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Combination of PCR analysis and sequencing on cytochrome-b gene *Canis lupus familiaris* for halal authentication

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ABSTRACT

The adulteration of beef with lower-priced meat, such as dog meat, is common to get economic profit. Dog meat is one type of meat that is not halal for consumption. There are several ways in which beef can be adulterated, including the use of dog meat. Specific primers for cytochrome-b (CYTBCA3-kh) can be used to identify the presence of dog meat contamination. This study aimed to identify dog meat using these primers. After conducting conventional PCR and agarose electrophoresis tests, the specificity of the primers was confirmed. Following this, the DNA base sequence was analyzed using a sequencing method to ensure accurate identification of dog meat contamination in beef. Specific dog primers tested on cattle, pigs, wild boars, goats, chickens, rabbits, and rats were confirmed using conventional PCR and agarose gel electrophoresis. Amplicon length verification was analyzed in a silico sequencing method using MUSCLE and BLAST NCBI software. The results showed that the primer CYTBCA3-kh amplified the canine Cyt-b mt-DNA gene specifically. The amplicon length obtained was 111 base pairs (bp), with a similarity value of 99.12% with *Canis lupus familiaris* mitochondrion, complete genome. The specific primer CYTBCA3-kh can be used to identify dog meat contamination in meatball products for halal authentication.
Keywords: CYTB gene, Halal Authentication, PCR, Sequencing, *Canis lupus familiaris*

INTRODUCTION

It's important to ensure that meat products are certified as halal before consumption, especially in countries where this is a major concern (Rahmati et al., 2016). Halal certification is necessary for food products to be consumable by Muslims. It's important to note that dog meat is not halal and should not be consumed by those who adhere to halal dietary laws. Authentication of halal status on various types of meat is a major concern in several countries (Rahman et al., 2014). Halal is one of the requirements that Muslims can consume food products. One type of meat that is not halal is dog meat. For Muslims, there is a prohibition on consuming food products containing dog meat because it is considered non-halal meat. Counterfeiting meat and non-meat products has become a common problem because these mixtures have lucrative economic value (Rohman & Che Man, 2012). The existence of a mixed product with non-halal components in any quantity causes the status of the product to become haram and prohibited for consumption (Ali et al., 2014). When purchasing food products, it is important to ensure they are Halal certified, especially for Islamic consumers. This certification ensures that the product meets the requirements for consumption according to Islamic dietary laws.

Non-halal mixtures in these food products are difficult to identify visually, thus requiring technology for their detection (Aida et al., 2005). The development of analytical methods for authenticating meat has been developed, such as Fourier transform infrared (FTIR) (Rohman & Che Man, 2012), spectroscopy, immune-electrophoresis, Real-time Polymerase Chain Reaction using specific primers (Abdel-Rahman & Saad Haggag, 2009), and real-time Polymerase Chain Reaction with Taqman (Kanthaswamy et al., 2012). The DNA-based method using PCR is an analytical method that is quite promising for the identification of non-halal mixed products (Nuraini et al., 2012), specific, sensitive, and fast for even in complicated processed meals, the identification of meat species (Amaral et al., 2015; Bottero & Dalmasso, 2011). The advantages of the PCR method are: (1) the DNA base

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sequence remains the same even though it undergoes a process that requires high temperatures, (2) it can be used in DNA sequences for all organisms, and (3) DNA is not damaged (Chhandak Basu, 2015; Chukwuemeka et al., 2020).

