		22	45	56,57	
5	Forward 5	5'AACATCCGAAAATCACACCC3'	415	20	45	57,03	1,65
	Reverse 5	5' AGAATGATATTTGTCCTCAGGG3'		22	40	55,38	
6	Forward 6	5'GCAACCCTCACACGATTCTT3'	158	20	50	57,31	1,86
	Reverse 6	5'GTGTAGTATGGGTGAAATGGAAT3'		23	55	55,45	
7	Forward 7	5'TGAAACTTCGGTTCCCTCTTAG3'	293	22	45	58,25	1,31
	Reverse 7	5'AAGGCTGTTGCTATAACGGTAA3'		22	40	56,94	

Table 2. Primer Candidates' Primer Properties Determination Results

Primer Pair	Sequence Primer	Hairpin (kcal/mol)	Self-dimer (kcal/mol)	Cross dimer (kcal/mol)
Forward 1	5'GCATTCATTGACCTCCCAGC3'	0	0	0
Reverse 1	5'TGTTCTACGGGTTGTCCTCC3'	0	0	
Forward 2	5'CAGCCCCCTCAAACATCTCA3'	0	0	-9,31
Reverse 2	5'GCGAGGGCGGTAATGATGAA3'	0	0	
Forward 3	5'ATGATGAAACTTCGGTTCCCTC3'	0	0	-8,73
Reverse 3	5'AAGGCGAAGAATCGTGTGAGG3'	-0,69	0	
Forward 4	5'AGCAACCCTCACACGATTCT3'	0	0	-6,19
Reverse 4	5'ATAGGCATTGACTTAGTGGTCG3'	0	-3,92	
Forward 5	5'AACATCCGAAAATCACACCC3'	0	0	-5,36
Reverse 5	5' AGAATGATATTTGTCCTCAGGG3'	-0,33	-5,67	
Forward 6	5'GCAACCCTCACACGATTCTT3'	0	0	-7,48
Reverse 6	5'GTGTAGTATGGGTGAAATGGAAT3'	0	0	
Forward 7	5'TGAAACTTCGGTTCCCTCTTAG3'	0	0	-4,54
Reverse 7	5'AAGGCTGTTGCTATAACGGTAA3'	0	-3,4	

The following factor to consider is the percentage of guanine-cytosine content (%GC). The GC percentage must also be taken into account as it might influence the melting temperature (Tm) of a primer due to the presence of guanine and cytosine bases. An effective primer typically contains a GC content ranging from 40% to 60% [23]. The results of the prepared primers indicate that all candidates fulfill the specified criteria within the range of 40-55% and also meet the %GC criterion. Primers with a low GC content might hinder PCR efficiency as they are unable to effectively compete with the template [24].

According to this research, the NetPrimer program exhibits varied outcomes for each primer pair. The results are presented as hairpin structures, cross dimers, and self-dimers. A hairpin is a structure formed by the pairing of complementary sequences inside a single strand of polynucleic acid, which can be either DNA or RNA. Avoiding the creation of loop/hairpin structures on primers is crucial, however it is quite challenging to find primers that are free from such structures [25]. Sasmito et al. [25] state that the acceptable range for free energy is ΔG more than -3 kcal/mol. A higher negative free energy will result in increased stability of the hairpin formation process. The data shown in Table 2 indicate the presence of a hairpin on reverse 3 at -0.69 and reverse 5 at -0.33. These findings are consistent with the expected hairpin value.

