Short Communication:

Wild Boar-Specific PCR Assay and Sequence Analysis Based on Mitochondrial Cytochrome-B Gene for Halal Authentication Studies

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Abstract: *Wild boar meat (WBM) is non-halal meat widely abused in Indonesia. The most common case is mixing beef with WBM either in raw or processed foods. Therefore, it is necessary to develop a detection method of WBM contamination. The objective of this study was to employ polymerase chain reaction (PCR) and sequence analysis using species specific primer (SSP) targeting on wild boar mitochondrial cytochrome-b (CYTBWB2-wb) gene for the identification of WBM in a meatball. The specificity of primer was tested, and the amplicon size was confirmed with conventional PCR and agarose electrophoresis. The base sequences were analyzed using GeneStudio software and subjected to BLAST using NCBI. CYTBWB2-wb primer was also used to test the reference meatballs made from beef and WBM using real-time PCR. The result showed that CYTBWB2-wb amplified wild boar Cyt-B mt-DNA gene specifically. The amplicon size was 194 base pair (bp) with a similarity of 93–98% toward gen Cyt-B mt-DNA of several wild boar types. The primer is able to detect WBM on the reference meatballs up to 0.1% wt/wt with efficiency value of 108.0% and coefficient of determination (R^2) of 0.970. The CYTBWB2-wb primer proved to be specific and could be used as a standard method to identify the presence of WBM contamination in meatball products for halal authentication studies.*

Keywords: *CYTBWB2-wb primer; meatball; halal authentication; sequencing; wild boar*

■ INTRODUCTION

Meat is a good source of protein and contains some important vitamins, nutrients, and essential amino acids needed for human's growth [1]. Beef is widely consumed with the consumption level of as much as 6.4 kg/capita in 2017 [2]. Beef-based foods such as meatballs and sausages become favorite food products because of their pleasant tastes and are easily processed into a variety of products. Due to the price difference, some unethical sellers try to blend beef having high price with lower price meat such as pork and wild boar meat (WBM) to get economic profits.

The Muslim community is prohibited from consuming food products containing WBM because WBM is considered as non-halal meat [3]. Halal is the main requirement for food products to be consumed by Muslims based on the provisions of Islam. The existence of a mixture or contamination of non-halal components in any amount will cause their status to become haram and prohibited to be consumed. The issue of

counterfeiting meat food products with non-halal components such as wild boar raises concerns among the Muslim community. Muslim consumers need halal authentication for food product they are purchased [1].

The mixture of non-halal components in a processed food product is difficult to identify visually and requires the application of technology for its detection [4]. Therefore, it is very important to develop analytical methods for authentication of beef and its beef-based product. Various methods for the detection of non-halal components have been developed, such as Fourier transform infrared (FTIR) spectroscopy [5], immune-electrophoresis [6-7], polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [8-9], real-time polymerase chain reaction using species specific [10-11], and Taqman-real time PCR [1-2]. Among these methods, DNA-based methods using PCR is quite promising for routine analysis of non-halal components. The advantages of DNA-based methods are: (1) DNA is not damaged, (2) the base sequence of DNA remains same even it is subjected to high temperature, and (3) it is present in almost all organisms [12]. DNA sequences are specific to certain organisms and are widely used in the analysis. Based on this, the DNA-based PCR method can be used to detect various types of non-halal components specifically and can be applied to food products which have mostly subjected to high-temperature processing.

Primer specificity is the main requirement for the identification of DNA using PCR techniques so that it can amplify one type of DNA target. The CYTBWB2-wb primer specificity was tested using real-time PCR and produced single amplification [13]. However, according to the European standards for molecular microbiology and molecular detection in genetically modified organisms, the amplification products in the target area need to be verified by their sequence to prove primer specificity [14]. The sequence analysis was intended to ensure that PCR products amplified with species specific primer (SSP) were the result of amplification in Cyt-B mt-DNA region [15]. Several PCR methods for detection of wild boars with specific primers have been reported previously, with targeted cytochrome-b [3], mitochondrial RNA [16], and mitochondrial d-loops [17-18]. However, the method developed has not added an amplification product test with DNA sequencing. In addition, WBM detection method developed by Parkanyi [18] and Mutalib et al. [16] using multilevel PCR and RFLP PCR have disadvantages where there is a possibility of mutations in the restriction site that can cause misinterpretation of test results [19]. PCR-RFLP is less suitable for identification of compositions of meat mixtures in processed products because the heat might degrade DNA and cause changes to the restriction site [20]. Meanwhile, the primer developed by Arini et al. [17] has not been able to distinguish between wild boar and pig. The CytbAG3A primer developed by Guntarti et al. [3] have been able to distinguish between boars and pigs, but the LOD obtained is still quite high at 48 pg, and several validation parameters are not met the criteria.