When using the PCR technique for DNA identification, primer specificity is the main requirement to strengthen the analysis of a particular DNA target. This ensures accurate and reliable results, crucial in various fields such as forensic science, medical research, and genetic testing. Using specific primers, the PCR technique can amplify and detect even the smallest amounts of DNA, making it an essential tool in modern molecular biology. Based on the testing of the CYTBCA3-kh primer using real-time PCR, it was found to have provided a single amplification (Kurniasih et al., 2020). To verify the specificity of the primer, the amplification product in the target area needs to be analyzed based on its sequence. This is an important step in ensuring accurate and reliable results in DNA identification using PCR techniques, particularly in fields such as forensic science, medical research, and genetic testing. The analysis aimed to verify that the PCR product amplified using a specific primer was the amplification result of cytochrome-b mtDNA (Mane et al., 2013; Kurniah et al., 2020). Previous studies have utilized the PCR technique with short amplicon lengths to identify the cytochrome-b region of dog meat in processed food products. The specificity of the primer used for amplification was confirmed through analysis of the amplification product based on its sequence. This is a critical step in ensuring reliable and accurate results in DNA identification, especially in fields such as genetic testing, medical research, and forensic science (Ali et al., 2014; Rahman et al., 2014). The newly developed method for detecting DNA from dog meat in beef meatballs was tested using species-specific primers targeting the mitochondrial cytochrome-b gene and real-time PCR. The amplification product was analyzed for its sequence to confirm the specificity of the primer used for amplification (Mohd Kashim et al., 2022). Although the method has not been tested for amplification products by DNA sequencing, the PCR approach has shown promising results in identifying the cytochrome-b region of dog meat in processed food products (Zhang et al., 2020). Through the use of real-time PCR amplification, we were able to successfully identify non-halal meat from dog meat in beef meatballs. We used a primer targeting the 105 base pair region of the mitochondrial cytochrome-b gene of *Canis lupus familiaris* for DNA detection (Kurniasih et al., 2020). This analytical approach provides reliable and accurate results in detecting non-halal meat in processed food products. Confirming amplicon length using a specific primer further ensures the specificity of the detection method.

RESEARCH METHOD

Materials

Analytical Balance (OHAUS), Glassware (Iwaki), Polymerase Chain Reaction (Bio-rad Gene Cycler), DNA Sequencer (Applied Biosystems 3500), Centrifuge (Hettich), UV transilluminator (Bio-rad), and water bath (Memmert). Dog meat is obtained from Bantul, Yogyakarta, Indonesia. Forward and reverse primers CYTBCA3-kh are specific primers specifically for dog DNA that have been validated, proteinase K (Invitrogen), lysis buffer (Invitrogen), TE buffer (Invitrogen), EvaGreen PCR master mix (Bio-rad), NFW (Bio-rad), and the TBE running buffer (Invitrogen).

Methods

1. DNA extraction

   DNA extracted with modification (Maryam et al., 2016). The lysis technique was carried out in a 2 mL microtube after 200 mg of meat was pulverized in a mortar. A vortex was used to homogenize mashed beef with 700 µL of lysis buffer and 30 µL of proteinase K. This combination was incubated for 2 hours at 55°C. After shaking for 30 minutes, 710 µL of phenol-chloroform-isoamyl alcohol was added to the mixture. For 5 minutes, samples were centrifuged at 12,000 rpm. The supernatant was discarded and replaced with cold 1:1 2-propanol. After 1 hour in a deep freezer, the mixture was centrifuged at 12,000 rpm for 5 minutes. The filtrate was collected, 500 µL of 70% ethanol was added, and the mixture was centrifuged for 5 minutes at 12,000 rpm. The supernatant was removed, and the residue was dried. After that, the dried DNA residue was dissolved in 100 µL TE buffer and incubated for 30 minutes at 50°C. DNA isolation was utilized for PCR analysis and can be kept in the freezer for 3 months at -20°C.

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2. Analysis with Conventional PCR

   PCR was used to examine the extracted DNA. The reagent mixture (25 µL) included 12.5 µL EvaGreen PCR master mix, 1.25 µL forward and reverse primers, 124 µL DNA template (50 ng/µL), and 8.75 µL NFW. Pre-denaturation at 95°C for 3 minutes (1 cycle), denaturation at 95°C for 15 seconds, annealing at an optimal temperature of 50.6°C for 20 seconds, extension at 72°C for 30 seconds (25 cycles), and elongation at 72°C for 5 minutes were the PCR conditions used. Gel electrophoresis was used to examine the PCR findings, which were obtained by adding 5 L of PCR product to a 2% agarose gel containing fluoro-safe dye. The DNA ladder is used as a marker. Electrophoresis was performed for 50 minutes at 80V with a TBE running buffer. Visualization of DNA bands was performed using a UV transilluminator.
3. PCR Result Purification

