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Research Article

Molecular Identification of Domestic and Wild Boars Using Cytochrome-b Specific Primers for Halal Authentication

[1]Rifqi, [1]Edi Suryanto, [1]Ari Surya Sukarno, [1]Rusman, [1]Nanung Agus Fitriyanto, [2]Abdul Rohman and [1,2]Yuny Erwanto

[1]Department of Animal Products, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Institute for Halal Industry and System, Universitas Gadjah Mada, Yogyakarta, Indonesia

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Corresponding Author:
Yuny Erwanto
Department of Animal Products, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia;
Institute for Halal Industry and System, Universitas Gadjah Mada, Yogyakarta, Indonesia
Email: yunyer@ugm.ac.id

Abstract: The subject of adulterating wild boar and pig meat products with other animal meat is delicate, particularly in the Muslim nation of Indonesia. In recent years, there has been a lot of concern over halal meat products that contain wild boar and pig meat due to Economically Motivated Adulteration (EMA). However, there are limited studies of PCR-DNA-based to discriminate between domestic pigs and wild boars. This study aims to design species-specific primers to identify wild boar in the cytochrome-B gen region. DNA from seven animals (wild boar, pig, cow, sheep, goat, chicken, and fish) was extracted. Both conventional and real-time PCR were used for qualitative and quantitative identification. The cytochrome-B gene of wild boar and pig was sequenced, and this region was then used to design primers using PrimeQuest. Designed primers were characterized by four criteria: specificity, sensitivity, limit detection, and repetition tests. The set of primers designed for amplification consisted of Cyt-B Forward171 5'CGAGACGTAAATTACGGATGAC'3 and Reverse-488 5'GGTAATGATGAAGGGCAGGATG'3. The results of this study showed the primer amplificated wild boar DNA, specifically with an annealing temperature of 53°C, performed in a 25 cycle RT-PCR system. Good recommendations were shown by the sensitivity test (R2 = 0.9817, slope = -3,4742, y-intercept = 30,625; and efficiency = 94%). In conclusion, the adulteration of wild boar meat in food products can be detected using a specially designed primer. The proposed primer can be used to identify wild boars in products sold on the commercial market, according to the results of qualitative and quantitative tests.

Keywords: Adulteration, DNA, Identification, Primer Design, Wild Boar

Introduction

Meat source identification is critical in food safety and assurance, particularly in Indonesia, a predominantly Muslim country where Halal authentication is essential. Beyond health and hygiene considerations, Halal certification is crucial for maintaining consumer trust and ensuring compliance with religious dietary laws. Studies on species identification are vital to prevent the falsification of Halal meat by mixing it with non-Halal meat, addressing both trade competition concerns and consumer satisfaction. Adequate consumer protection necessitates using sensitive, accurate, and up-to-date methods for identifying intra- and inter-species adulteration in meat products (Erwanto *et al.*, 2014; Siswara *et al.*, 2022).

Economic motivations often lead to adulterating meat products with lower-cost alternatives, such as wild boar mixed with pork or beef, as Danezis *et al.* (2016) reported. For example, wild boar meat, being less expensive, is sometimes intentionally blended into processed meat products to increase profits while deceiving consumers about the authenticity of the meat. Such practices undermine consumer trust and pose ethical and safety concerns, especially in regions where religious dietary laws and food authenticity are highly prioritized. Wild boar meat's presence in food products must be identified to uphold consumer rights and ensure product safety.

Unlike domesticated pork, which is easier to monitor and control throughout the supply chain, wild boar meat often comes from poaching, introducing additional food safety risks. These risks arise from the potential contamination of the meat with zoonotic parasites and bacteria, which can harm human health (Fredriksson-Ahomaa, 2019; Meng *et al.*, 2009). Wild boars and other

wild animals frequently serve as reservoirs for several infections that humans can contract by eating contaminated meat or by direct contact. (Danezis *et al.*, 2016). Consuming undercooked or badly processed wild animal meat has been connected to viral infections like Nipah and Ebola as well as diseases like trichinosis, which is caused by Trichinella sp. (Cantlay *et al.*, 2017). In particular, wild boars are known to carry zoonotic parasites like Toxoplasma gondii and bacteria such as Salmonella sp. and Yersinia enterocolitica (Fredriksson-Ahomaa, 2019; Meng *et al.*, 2009). These health risks underscore the urgent need for reliable methods to detect wild boar meat in food products, ensuring consumer safety and compliance with food safety standards. Additionally, research by Cantlay *et al.* (2017) shows that the consumption of bushmeat further heightens the risk of zoonotic disease transmission, exacerbating food adulteration concerns and posing a significant threat to global food security.