The combination of PCR amplification and DNA sequencing were proved to be more precise and authentic tools for detection of the species origin of meat. Both genomic and mitochondrial genes were targeted to identify species origin of meat by PCR amplification followed by sequencing [21]. The objective of this study was to employ PCR and sequence analysis using SSP targeting on mitochondrial cytochrome-b of wild boar species (CYTBWB2-wb) gene for the identification of non-halal meat of wild boar meat (WBM).

■ EXPERIMENTAL SECTION

Materials

Wild boar meat was obtained from hunters in sub-district Baamang, district Kotawaringin Timur, Central Kalimantan Province, Indonesia. The others meat obtained from Yogyakarta, Indonesia, in which beef, goat and chicken were obtained from sub-district Mlati, Sleman, Yogyakarta, pork from Godean, Sleman, Yogyakarta, rabbit from Kaliurang, Sleman, Yogyakarta, and dog meat was obtained from Bantul.

Procedure

Primers design

Forward (F) and Reversed (R) primers of CYTBWB2-wb were designed using PrimerQuest software from Integrated DNA Technologies. The DNA sequence of wild boar mitochondrion complete genome (NC_026992) used for primer design was retrieved from NCBI GenBank. The primer specificity was checked *in silico* using primer BLAST compared with beef, pork, goat, chicken, rabbit, and dog. The designed primers used were:
Forward: CGG TTC CCT CTT AGG CAT TT
Reverse: GGA TGA ACA GGC AGA TGA AGA

The primers have the characteristics of melting temperature of 60 °C (F and R), GC contents of 50% (F) and 47.6% (R) with amplicon product of 191 base pair (bp) [13].

The extraction and evaluation of DNA

The DNAs used for PCR assay were extracted manually using Phenol-chloroform, isoamyl alcohol (CIAA) methods. The purity of DNA was quantitatively analyzed with NanoQuant Spark Tecan (Switzerland) and was visualized with electrophoresis procedure (i-Mupid J Cosmo Bio Co., Tokyo, Japan). DNA purity was calculated based on the ratio (R) of absorbance values at wavelengths (λ) of 260 and 280 nm, respectively. DNAs with R values of 1.8–2.0 were considered as pure [22].

Amplification analysis of DNA using PCR

The extracted DNA was analyzed using PCR. The reagent composition (20 µL) consisted of 10 µL EvaGreen PCR master mix, 1 µL forward primer and reverse primer, 1 µL DNA template (50 ng/µL), and 7 µL free nuclease water. The conditions used were: pre-denaturation at 95 °C for 3 min (1 cycle), denaturation at 95 °C for 10 sec, annealing at optimized temperature of 59 °C for 20 sec, extension or amplification at 72 °C for 45 sec (30 cycles, respectively), and elongation at 72 °C for 5 min to stabilize the amplification product.

Evaluation of PCR product

The PCR products were visualized with electrophoresis (i-Mupid J Cosmo Bio Co., Tokyo, Japan) using agarose 2% in TBE 1× for 45 min at 100 V to see if the DNA was successfully amplified using designed SSP. We used 5 µL for each type of DNA with a concentration of 50 ng/µL. The result was recorded using transilluminator (Syngene, Synoptics Ltd., England). The band formed was compared with the DNA marker to determine the size of the amplicon. The DNA marker used was the DNA ladder 100 bp (GeneAid, Taiwan) [23].