   PCR results were purified using a Purification Kit, namely ExoSAP-ITTM Express PCR Product Clean-up. The PCR amplification results were taken as much as 5 µL and 2 µL of ExoSAP-ITTM reagent added. The mixture was incubated at 37°C for 15 minutes and then at 80°C for 15 minutes. The purified PCR product can be used in Cycle sequencing for DNA analysis and stored at -20°C.
4. Sequencing

   The sequencing process The BigDye® Terminator V3.1 Cycle Sequencing Kit was used. protocol using a PCR tool. The cycle sequencing procedure begins with 44 seconds of pre-denaturation at 96°C (1 cycle), followed by 25 seconds of denaturation at 96°C, 5 seconds of annealing at 50°C, and 4 minutes of extension at 60°C. After that, the PCR cycle sequencing product was purified with the BigDye X-terminator Kit. DNA chromatograms from the sequencing results were edited and analyzed using Banser DNA software. After the alignment of the DNA sequences coding for the cytochrome-b CYTBCA3-kh is known, the sequences are compared to the sequences in the Genebank database using the BLAST program at NCBI (Najmi Mohammad Fauzi et al., n.d.).

Data Analysis

The specific dog primers (CYTBCA3-kh) carried out by (Kurniasih et al., 2020) must be confirmed by conventional PCR and gel electrophoresis. After obtaining pure results on PCR with gel electrophoresis, the next step is to perform a sequencing test to determine the DNA base sequence. The results of sequencing can be used to determine the kinship between species. This approach can provide more accurate and reliable results in identifying the presence of dog meat in processed food products.

RESULT AND DISCUSSION

The first step to identify fresh meat's species origin was DNA extraction. The results (Figure 1) indicated that DNAs extracted from fresh meats such as *Sus scrofa* (wild boar), *Sus scrofa domestica* (pork), *Bos taurus* (beef), *Capra hircus* (sheep), *Gallus gallus* (chicken), *Leporidae* (rabbit), and *Canis lupus familiaris* (dog). Electrophoresis on gels containing 0.8% agarose was used to observe the quality of the extracted DNAs, and UV light was used to visualize the resultsThe purity of the extracted DNA was then determined by measuring the absorbance values of DNA-containing solutions at 280 and 260 nm. The purity index and concentration of isolated DNAs are summarized in Table 1. DNAs with R values ranging from 1.7 to 2.0 were judged pure. (Arini et al., 2018). The ratio of absorbance values of DNA extracted from meat was between 1.8 and 2.0, which was significantly higher than the ratio of absorbance values extracted from processed items. Pure DNA molecules have a 260/280 ratio between 1.8 and 2.0 (Rosyid et al., 2023). These results showed that meat has a higher amount of pure DNA. The optimal concentration of template DNA for PCR activities, according to Nugroho et al., (2017), was between 10 and 100 ng/mL. According to this study, the amount of DNA in wild boar, hog, beef, goat, rabbit, and dog samples was extremely high. This is because the extracted sample was a fresh piece of meat containing no other materials.

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Table 1. The purity index of DNAs extracted from fresh meats

Meats	Concentration (ng/µL)	Ratio 260/280
Pork	1952.68	2.00
Wild boar	3150.23	1.87
Beef	105.98	1.70
Canine	2787.02	1.82
Chicken	3600.84	1.80
Rabbit	155.21	1.75
Goat	275.13	1.80

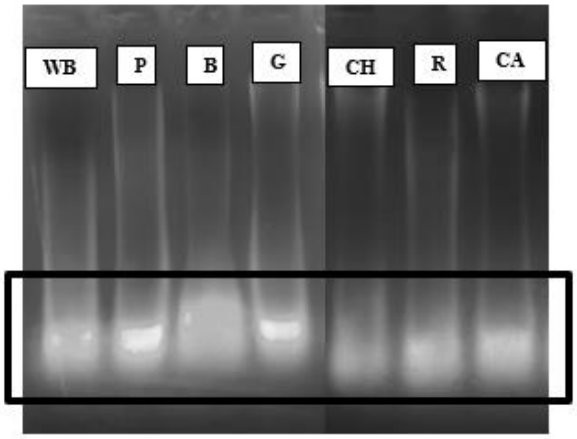

Figure 1. The evaluation of DNAs extracted using agarose gel electrophoresis from fresh meat of wild boar (WB), pork (P), beef (B), goat (G), chicken (CH), rabbit (R), and canine (CA) on the agarose gel 0.8%