In a halal context, the presence of wild boar meat in food products is a significant problem because it is not permitted in Islamic teachings. This is exacerbated by the difficulty of detecting wild boar meat visually because its physical characteristics are similar to domestic pork and several other types of red meat, such as beef or goat. Therefore, developing a DNA-based identification method is an important solution to ensure the halalness of a food product (Aida *et al.*, 2005; Ali *et al.*, 2012).

PCR have proven to be the most reliable approach for food authentication, as these methods can detect DNA traces even in minimal amounts or after intensive food processing (Rahmati *et al.*, 2016; Tanabe *et al.*, 2007). In order to distinguish wild boars from domestic pigs and other frequently consumed animals, this study created primers specifically for the cytochrome-B gene. This technique can be applied in the food business to guarantee adherence to halal standards and stop fraud in the food supply chain, with a detection sensitivity of up to 0.5% in meat mixes. Additionally, real-time PCR test findings demonstrate that this primer has a high specificity for wild pig DNA and does not cross-react with other species, making it a potentially trustworthy tool for halal testing in the commercial sector.

An accurate DNA identification method is very important to support global halal regulations, including those implemented by the Indonesian Ulama Council (MUI), Jabatan Progress Islam Malaysia (JAKIM), and other international halal certification bodies. The development of more precise, quick, and targeted detection techniques, like those created in this study, will help preserve the integrity and openness of the halal food sector as consumer awareness of halal food items grows.

Previous studies have widely investigated pork adulteration in food using porcine-specific primers (Ali *et al.*, 2011; Cahyadi *et al.*, 2020; Martin *et al.*, 2009). However, studies developing specific primers for wild boars remain limited. Notably, Aina *et al.* (2019) and Arini *et al.* (2018) reported because of the tight genetic ties between the two subspecies, primers were unable to differentiate between pig and wild boar DNA. Due to the strongly matched DNA sequences produced by this genetic resemblance, trustworthy techniques for identifying adulterated wild boar meat through DNA analysis must be developed.

Because of its sensitivity, speed, and capacity to distinguish between closely related species, polymerase chain reaction, or PCR, has emerged as the method of choice for species identification. In contrast to conventional techniques like SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) (He *et al.*, 2018), lipid-based methods (Szabó *et al.*, 2007), and enzyme-linked immunosorbent assay (ELISA) (Patterson *et al.*, 1984) which are time-consuming and prone to inaccuracies, PCR offers a precise approach to detecting DNA even in degraded samples. Additionally, DNA stability during food processing, compared to often unstable proteins under such conditions, further establishes PCR as the most reliable technique for identifying species in processed food products. DNA, unlike proteins, remains intact even in highly degraded samples, making DNA-based PCR a robust tool for species identification (Xu *et al.*, 2018). Furthermore, mitochondrial DNA (mtDNA) analysis offers advantages due to its abundance in cells and natural amplification, making it a reliable genetic marker (Erwanto *et al.*, 2014). Among mtDNA regions, the cytochrome-B gene has been widely used for species-specific primer design due to its high variability and suitability for identifying closely related species.

Previous studies used specific primer identification through an RT-PCR system to detect meat-based food products such as meatballs (Aina *et al.*, 2019). Economically motivated adulteration (EMA) is common, especially in developing countries. Adulterating cow meat products with wild boar or pig meat is the most frequent case of EMA (Ballin *et al.*, 2009). In addition, religious concerns are an important aspect of meat adulteration triggers after economic motivation (Qin *et al.*, 2019).

This study designed new primers (F-171 and R-488) based on mitochondrial cytochrome-B DNA sequences. The primary objective was to validate the specificity and sensitivity of these primers in detecting wild boar DNA. DNA templates from seven species—wild boar (*Sus scrofa*), domestic pig (*Sus scrofa* domestica), cow (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra*), chicken (*Gallus gallus* domesticus), and catfish (Clarias)—were tested to evaluate the primers' performance in distinguishing wild boar meat adulteration.

Materials and Methods

Sample Preparation and DNA Extraction

Seven distinct wild animals were used to produce the meat products: boar, pig, cow, sheep, goat, chicken, and

fish for genomic DNA preparation. All of the raw meat was bought from the local market in Yogyakarta, Indonesia. Every sample was individually handled to prevent cross-contamination and stored at –20°C. DNA Tissue preparation and extraction were carried out according to the protocol of the Animal DNA Extraction Kit (Geneaid Biotech Ltd). The DNA was diluted with a pre-heated elution buffer. Wild boar meat was obtained from the market selected for DNA isolation, followed by species-specific primer development at the end of this study.