Reference meatball testing

The reference samples used in this study were meatballs made from beef and wild boar with known concentrations, as shown in Table 1. Fresh beef and wild boar meat were washed and drained. The prepared meat (beef, beef-WBM, WBM) as much as 90% was mashed and mixed with wheat (10%), salt (0.01% wt/wt), and other herbs commonly used in making meatballs. The mixture was then formed into a ball and then boiled in boiling water until cooked. The DNA of reference meatball was extracted and then tested using q-PCR. The condition of q-PCR methods used was according to the manufacturer's procedure given (Biotium Inc., Bio-Rad Laboratories, CA, USA). In reaction tube, 10 µL of EvaGreen, 7 µL nuclease-free water, 1 µL forward primer 10.0 µM, 1 µL reverse primer 10 µM, and 1 µL of extracted DNA 50 ng were mixed and analyzed using q-PCR instrument (CFX96 Touch Real-Time PCR Detection System, Biorad USA). The q-PCR procedure was as follows: pre-denaturation at 95 °C for 3 min (1 cycle), denaturation at 95 °C for 10 sec (30 cycles), annealing at 59 °C for 20 sec and extension or amplification at 72 °C

Table 1. Composition of meatball reference samples containing beef (B) and wild boar meat (WBM)

No	Sample	WBM (%)	Beef (%)
1	Beef meatball without WBM	0	100
2	Beef meatball + WBM 0.1%	0.1	99.9
3	Beef meatball + WBM 0.7%	0.7	99.3
4	Beef meatball + WBM 1%	1	99
5	Beef meatball + WBM 2%	2	98
6	Beef meatball + WBM 3%	3	97
7	Beef meatball + WBM 5%	5	95
8	Beef meatball + WBM 10%	10	90
9	Beef meatball + WBM 25%	25	75
10	Beef meatball + WBM 50%	50	50
11	Beef meatball + WBM 75%	75	25
12	Wild boar meatball 100%	100	0

for 45 sec. The relative fluorescence signal was performed automatically at each cycle ends. For analysis of the melting curve, the temperature was set 65–95 °C with increasing temperature of 0.5 °C/5 sec. The evaluation included linearity, efficiency, and sensitivity. Data were processed and analyzed using CFX Maestro™ software included in q- PCR instrument.

DNA sequencing

This stage was started with the purification of DNA using Illustra™ GFX™ PCR DNA and gel band purification kit. The method used followed the procedure of the kit provided by the manufacturer. A-10 µL PCR product and 500 µL of type 3 buffer were added to the filter and centrifuged at 16000 × g for 30 sec. The sample was washed with wash buffer type 1 and then centrifuged at 16000 × g for 30 sec. Furthermore, A-20 µL type 6 elution buffer was added, stand for 60 sec, and the sample was centrifuged at 16000 × g for 60 sec. The mixture consisting of 4 µL buffer, 2 µL big dye, 1 µL forward or reverse primer, 1 µL purified DNA, 12 µL NFW was run in PCR thermocycler with initial denaturation conditions of 96 °C for 1 min, denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec, extension at 60 °C for 4 min in 25 cycles. Then, the mixture was added with 90 µL SAM, 20 µL terminator, and 10 µL DNA, vortexed for 30 sec and centrifuged for 3 min. A-20 µL of the sample was inserted into the plate and centrifuged at 16000 × g for 60 sec. The sample sequenced using Applied Biosystem 3500 Genetic Analyzer, and the result was analyzed using GeneStudio software.

■ RESULTS AND DISCUSSION

PCR Analysis

Wild boar is one of the non-halal meats which is widely misused as a raw material for beef. Wild boar meat (WBM) has a texture and color similar to that of beef. In meatball products, WBM is preferred over pork because the texture of WBM is similar to that of beef meatballs. Therefore, WBM is very potential meat adulterant in beef meatballs. PCR is an analytical technique based on DNA exponential amplification through *in vitro* enzymatic reaction on thermocyclers similar to that of *in vivo* replication [24]. This study use species specific primary (SSP)-PCR combined with sequence analysis to detect the presence of WBM contamination in beef meatballs. The SSP-PCR method is very simple, sensitive, and rapid compared to other PCR assays and has proven to be powerful for identifying species in a mixture of food ingredients either in raw or in processed food [25]. Meanwhile, the sequence analysis is used to ensure the correctness of the PCR products from DNA amplification with SSP. The combination of both methods will produce valid data for identifying species on a product. In this study, DNAs from WBM and reference meatballs were extracted using the phenol-chloroform method. The purity of DNA was visualized by agarose electrophoresis and was measured quantitatively using Nanoquant Tecan Spark. The results of agarose electrophoresis showed that DNA was well extracted without being degraded. Quantitative measurements showed high purity of DNA with absorbance ratio (R) values at 280 and 260 nm ratio of 1.8–2.0 for all DNA used.