The selection of forward and reverse mitochondrial cytochrome-b CYTBCA3-kh primers was based on published data on the identification of different DNA taken from non-halal meat (wild boar, hog, and dog) (Kurniasih et al., 2020). The primer has been specifically validated to provide a single amplification of dog meat. Specific dog primers must be confirmed using conventional PCR and agarose gel electrophoresis. It is important to verify the amplification product in the target area based on the sequence to prove the specificity of the primer. The PCR process involves three important steps, namely, the denaturation of the template DNA chain containing the target sequence, the annealing of the primer pairs on the target DNA, and the extension or polymerization reaction, which will be catalyzed by DNA polymerase (Buh Gašparič et al., 2010; Rosyid et al., 2023). After performing amplification by PCR, it is important to analyze the results carefully to determine if there are any other products besides the target product (Indriati et al., 2019). One way to identify the target product is to look at the electropherogram with the primer CYTBCA3-kh, which should only show one band. The primer sequences used were forward primer -CCT TAG CCA ATG CCT ATT C- and reverse primer -GCG ACT TGT CCG ATA ATG-. Based on the primer sequence used, the target amplicon length for this PCR amplification is 105 bp. Figure 2 shows the results of the amplification visualization.

Based on the analysis of Figure 2, it is clear that the PCR product results exhibit a single band on the 2% agarose electrophoresis. This indicates that both pairs of primers used in the experiment could amplify the desired DNA fragment with specificity. The experiment was successful in achieving the desired outcome.

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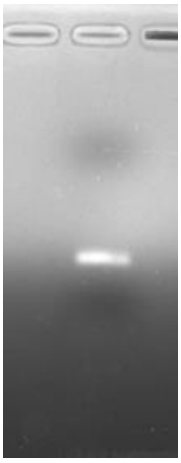
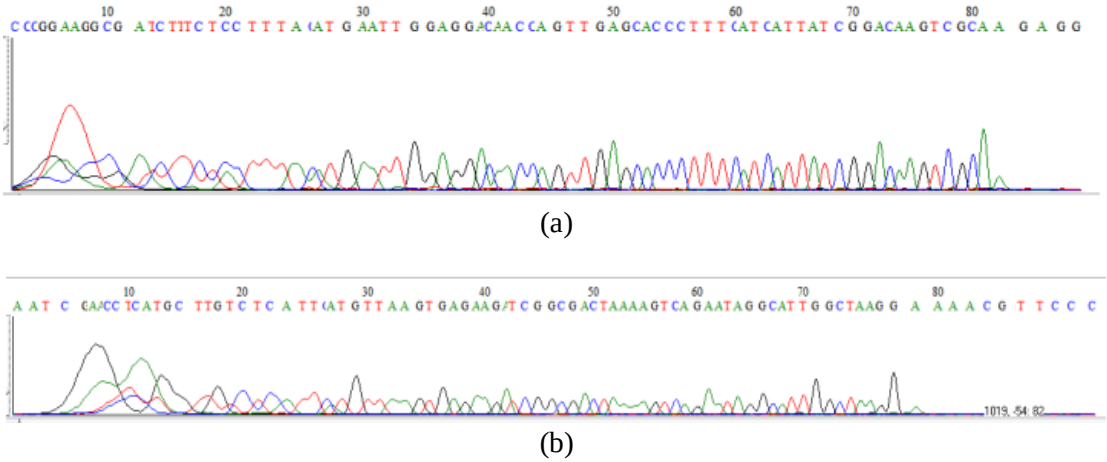


Figure 2. Visualization of PCR amplification results using primer CYTBCA3-kh on dog meat