Primer Design

The PrimeQuest online program from Integrated DNA Technologies was used to design the forward (F-171) and reverse (R-488) primers for the cytochrome-B mitochondrial region of wild boar. First, a universal cytochrome-B primer was used to amp up DNA templates from domestic pigs and wild boar. Using a thermocycler (Labnet International Inc.), the amplification procedure was carried out as follows: 40 cycles of pre-denaturation at 94°C for two minutes, denaturation at 94°C for one minute, annealing at 63°C for one minute, and extension at 72°C for one minute, followed by a final extension at 72°C for five minutes. Using the NCBI GenBank primer BLAST program, the specificity of the primers for the DNA templates of pigs and wild boar was confirmed in silico. The universal cytochrome-B mitochondrial DNA sequences for wild boar and pigs, as referenced by Kocher *et al.* (1989), were used as a positive control. The sequencing results served as the basis for developing wild boar-specific primers to distinguish between species and intra-species variations.

PCR Reactions

The amplifications were carried out in 10µL volumes, composed of the following components: 5µL of PCR master mix (SMOBIO-ExcelTaq™ 5X PCR Master Dye Mix), 2µL of ddH2O, forward primer (1µL) , reverse primer (1µL), and 1µL of each DNA template. PCR amplification was conducted using a thermocycler following the specific master mix and primer protocols. After amplification, the PCR products were analyzed through gel electrophoresis using a 1.5% agarose gel (Sigma Aldrich-low EEO) with Florosafe DNA staining (1st Base). The final visualization of the results was performed under UV light.

Sequencing and Bioinformatic Analysis

To create a DNA amplicon, the extracted DNA template was amplified using universal primers that target the mitochondrial DNA's cytochrome-B region. Sanger DNA sequencing was used to sequence the PCR products from both ends (Agilent, USA). The chromatograms of the amplicons were analyzed using MEGA 11, which was used to generate the sequence assembly (Tamura *et al.*, 2021). The nucleotide sequence that was produced was then used to design primers in the same area. Furthermore, the BLAST tool at the National Center for Biotechnology Information (NCBI) GenBank was used to compare the nucleotide sequences with those of other sub-species, such as the SS Agl (GU135705.1), the Vietnam pig (KX982653.1), the Large White (AY920909.1), the Baoshan (KT194217.1), Yuxi (MK858173.1), and the Vietnam pig (KX982653.1).

Real-Time PCR Reactions

Ten microliters were used for the RT-PCR, which contained one microliter of template DNA, one microliter of each forward and reverse primer, two microliters of ddH_2O, and six microliters of SYBR Green Premix Ex Taq (Japan). Pre-denaturation at 95°C for two minutes, denaturation at 95°C for fifteen seconds, annealing at 53°C for fifteen seconds, and extension at 72°C for one minute were the conditions under which the RT-PCR was carried out on the Real-Time PCR QuantStudio 3 (Applied Biosystems, USA). A melt curve analysis from 53°C to 95°C with an increment of 0.1 degrees per second was performed after this cycle was repeated 25 times.

Results

DNA Extraction Quality

A NanoDrop 2000 spectrophotometer, which calculates the ratio of absorbance at wavelengths 260 nm and 280 nm, was used to assess the concentration and purity of the extraction products. Pure DNA is indicated by a ratio of 1.8 to 2.0, whereas lower values imply protein contamination (Olson & Morrow, 2012). The quantitative results in Table 1 demonstrate that the A260/A280 ratios for the seven samples ranged from 1.2 to 1.35, which is consistent with protein contamination. Although protein contamination was observed, the absence of RNA contamination ensured the DNA's usability. The smearing in electrophoresis gels, while indicative of fragmentation, did not impede amplification, underscoring the robustness of the extraction process. Similar findings have been documented in studies where low purity ratios were offset by successful amplification, highlighting the flexibility of downstream applications (Imbeaud *et al.*, 2005). The remaining proteins in the sample can inhibit the action of the Taq DNA polymerase enzyme, thereby reducing the efficiency of PCR amplification and increasing the possibility of weak or inconsistent amplification results.

Furthermore, the visualization for DNA extract results showed clear DNA bands without indications of significant degradation, although there was a slight smear indicating the possibility of minor fragmentation due to the extraction process (Figure 1). DNA was isolated from different animal species using the Animal DNA Extraction Kit (Geneaid Biotech Ltd.). To confirm the success of the extraction, the DNA samples were

electrophoresed on a 1.5% agarose gel stained with Gold View nucleic acid dye. The gel was run at 100V for 30 minutes in 1× TBE buffer and subsequently visualized under UV light. DNA purity can be enhanced by refining extraction methods, incorporating additional purification steps such as the use of RNase, proteinase K, or spin column-based extraction techniques (Imbeaud *et al.*, 2005). The successful amplification of the primers that have been validated in this study shows that even though there is protein contamination, the primers still have high enough sensitivity to detect wild boar DNA in the meat samples tested (Figure 2). Therefore, DNA was considered sufficient for subsequent cytochrome-B amplification despite evidence of protein contamination.