The species-specific primer (SSP) used was CYTBWB2-wb, previously designed using PrimerQuest software, targeting on wild boar cyt-B mt-DNA. This specificity primer was evaluated toward DNAs extracted from meats of wild boar, pig, cow, dog, chicken, rabbit, and goat. Based on our previous report, CYTBWB2-wb primer was specific to wild boar DNA [13]. The amplification curve from q-PCR testing showed that only wild boar DNA was amplified while the other DNAs and negative control or no template control (NTC) were not amplified. The melt curve of the PCR product showed a melting temperature at 80 °C. The sensitivity assay resulted that the used primer had a very low limit of detection (LoD) value of 5 pg/µL of wild boar DNA.

The primer specificity of CYTBWB2-wb is also proven by electropherogram of PCR product. Wild boar DNA and comparative DNA were amplified with CYTBWB2-wb primer using conventional PCR. This test also became the initial stage of sequence analysis in which the amplification product was compared with a DNA ladder of 100 bp marker to confirm the size of the amplicon formed. The amplicon size is one of the important data for ensuring the correctness of amplification products.

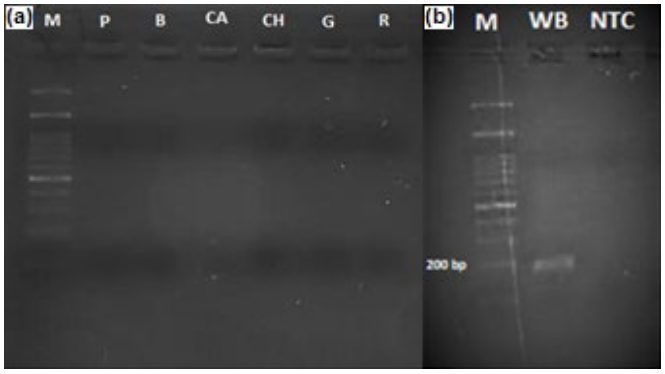

Fig 1. The gel agarose electropherogram of PCR amplification products from (a) pork (P), beef (B), canine (CA), chicken (CH), goat (G), and rabbit (R) (b) wild boar (WB), and no template control (NTC) using primer CYTBWB2-wb. M is DNA marker (DNA ladder 100 bp). Only wild boar DNA that is amplified of around 200 bp

PCR assay using CYTBWB2-wb primer was carried out at an annealing temperature of 59 °C, as optimized by Aina et al. [13]. PCR assay using CYTBWB2-wb primer gave a positive result. The SSP amplified wild boar DNA specifically compared to 6 other DNAs extracted from pig, cow, dog, goat, rabbit, and chicken. Agarose electrophoresis exhibited that only wild boar DNA could be amplified with an amplicon size of around 200 bp (Fig. 1). The other DNAs were not amplified as indicated by the absence of amplification bands. This result in conventional PCR was in agreement with that of q-PCR. The amplicon size formed is also in accordance with the initial design *in-silico* in which the amplicon size was estimated at 191 bp. This indicated that primer CYTBWB2-wb could amplify wild boar DNA specifically. A single and clear band indicated that the PCR condition was suitable so that it can be processed to the sequencing process.