To ensure optimal sequencing results, it is recommended to carry out PCR product purification after analyzing Figure 2. The ExoSAP-IT™ Express method can eliminate any enzymatic residue in the PCR amplification product, resulting in high-quality sequencing results. ExoSAP-IT™ can purify PCR products from amplicon lengths of less than 20 bp to more than 100 bp without losing the sample DNA (BioTechniques, 2016). Now that we've completed the PCR step, the next step is the cycle sequencing process. We'll use the BigDye® Terminator protocol during this stage to produce a termination sequence at 96°C. It's important to ensure that the DNA template is completely free from any inhibitors and any remaining PCR reagents. In addition, it is necessary to ensure that all required reagents are in sufficient quantities to successfully carry out the sequencing process. After completing the cycle sequencing process, the next step is to purify the results to remove any residual reagents that could cause disturbances during the sequencing process. This includes removing dNTP, ddNTP, DNA polymerase, and salts. Once purified, the resulting supernatant can be injected directly into the sequencing instrument. It's important to ensure the sequencing results are as accurate as possible. When working with PCR products, verifying their validity is important to ensure accurate results. One method for verification is DNA sequencing, which allows for the determination of the DNA base sequence of a fragment (Gaffar, 2007; (Zhang et al., 2020)). Maede (2006) suggests three methods to verify the validity of PCR products. One of these methods is DNA sequencing, which allows for the determination of the DNA base sequence of a fragment (Yang et al., 2014).



(a)

(b)

Figure 3. Sequence alignment analysis using the forward primer (A) CYTBCA3-kh and reverse primer (B).

The initial step in DNA sequencing is determining the amplicon length. DNA that PCR has amplified can be determined by sequencing. The sequencing process is carried out using an Automated

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Capillary Sequencer; separating the fragments and reading the DNA base sequence can be done automatically. The results of the sequencer machine readings are called electropherograms, which are colored peaks that show the sequence of DNA bases (Brown T.A., 2010). The electropherogram of good sequencing is that the peaks produced are clear and do not overlap between one peak and another (Mane et al., 2013). The results of DNA base sequencing using forward and reverse primers were 86 bp and 79 bp, respectively (Figure 3). After analyzing the nucleotide base sequence from the CYTBCA3-kh PCR results in Figure 3, we performed a sequence alignment analysis using the MUSCLE software online to obtain amplicon sequence data. The alignment results can be seen in Figure 4.

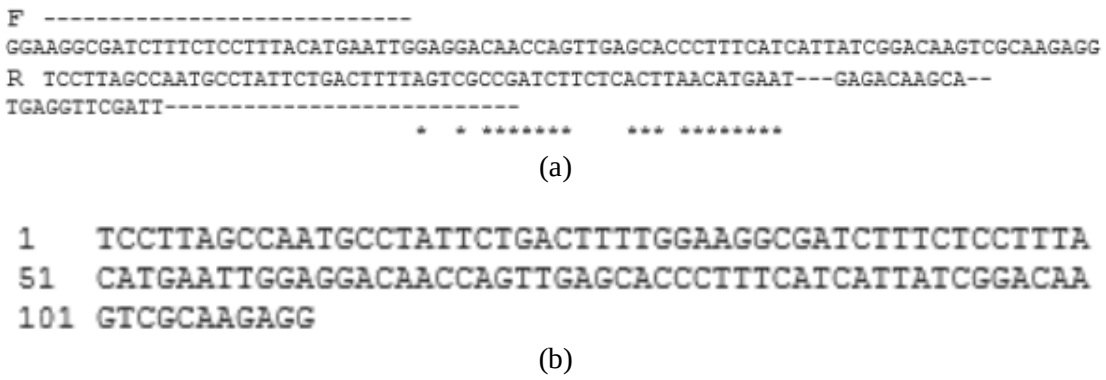

Figure 4. A sequence of DNA fragments (A) results of aligning primer F with primer R using MUSCLE software and (B) results after alignment to obtain a size of 111 bp