Table 1: Concentration and purity of DNA templates were extracted

No	Sample	Concentration (µg/mL)	Purity (A_{260}/A_{280})
1	Pig	32,2	1,262
2	Wild boar	31,7	1,257
3	Cow	72,8	1,276
4	Chicken	66	1,288
5	Goat	87,4	1,298
6	Sheep	131,6	1,339
7	Catfish	146,8	1,301

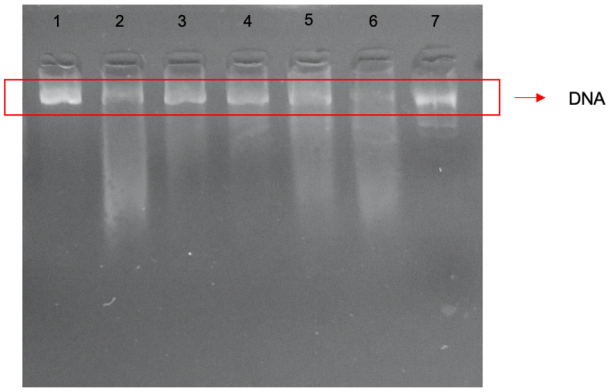

Fig. 1: Agarose gel electrophoresis of DNA from different animal species. Lanes: 1, Pig (*Sus scrofa domestica*); 2, Wild boar (*Sus scrofa*); 3, Cow (*Bos taurus*); 4, Chicken (*Gallus gallus domesticus*); 5, Goat (*Capra hircus*); 6, Sheep (*Ovis aries*); 7, Fish (*Clarias* sp.)

Primer Design

The presence of mitochondrial DNA in each sample was verified by PCR amplification using universal cytochrome-B primers. The 25 µL reaction mixture consisted of 12.5 µL PCR master mix (SMOBIO), 1 µL of each primer (10 µM), 2 µL DNA template (20 ng/µL), and 8.5 µL nuclease-free water. Amplification was performed under the following conditions: initial denaturation at 95°C for 2 minutes; 40 cycles of 95°C for 15 seconds, 53°C for 15 seconds, and 72°C for 1 minute. The successful amplification, which generated a ~500 bp amplicon from wild boar and pig templates (Figure 2), confirmed that the target mitochondrial sequence was present. This verification step was conducted prior to the design of species-specific primers and confirmed that the universal target sequence was a suitable basis for developing primers capable of distinguishing wild boar from other species.

Comparative DNA sequence analysis was conducted to confirm the ability of the designed primers to differentiate wild boars from domestic pigs. A multiple sequence alignment of cytochrome-B sequences from wild boar, domestic pig, and related *Sus* species was performed using MEGA 11 software with reference sequences from GenBank: Vietnam pig (KX982653.1), Large White (AY920909.1), Baoshan pig (KT194217.1), Yuxi pig (MK858173.1), and *Sus scrofa* strain Agl (GU135705.1) (Figure 3).

This alignment highlighted interspecies nucleotide polymorphisms, confirming sufficient variation for specific primer design. Based on these polymorphisms, the primers Forward-171 (5’ CGAGACGTAAATTACGGATGAC 3’) and Reverse-488 (5’ GGTAATGATGAAGGGCAGGATG 3’) were designed. The primers had melting temperatures (Tm) of 61°C and 63°C, respectively, with GC contents of 45.5% and 50%. *In silico* analysis confirmed their specificity for wild boar DNA, yielding an expected amplicon of 361 bp.

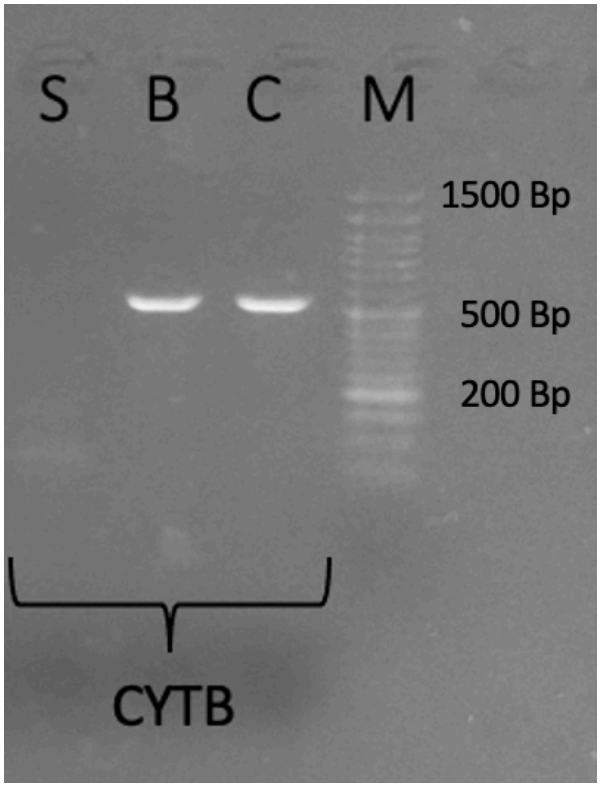

Fig. 2: Verification of mitochondrial DNA by cytochrome-B PCR. Gel lanes show amplification products from: S: Cow (*Bos taurus*) B: Domestic pig (*Sus scrofa domestica*); C: Wild boar (*Sus scrofa*); Lane M contains a DNA marker