The evaluation of primer performance during amplification of wild boar DNA extracted from reference meatballs consisting of beef and WBM was conducted using real-time PCR. Linearity and efficiency tests were carried out on 100% wild boar meatball reference meatballs while sensitivity test used multilevel concentrations of WBM. Evaluation of primer performance on reference meatballs showed good results and met the specified parameters. The efficiency value (E) and linearity obtained were in the required range with E of 108.0% and R^2 of 0.970 (Fig. 2). Meanwhile, the sensitivity test showed that CYTBWB2-wb primer still able to detect WBM on meatballs up to 0.1% wt/wt (Fig. 3). Based on Ct values analysis, the shift

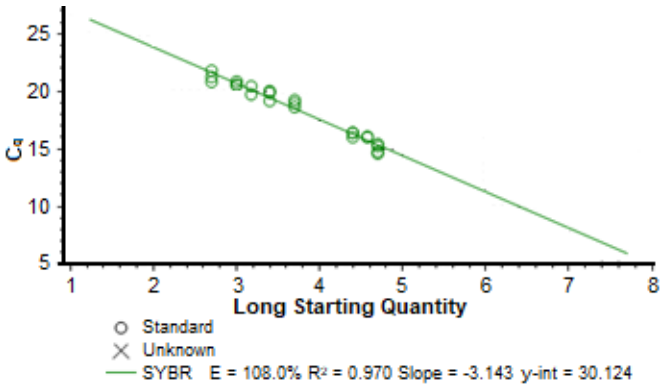

Fig 2. Standard curves for linear relationship between Ct values (y-axis) vs. the log10 of the copy number of (DNA) extracted from meatball containing 100% wild boar meat, also used for calculating efficiency of amplification (E)

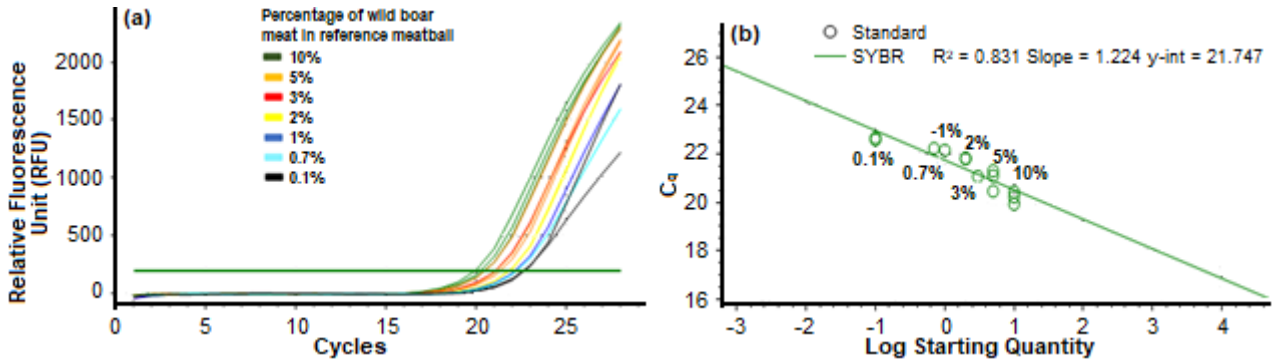

Fig 3. The sensitivity test for determining of limit of detection (LoD) of DNA extracted from reference meatball. (a) = the amplification curve; (b) = the relationship between log starting concentration (x-axis) with Ct values (y-axis)

in Ct values at low WBM concentrations could still be observed. The Ct value increases with the decrease of WBM concentration in reference meatball, as shown in Fig. 3(a). Although the change in Ct value at WBM levels of 1%, 0.7%, and 0.1% is very small, it can still be appropriately detected using this method. This can be confirmed by linear correlation between Cycle threshold (Ct) or Cycle quantitation (Cq) values and log starting concentration of DNA (Fig. 3(b)). These results indicated that CYTBWB2-wb primer could be used to detect the presence of WBM contamination on meatball products.