Additionally, the primer characteristic that has to be examined is the existence of secondary structures in the primer. The secondary structure of the primer can impact the yield and integrity of PCR products, as it diminishes the primer's capacity to bind to the template. The NetPrimer tool evaluates the quality of the primer by analyzing the secondary structure in the form of self-dimers and cross dimers. The stability of secondary structure is dictated by the free energy (ΔG), which is utilized to disrupt the secondary structure. Self-dimers arise from intermolecular interactions between two

molecules of the same primer, such as the forward primer with other forward primers and the reverse primer with other reverse primers. The parameter examined in self dimers is the free energy (ΔG). A self-dimer with a free energy change (ΔG) of -6 kcal/mol is still within an acceptable range [25]. According to the findings of the self-dimer analysis presented in Table 2, all potential primer pairs meet the necessary criteria. This is because they exhibit a ΔG value for the self-dimer that is greater than -6 kcal/mol. This higher value ensures that the secondary structure of the self-dimer is less stable, which prevents it from interfering with the primer attachment process (annealing).

Primers that form a binding interaction with their complementary partner primers, including the reverse and forward primers, are referred to as cross dimers. Cross dimers with a free energy change (ΔG) of -6 kcal/mol can still be tolerated. If the value of ΔG is more than -6 kcal/mol, it will be challenging to disrupt the secondary structure that forms, making it difficult for the primer to bind to the intended location [25]. The ΔG value of the cross dimer formed by the candidate primer pair reverse 2, reverse 3, reverse 4, and reverse 6 is more negative than -6 kcal/mol. This indicates that the prospective primer pair does not match the requirements as it can create a stable secondary structure known as a cross dimer.

3.4 Evaluation of Primer Pairing Candidates using SnapGene Viewer

Annotation of the *Cyt b* gene sequence using SnapGene Viewer software shows that the sequence begins with the start codon with the code ATG with a nucleotide length of 1140 base pairs.

According to Figure 2, it is determined that all seven possible primer pairs bind to conservative areas. It is crucial to connect all primer candidates to conservative sections. This will enable the primer to identify 7 primer pairs of *Cyt b* genes and produce precise results for halal authenticity detection using PCR. The amplicon lengths acquired by mapping using SnapGene Viewer program using 7 pairs of primers are variable. The first primer pair has an amplicon length of 986 bp, the second primer pair has an amplicon length of 514 bp, the third primer pair has an amplicon length of 456 bp, the fourth primer pair has an amplicon length of 458 bp, and the fifth primer pair has an amplicon length of 415 bp. The sixth primer pair has an amplicon length of 158 bp, whereas the seventh primer pair has an amplicon length of 293 bp.

3.5 Genomic DNA Analysis Results

The genomic DNA in meat is isolated by the sample extraction method utilizing the Geneaid Genomic DNA Mini Kit (Tissue). This kit is utilized because to its superior time efficiency and straightforward preparation in comparison to other isolation procedures, such as chloroform and phenol extraction methods [26]. The investigation involved the extraction of DNA from samples of pigs, chickens, and cows. Pork samples were employed as the desired reference for porcine DNA material, while chicken and cow samples were utilized as non-target DNA templates for isolation.

The DNA molecules that were separated from other substances were subsequently examined for their purity using electrophoresis. Prior to the electrophoresis procedure, DNA is combined with loading dye or a colored charge buffer. The use of this dye enhances the density, causing the DNA to settle to the bottom of the well. Furthermore, this dye aids in the positioning of DNA in the well and indicates the movement of DNA [27].

Figure 3 clearly demonstrates the presence of a DNA molecule, which is shown by a band highlighted with a red color box. This photo depicts the presence of smudging. A smear refers to a band of DNA that appears as elongated spots on an electrophoresis gel [28]. The existence of smear is attributed to DNA degradation or fragmented DNA, rather than being intact. Therefore, obtaining thicker, tougher, and undamaged DNA will improve the quantity of DNA [28].

3.6 PCR Amplification Results

Furthermore, the results of DNA isolation serve as templates in the PCR amplification process. The purpose of the PCR process is to replicate a DNA molecule.