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Fig. 3: Multiple sequence alignment of cytochrome-B gene sequences from wild boar, domestic pig, and related *Sus* species showing nucleotide variations used for primer design

Primer Quality Test

Specificity tests were performed using thermocycling, which optimized the annealing temperature to 53°C with 25 cycles. Visualization on an agarose gel confirmed the specific amplification of wild boar DNA, consistent with the results obtained from both conventional and real-time PCR (Figure 4). The cycle threshold (Ct) values indicated the specificity of the primers, with wild boar DNA showing a Ct value of 13.25 and pig DNA showing a Ct value of 24.818. Melt peak analysis further supported this finding (Figure 6), revealing a single peak for wild boar DNA and multiple peaks for other species. Sensitivity tests (Figure 7) demonstrated that the lowest detectable DNA concentration for wild boar was 2.03 ng/µL, resulting in a Ct value of 28.467. The dilution concentrations and their corresponding Ct values are detailed in Table 2, showing a consistent increase in Ct value with decreasing DNA concentration. The efficiency of the PCR was calculated to be 94%, in line with the criteria established by Broeders *et al.* (2014). Detection limit tests indicated that the primers could detect wild boar DNA at 0.5% concentration within pig DNA templates, demonstrated by a Ct value of 31.66 (Figure 9). Reproducibility tests showed consistent amplification with low standard deviations and strong correlation coefficients, validating the reliability of the designed primers (Figure 10 and Table 4).

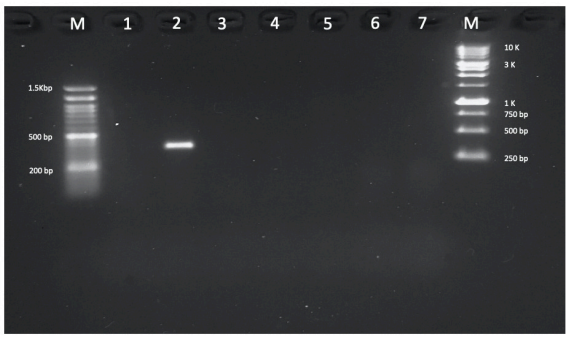

Fig. 4: PCR amplification for the detection of wild boars utilizing species-specific primers. Lanes: (1) Pig; (2) Wild boar; (3) Cow; (4) Chicken; (5) Goat; (6) Sheep; (7) Fish

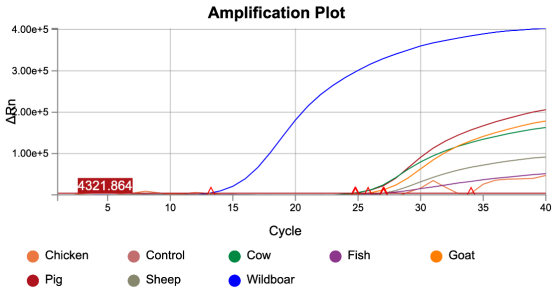

Fig. 5: Real-time PCR amplification plot of wild boar DNA using species-specific primers

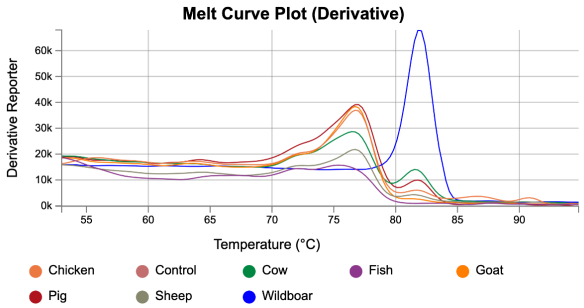

Fig. 6: DNA from wild boars was analyzed using a melt curve and species-specific primers