Sequence Analysis

According to Maede [21], three methods can be used to verify the correctness of PCR products, namely, restriction analysis using restriction endonuclease enzymes, probe hybridization, and DNA sequencing. Among these methods, DNA sequencing is mostly chosen because it provides reliable results of the base sequence. The initial stage of the sequencing process is the determination of the amplicon length as explained in the PCR analysis section. The next stage is the DNA sequence. Each fragment was sequenced twice using forward and reverse primer respectively so that the DNA sequence obtained was more accurate because of twice reading from the front and back of the fragment. The sequencing of purified amplification product using forward primer produced an amplicon of 159 bp, while the reverse primer produced an amplicon of 162 bp in the following order (Fig. 4). The data were processed by alignment analysis using GeneStudio software to obtain the complete amplicon sequence data. Before an alignment analysis was carried out, one of the base sequences above must be reversed and complemented by the corresponding nucleotide pair so that the two basic sequences are in the same position. Basically, the forward primary sequence is in position 5' → 3', while the order of the reverse primer is the opposite of position 3' → 5'. The results obtained were shown in Fig. 5. This sequence is a complete sequence of DNA fragments measuring 194 bp. The same base was marked with a point (.), while an unidentified base was indicated by a stripe (-). The base sequence was similar, which indicated that forward and reverse-primers could amplify

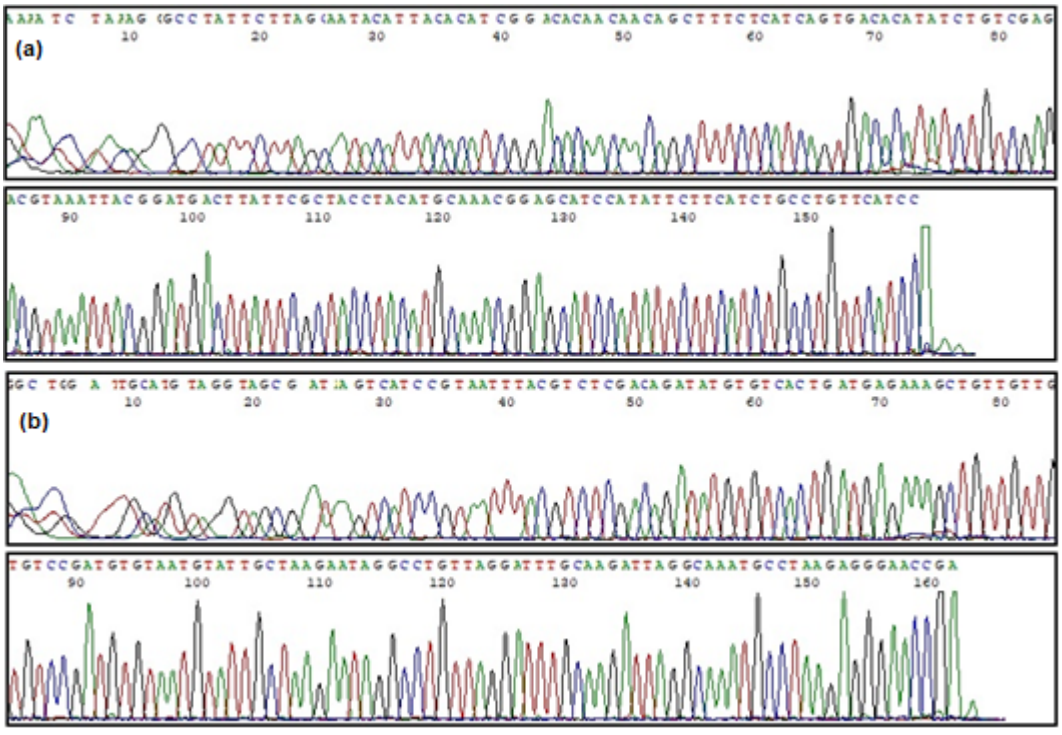

Fig 4. The result of DNA sequencing using (a) forward primer of CYTBWB2-wb (b) reverse primer of CYTBWB2-wb, before sequence compliment is performed