Figure 2. Primer Candidate Attachment

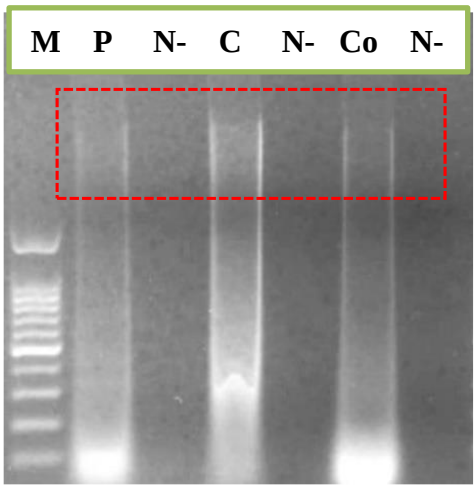

Figure 3. Genomic DNA electrophoresis results. M= Marker (ladder); P= Pig sample; C= Chicken sample; Co= Cow sample; N-=Negative control

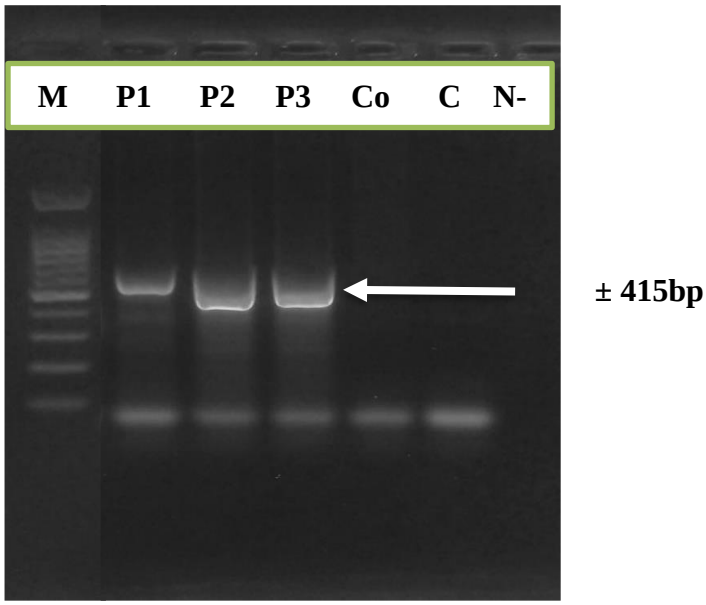

Figure 4. PCR amplification results the combination of primers Forward 5 – Reverse 5, amplicon length = ± 415 bp. M= Marker (ladder); P1= Pig sample 1; P2= Pig sample 2; P3= Pig sample 3; Co= Cow sample; C= Chicken sample; N- = Negative control

Visualization of the amplification results of the target gene DNA fragment was seen using electrophoresis process and observed with UV transilluminator. In this electrophoresis, a 1 kb ladder marker is used as a reference to determine the size of the amplified DNA base of the migrating DNA molecule. Based on the results of this visualization, it can be seen that the combination of primers Forward 5 – Reverse 5 there is a firm band in accordance with the estimated product of ± 415 bp with optimization of annealing temperature of 58°C. This shows that the quality and purity of DNA greatly affects the amplification process in DNA.

4 Conclusions

During the computational simulation, a total of 7 sets of potential primers were found. These candidates can now be evaluated using wet lab techniques. The experimental findings from the wet lab demonstrate that the primer pair 5, comprising of the forward sequence 5'-AACATCCGAAAATCACACCC-3' and the reverse sequence 5'-AGAATGATATTTGTCCTCAGGG-3', effectively amplified the DNA material. The estimated size of the result was around 415 base pairs, and the temperature at which the DNA strands were allowed to bind together was 58°C. This primer combination has the ability to identify the *Cyt b* gene of the *Sus scrofa* species.

5 Declarations

5.1 Acknowledgements

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This research did not receive funding from any source.

5.3 Author contribution

The names of the authors listed in this journal contributed to this research.

5.4 Conflict of Interest

All authors declare no conflict of interest in this manuscript.

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