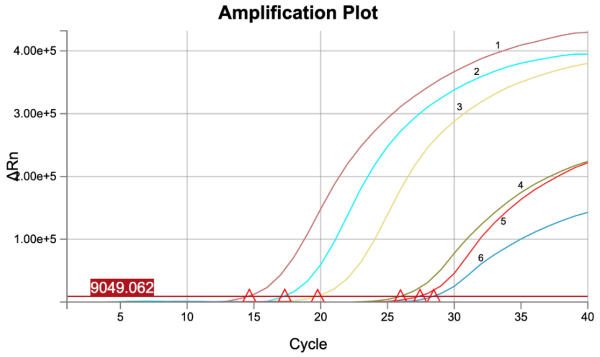

Fig. 7: Amplification curves from real-time PCR analysis of wild boar DNA serial dilutions using primers F1 and R3

Table 2: Six level of dilution of wild boar's DNA, dilution logarithm, and CT value of each sample

No	Dilution concentration	Log	CT Value
1	31700 ng/mL	4,50	14,66
2	6340 ng/mL	3,80	17,31
3	1268 ng/mL	3,10	19,77
4	50,72 ng/mL	1,70	25,975
5	10,14 ng/mL	1,01	27,437
6	2,03 ng/mL	0,31	28,47

Discussion

This study designed primers specifically for DNA identification of wild boar (Sus scrofa), considering potential cross-reactivity with domestic pigs (Sus scrofa domestica) due to genetic similarities. Previous studies, such as Aina *et al.* (2019), reported that primers developed for wild boar could still amplify domestic pig DNA, highlighting the challenge of differentiation. To address this, we validated the primers in silico by comparing wild boar sequences with various pig subspecies in GenBank. Melt curve analysis from real-time PCR showed a single peak for wild boar DNA, while other species exhibited double peaks or no amplification, indicating high specificity.

For validation through experimentation, cross-reactivity assessments were performed using DNA samples from wild boars (*Sus scrofa*), domesticated pigs (*Sus scrofa domestica*; Landrace, Duroc, Berkshire), and feral swine. Results from conventional PCR indicated that while the primers effectively amplified wild boar DNA, weak bands of amplification were also noted in Landrace and Duroc pig samples, which suggests possible cross-reactivity. Analysis through real-time PCR supported this observation, as wild boar DNA displayed a robust signal at a Ct of approximately 14, in contrast with Landrace and Duroc that showed weak amplification at Ct values around 32 until 35. Melt curve evaluations indicated a minor overlap in melting temperatures between the wild boar (82.4°C) and the Duroc pig (81.9°C), implying partial homology in sequences. In silico alignment of cytochrome-b sequences revealed a mismatch of 2-4 nucleotides in some domestic pig breeds, which could lead to non-specific binding under less-than-ideal PCR conditions. These results are consistent with earlier research by Aina *et al.* (2019) and Gupta *et al.* (2021), which noted that mitochondrial DNA markers may demonstrate cross-reactivity because of shared maternal lineages. Likewise, Erwanto *et al.* (2014) and Karabasanavar *et al.* (2014) observed that cytochrome-b primers typically amplify both wild and domestic pigs due to the high conservation of their sequences.

This study focused on amplifying mitochondrial DNA in the cytochrome-B gene, which is more stable than genomic DNA due to its higher copy number within cells (Tanabe *et al.*, 2007). Unajak *et al.* (2011) highlighted mitochondrial DNA advantages for species differentiation due to its abundance and variability. Tanabe *et al.* (2007) further emphasized mitochondrial markers' utility due to maternal inheritance and lack of recombination. Gupta *et al.* (2021) confirmed distinct cytochrome-B variations between wild boars and pigs, validating the approach used in this study. Previous studies, reported by Erwanto *et al.* (2014), have shown that mitochondrial DNA remains detectable after high-heat treatments. However, DNA degradation may impact amplification, particularly if the primer-binding region is compromised. Therefore, the primer design in this study incorporated a relatively short amplicon size to improve the amplification of degraded DNA.

DNA-based food authenticity and adulteration detection depend on the creation of species-specific primers. In this work, DNA from seven distinct animal species that are frequently eaten as food was used to assess primer specificity using traditional PCR. Electrophoresis results confirmed that amplification occurred exclusively in wild boar DNA, matching the expected amplicon size of approximately 361 bp and demonstrating high specificity (Figure 4). Previous studies have employed various methods to amplify pork DNA in food products. Erwanto *et al.* (2011) amplified a 359 bp cytochrome b fragment using PCR-RFLP, and then I digested it with restriction enzymes to distinguish between species. Fibriana *et al.* (2012) targeted the PRE-1 locus (481 bp) to detect pig DNA in food products,

while (Tanabe *et al.*, 2007) designed a shorter 130 bp amplicon for processed food detection. Karabasanavar *et al.* (2014) also developed a highly specific PCR assay targeting the mitochondrial D-loop region to identify pork in food products. This assay yielded a unique 712 bp amplicon specific to pig DNA, with no cross-amplification observed in 24 other animal species. The assay effectively detected pork in raw, cooked, autoclaved, and microwave-processed samples, with a detection limit of 0.1% pork content and a 10 pg of pig DNA sensitivity. However, these approaches did not specifically differentiate between pig and wild boar in food samples. The precision of primer design, particularly at the 3' end sequence, as used in this study, may enhance species discrimination in cases of high genetic similarity.