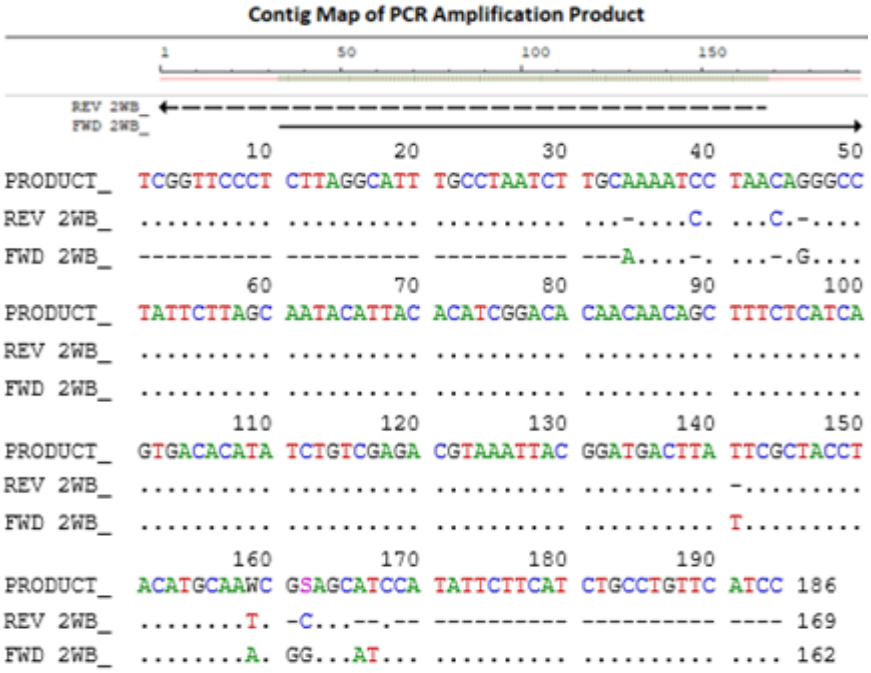

Fig 5. The bases sequence of Cyt-B mt-DNA fragment (194 bp) after alignment using GeneStudio software. Dot (.) indicated the identical result and stripe (-) indicated non identified bases

the same area. The amplicon length formed was 194 bp. However, only 186 bases can be read while the other (8 bases) are unreadable during the sequencing process. This can be caused by the presence of a mismatch (attaching dNTP which is not compatible with DNA polymerase) which can occur naturally. This phenomenon could be described as DNA polymerase enzyme does not have an exonuclease 3' to 5' proofreading activity so that it can cause a base installation error of about 1 in 9000 nucleotides. This amount is slightly different from the results of in silico design where the amplicon size is 191 bp. The sequencing result showed that the forward and reverse primers could attach perfectly to the target DNA. Whole bases of the primer were fully identified, which means that the annealing process runs at optimum conditions. Therefore, the amplification process can run well.

The amplification products were subjected to BLAST to obtain the base sequence obtained using the software provided by NCBI. The sequences obtained were compared to the wild boar sequences. The alignment score got a high value (≥ 200). Based on the BLAST results, it could be stated that the sequence of 194 bp fragments from the amplification results had filled the area corresponding to the estimated amplification area. The maximum score was 329, the total score of 329 with a query cover of 100%. This showed that there was only a single alignment. Most primers were perfectly attached to the printed DNA sequence with a high suitability value (≥ 200) which means that from the end of 5' to the end of 3' the primer attached to the printed DNA sequence.

The amplification product of CYTBWB2-wb primer has very high similarity with some wild boar species that live in Indonesia, which is 98% with the Cyt-B mt-DNA gene of *Sus barbatus* which lives in the Malay Peninsula, Sumatra, Kalimantan, and the Sulu Islands; 98% with Cyt-B mt-DNA of *Sus verrucosus* gene which is endemic to Java, Bawean, and Madura islands; 94% with Cyt-B mt-DNA gene of *Sus scrofa* which is widely found in Northern Kalimantan; and 94% with Cyt-B mt-DNA gene of *Sus celebensis* endemic to Sulawesi. In addition, the amplification products also have a fairly high resemblance to the Cyt-B gene from several species of wild boar from abroad such as *Sus scrofa riukiuanus* from Japan, *Sus scrofa cristatus* from Myanmar and Thailand, and *Sus cebifrons* from the Philippines at 93%