According to Figure 4, the alignment of the forward and reverse primers results in a complete size of 111 bp DNA fragments. It is important to note that the mismatch of the primers is due to the absence of a proofreading mechanism from the 3' to 5' ends by the DNA polymerase enzyme, which can cause errors in base pairing. Therefore, it is crucial to ensure accurate and precise sequencing to achieve the desired outcome when using these primers (Binnewies et al., 2006; Ishak & Lazim, n.d.). Meanwhile, differences in the sequencing results of the number of amplicons resulting from the in silico design, where the size was 105 bp, could be caused by a mismatch in the attachment of the dNTP by DNA polymerase (Drancourt et al., 2000). Based on the sequencing results, both the forward and reverse primers have successfully attached to the target DNA. This indicates that the desired DNA sequence has been amplified and can be further analyzed. After analyzing the DNA sequencing results, the annealing process is running optimally, and all DNA bases can be identified. The homology analysis of the DNA sequence coding for cytochrome-b CYTBCA3-kh using BLAST software on GeneBank (NCBI) has shown that most of the primers have attached perfectly to the DNA sequence with a high alignment value (> 200). This indicates that the DNA template can adhere from the 5' test to the 3' end of the primer, and further analysis can be carried out with confidence (Aina et al., 2020). After comparing the sequence results with the sequence of dogs (*Canis lupus familiaris*), it can be seen that the dog meat isolation has a similarity percentage of 99.12% with the *Canis lupus familiaris* mitochondrion complete genome sequence, according to the results of BLAST. The NCBI BLAST GenBank analysis using the CYTBCA3-kh primer revealed interesting details about the relationship between *Canis lupus familiaris* DNA and other species. Figure 5 displays the phylogeny tree of *Canis lupus familiaris*, revealing its close kinship with various species. The Cyt-b DNA gene Canis lupus dingo, found in India, shares a similarity of 91.69% with *Canis lupus familiaris*. Arctic foxes with the Cytb-b DNA Vulpes lagopus gene have an 83.60% similarity, and the Cyt-b DNA Vulpes vulpes gene,

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which belongs to the red fox, has an 82.98% similarity. These fascinating results provide insight into the genetic connections between different species.

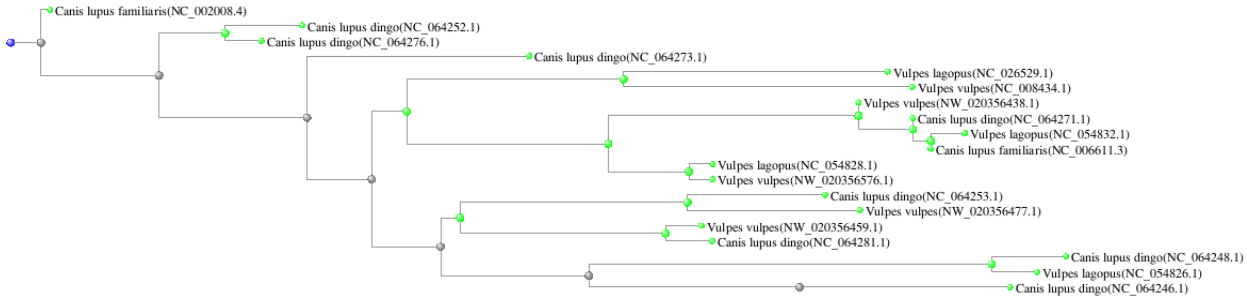

Figure 5. Phylogenic Tree of Relationship between *Canis lupus familiaris* DNA and Several Species in the NCBI BLAST GenBank Using CYTBCA3-kh Primer

The DNA sequencing assay method is rapid, time-efficient, highly sensitive, and reproducible. However, inhibitors in the meat matrix reduce the effectiveness of this sequencing assay. The quality of the DNA is a critical factor in the PCR process, which must be optimized for the efficient generation of accurate sequencing data. The sequencing test can't be done if the DNA is of bad quality and the primers can't amplify the target gene in the PCR test (Ishak & Lazim, 2022).

CONCLUSION

It has been discovered through research that the CYBCA3-kh primer is capable of targeting and amplifying DNA that is unique to canines. Using the CYTBCA3-kh primer can effectively identify contaminated dog DNA in food for halal authentication purposes. This is due to the primer's ability to accurately amplify the unique target sequence found in canines, as evidenced by its 92.12% resemblance to the Cyt-b mt-DNA of a dog. It is possible to argue that this primer effectively targets the desired sequence.

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