High primer specificity was demonstrated by real-time PCR validation, which verified that only wild boar DNA showed significant amplification (Figure 5). Even in combinations containing domestic pig DNA, the detection limit analysis demonstrated that the proposed primers could detect wild boar DNA at quantities as low as 0.5% (Figure 9 and Table 3). The technique is helpful for halal verification and preventing food fraud because of its high sensitivity, which guarantees that even trace amounts of wild boar contamination may be detected in commercial beef products. Unlike prior studies, including that of Aina *et al.* (2019), which obtained a sensitivity of 5 pg/µL, our approach offers dependable detection with little cross-reactivity, which makes it ideal for regulatory enforcement.

Table 3: Wild boar and pig DNA templates mixing series for detection limit test

No	Percentage		CT Value
	Wild boar	Pig	
1	0,5%	99,5%	31,66
2	1%	99%	29,87
3	25%	75%	16,57
4	50%	50%	15,47
5	75%	75%	14,2
6	100%	0%	14,56

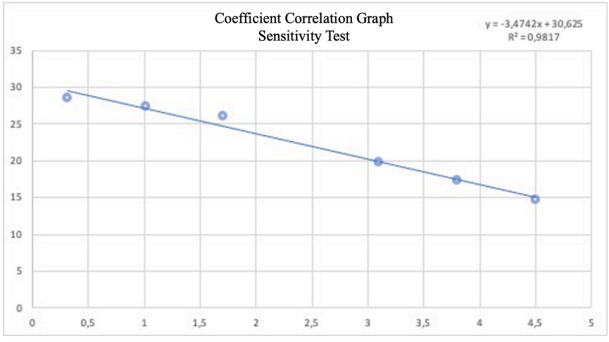

Fig. 8: Correlation analysis between DNA concentration and Ct value for wild boar DNA detection using primers F1 and R3 in real-time PCR

The method's promise for quantitative food authentication is further supported by the significant connection between Ct values and DNA concentration (R2 = 0.9817; Figure 8), which enables both the detection and calculation of wild boar contamination levels in food products. For halal certification organizations, food safety regulators, and industry participants, where accurate measurement of non-halal meat contamination is essential for compliance and customer confidence, this capacity is especially pertinent. The assay functions within the ideal range for real-time PCR applications, as evidenced by the 94% PCR efficiency, which is consistent with Broeders *et al.* (2014).

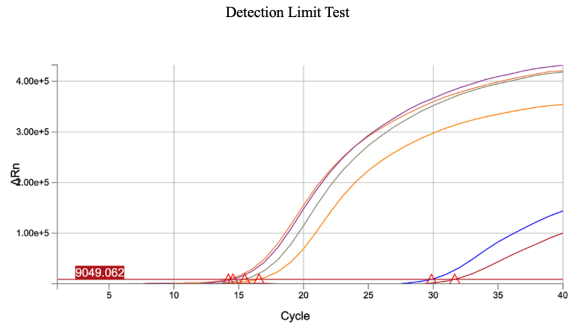

Fig. 9: Results of detection limit test by mixing wild boar and pig DNA templates series

This technique has wider uses in biosecurity surveillance and wildlife forensics than just food verification. Trace amounts of wild boar DNA can be used to track unlawful hunting and the meat trade, especially in areas where wild boars are exploited for human consumption. Furthermore, the technique may be used in zoonotic disease surveillance to detect populations of wild boar that may act as hosts for diseases like African Swine Fever (ASF) and Classical Swine Fever (CSF) (Gupta *et al.*, 2021).

The primers' specificity was further confirmed by melt curve analysis, which showed a clear peak for wild boar DNA with little overlap with domestic pig DNA (Figure 5). The crisp and stable melt peaks found in this study imply that this method is still useful even for analyzing processed meat, despite earlier research by Tanabe *et al.* (2007) noting that DNA degradation from high-temperature processing could affect detection accuracy. This is especially important for making sure that halal certification, processed food surveillance, and meat product labeling all comply.

Further validation using highly processed food samples is advised to further establish real-world applicability, even if the results show great specificity and sensitivity. Furthermore, multiplex PCR techniques may be used in the future to detect many species simultaneously in a single reaction, improving supply chain traceability and large-scale food authenticity verification.