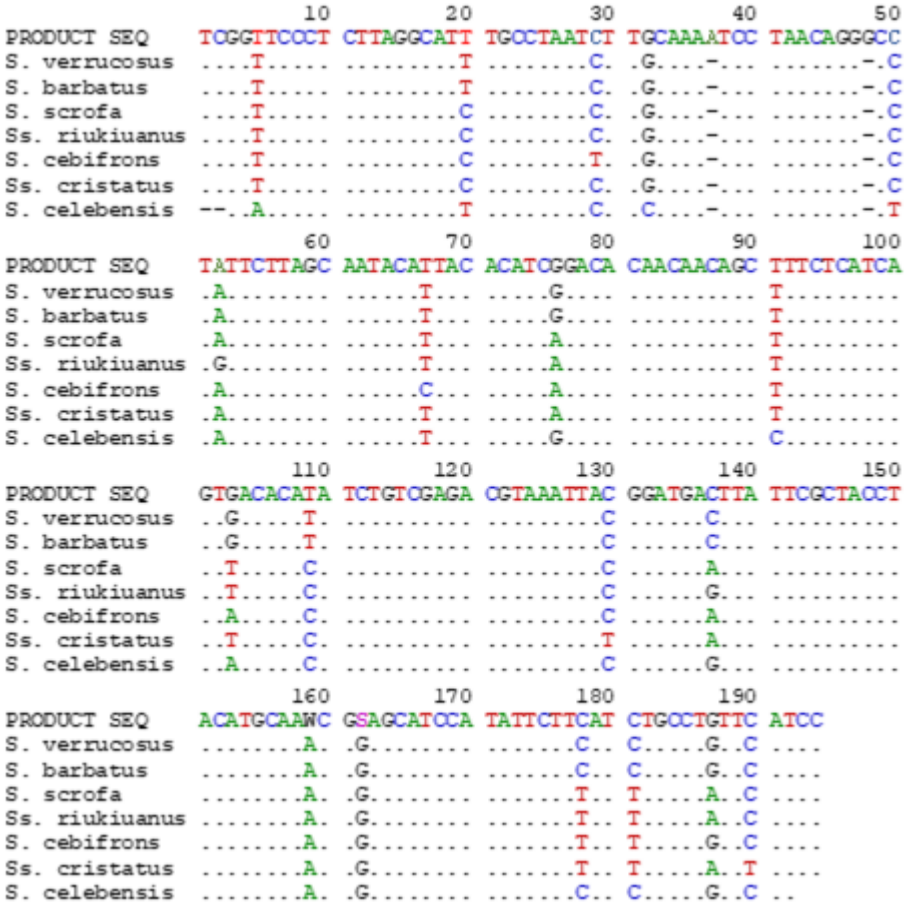

Fig 6. The alignment of Cyt-B mt-DNA fragment (194 bp) and the wild boar gene from NCBI GenBank

Table 2. Percent identities and gaps result of nucleotide BLAST analysis between the Cyt-B mt-DNA fragments (194 bp) with the wild boar gene from NCBI GenBank

Organisms	Sequence ID	Identities (%)	Gaps (%)
Sus verrucosus	GQ338963.1	98	1
Sus barbatus	AM492661.1	98	1
Sus celebensis	AY534298.1	94	1
Sus scrofa	EU531834.1	94	1
Sus scrofa riukiuanus	LC133269.1	93	1
Sus cebifrons	KF952600.1	93	1
Sus scrofa cristatus	MG725631.1	93	1

(Table 2 and Fig. 6). Similarities with the Cyt-B gene in wild boar mitochondrial DNA (93–98%) indicated that CYTBWB2-wb primer attached specifically to the target gene, Cyt-B mt-DNA. This has many advantages in the use of CYTBWB2-wb primer for the analysis of wild boar contamination. The high similarity with many types of wild boar causes this primer can be used for the analysis of wild boars in many areas both in Indonesia and abroad where each region has a different species of wild boar. This result is beneficial, considering that there are quite a lot of Muslim communities in many countries, which also requires halal authentication in food products.

■ CONCLUSION

The results of this study show that the CYTBWB2-wb primer can specifically amplify wild boar

contamination among 6 comparable species. DNA bases of PCR amplification with primers have a similarity of 93–98% to the Cyt-B mt-DNA gene from several types of wild boar indicating that the CYTBWB2-wb primer can specifically amplify the target sequence. The primer is able to detect the mixture of wild boar meat on the reference meatballs up to 0.1% w/w with efficiency value of 108.0% and R^2 value of 0.970. It can be concluded that the CYTBWB2-wb primer proved to be specific and suitable to identify the presence of wild boar contamination in meatball products for halal authentication studies.

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