Real-time PCR reproducibility analysis for wild boar DNA detection showed minimal Ct variation, with a standard deviation of 0.4286 and a coefficient of variation (CV) of 2.99% (Figure 10 and Table 4), demonstrating high precision. The low CV further confirms primer stability and reproducibility, aligning with Kang (2019), who mentioned that a CV below 5% ensures amplification validity. Reproducibility tests confirmed the reliability of the primers across multiple runs, demonstrating their consistency for research and industry applications. Similarly, Rahmati *et al.* (2016) used real-time PCR to amplify pork DNA in spiked meatball samples, reporting low Ct variability across multiple runs. The approach maintained consistent pork DNA detection in processed foods, though dependence on specific conditions restricted applicability to highly processed samples. Montowska and Pospiech (2012) also validated DNA-based meat authentication using conventional and real-time PCR, confirming reliable amplification in sausage and meatball samples but not addressing wild boar differentiation.

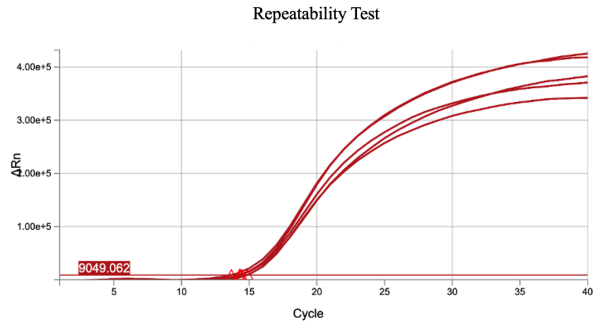

Fig. 10: Amplification plot of the repeatability test for real-time PCR-based wild boar DNA detection

Table 4: Repeatability test result

Sample	Ct	ΣCt	STD	CV (%)
Wild boar's DNA template with concentration of 31,7 µg/mL	14,88 14,28 13,68 14,32 14,42	14,32	0,4286	2,9937

The primary method for identifying food adulteration is now the use of DNA-based PCR techniques in food authentication. Numerous investigations have demonstrated that the PCR technique may successfully identify non-halal meat mixture in processed foods (Aida *et al.*, 2005; Rahmati *et al.*, 2016). Additionally, it has been demonstrated that DNA-based food detection is more effective than conventional techniques like lipid-based analysis or enzyme-linked immunosorbent assay (ELISA), which are frequently less sensitive for processed food samples (Bottero & Dalmasso, 2011; Xu *et al.*, 2018). Some research showed that DNA can still be detected in processed products even though they have experienced degradation due to high heating or extreme processing, making PCR a more reliable method in food testing (Karabasanavar *et al.*, 2014; Tanabe *et al.*, 2007). Therefore, with the high sensitivity and specific detection validated in this study, the developed cytochrome-B primers can effectively prevent food adulteration and support halal regulations and global food safety.

The current research contributes significantly to the field by designing and validating primers that can effectively distinguish wild boar DNA from pig DNA, even in mixed and processed food products. This innovation addresses a critical gap present in earlier studies, thereby enhancing the detection of species adulteration. Additionally, the primers exhibit high sensitivity, specificity, and reproducibility, meeting stringent requirements for identifying food adulteration. Future applications of this research may include market surveillance and the development of standardized protocols for broader species differentiation, especially in cases involving closely related species. The method can also be adapted for routine testing in food industry laboratories and has the potential to be integrated with multiplex-PCR techniques, allowing for the simultaneous detection of multiple species in a single reaction (Ali *et al.*, 2012; Qin *et al.*, 2019).

Conclusion

This study successfully developed and validated specific primers (F1 and R3) for wild boar DNA with an optimized primer attachment temperature protocol of 53°C and 25 PCR cycles, producing a 361 bp amplicon. The primers demonstrated high specificity, sensitivity, and reproducibility, with a 0.5% wild boar DNA detection limit in pig DNA mixtures. These findings address the limitations of previous studies by enabling reliable differentiation between wild boar and pig DNA, even in processed food matrices. Thus, the designed primers can be robust genetic markers for identifying wild boar meat adulteration, contributing to stricter Halal and Kosher food authentication standards and broader food safety applications.

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Conflict of Interest

The authors affirm that they have no conflicts of interest.

Authors Contributions

Edi Suryanto: Conceptualized and designed the study, performed data curation and formal analysis, and prepared the original draft of the manuscript.

Rifqi and Ari Surya Sukarno: Conducted the experiments, contributed to data interpretation, and

participated in writing, reviewing, and editing the manuscript.

Rusman, Nanung Agus Fitriyanto, and Abdul Rohman: Provided supervision, contributed to conceptualization, and managed project administration.

Yuny Erwanto: Proposed the research idea, supervised the experimental work, acquired funding, and provided final approval for the submitted and revised versions of the manuscript.

All authors discussed the results, reviewed the manuscript critically, and approved the final version for publication.

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