Primer Pairs Specificity Test for Frog Meat Identification Using PCR Technique

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Abstract: *Halal food assurance is becoming more important with the growth of the halal industry globally. Adulteration of halal meat products using non-halal sources such as pork, dog, boar, and even frog meat has become a major problem for moslems. The purpose of this study is to initiate the method for frog meat identification using polymerase chain reaction (PCR) technique. In this study, three primer pairs (Fk1-Rk1, Fk2-Rk2, Fk3-Rk3) were analyzed for their specificity toward frog meat against other common halal meat sources such as beef, chicken, shrimp, squid, and mackerel. The visualization of DNA amplification showed that primer pair Fk1-Rk1 produced primer-dimer, thus cannot be used for this circumstance. Primer pair Fk2-Rk2 showed a better result where DNA amplicon was produced at ~100 bp for frog meat and no amplicons for other meat. Primer pair Fk3-Rk3 showed a different pattern of DNA amplification for all the meat tested, where the amplicon of frog meat was shown at ~100 bp, while the other meat showed multiple amplicons or none. In conclusion, primer pairs Fk2-Rk2 and Fk3-Rk3 showed their potential as primer pairs for frog meat identification using PCR for implementing halal food assurance, although sensitivity analysis needs to be investigated.*

Keywords: *halal food assurance; adulteration; frog meat; polymerase chain reaction*

■ INTRODUCTION

Most people, especially moslems, are now more aware of halal food assurance because moslems are not allowed to consume non-halal food [1-2]. This assurance might be based on the source, the additives, and the procedures used during the product's preparation. This is in line with the growth of the halal food industry, which contributes 1.6 billion USD worldwide [3-5]. Due to the huge economic growth, halal food and beverages industries are susceptible to adulteration by cheaper and

even non-halal sources. Many sources such as dog, pork, and boar meat are even used as the replacement or addition for beef products for the producer to gain more profit [6]. As a result, the method to identify contaminated non-halal meat in the food product is greatly developed.

One of the meat sources that belong to non-halal food is frog meat. This is based on Fatwa number 4 year 2003, issued by Indonesia Ulema Council (*Majelis Ulama Indonesia*), stating that it is forbidden for moslems to consume frog meat, making it a non-halal food source. On the other hand, Indonesia is one of the biggest frog meat exporters in the world, making it prone to be used as meat substitution for halal food products. Although such a case rarely to none happens, a method for identifying frog meat should be established for precaution. A method that was used to analyze the presence of frog meat in the food products is based on the difference in spectrum profile from frog oil compared to other oils using Fourier transform infrared spectroscopy (FTIR) [7]. Having said that, the investigation did not reveal the comparison between the frog oil spectrum against another oil spectrum.

Another approach in doing determination of meat species is based on the volatile organic compounds (VOCs). A different source of meats produces a different ratio of the VOCs (e.g., hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, etc.) that can be analyzed using gas chromatography-mass spectrometry (GC-MS) and even sensory device such as electronic nose because the different ratio of VOCs resulting different odor [8-9]. However, the preparation and analysis of oil using FTIR and VOCs using GC-MS are not quite practical. The cooking process and post-mortem treatment of the meat also could contribute to varying those ratios.

The most popular method to make identification of meat sources is based on protein and DNA analysis. Although protein-based analysis has some edges, particularly in the practical sense, such as the availability of various protein kit analyses and relatively affordable, it has a major drawback when compared to DNA-based analysis, especially for products that have been extensively processed [10]. This is because DNA is much more stable under extreme conditions and not easily denatured [11]. Therefore, DNA-based analysis using polymerase chain reaction (PCR) remains the most popular method to distinguish contamination of non-halal meat in food products.

Identification of the presence of dog meat in beef meatballs was successfully investigated using real-time PCR using the primer pair designed out of cytochrome-*b* nucleotides [12]. Another PCR method, namely two direct-triplex PCR, was used to identify various meat ranging from pork, beef, horse, chicken, turkey, dog, lamb, and buffalo meats, which later developed into direct pentaplex PCR [13-14]. However, the investigation to identify frog meat using the PCR method has yet to be carried out. Therefore, this study aimed to develop the method of frog meat identification using the PCR technique to support the halal food assurance implemented by the government. This study focuses on the selection of primer candidates that can be used for frog meat identification using PCR.

■ EXPERIMENTAL SECTION

Materials

Materials used include various meat such as frog (K), beef (S), chicken (A), shrimp (U), squid (C), mackerel (T), 70% alcohol, sterile distilled water (SDW), DNA isolation reagent DNEasy Blood & Tissue Kit (Qiagen), Dreamtaq Green PCR master Mix 2x (Thermo Fisher Scientific), and three pairs of primers based on cytochrome b (cyt-b) target gene (primer Fk1-Rk1, Fk2-Rk2, and Fk3-Rk3).

Instrumentation

The instrumentation used were analytical balance (Kern ABJ-2204NM), microcentrifuge (Tomy), NanoDrop Spectrophotometer (Thermo Fisher Scientific), electrophoresis machine (MUPID-eXU), UV transilluminator (Uvitec Firereader V10 Plus), and Mastercycler Nexus PCR Cycler (Eppendorf).

Procedure

Primer design

Primer design was carried out using BioEdit version 7.0.4.1 software. The cyt-*b* genes from eight frog species

and three other cyt-*b* genes from cow, chicken, and goat were aligned to tableure out the conserved nucleotide region amongst them. The chosen primer pair (later called Fk1 and Rk1) was based on the similarity between eight frog species and the most differences with the other three. All of the genes were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) with the accession number as follows: L08376.1 (*Gallus gallus*), D34635.1 (*Bos taurus*), D84201.1 (*Capra hircus*), KU246049.1 (*Rana kukunoris*), MF370348.1 (*Rana amurensis*), KX686108.1 (*Rana catesbeiana*), AF205091.1 (*Rana dybowskii*), AF205087.1 (*Rana nigromaculata*), AF205088.1, (*Rana plancyi*), AF205093.1 (*Rana rugosa*), and NC_042226.1 (*Rana temporaria*). The second and third primer pairs (Fk2, Rk2, Fk3, and Rk3) were microsatellite markers in the common frog (*R. temporaria*) previously used for investigations of population structure and reproductive behavior in *R. temporaria* [15]. The sequence of all primer pairs is shown in Table 1.

DNA isolation and quantification

Frog meat (*R. catesbeiana*) as well as beef, chicken, shrimp, squid, and mackerel meat, were weighed for 100 mg and transferred to a 2 mL microtube. The meat was crushed using micropistil, and the DNA was isolated using DNEasy Blood & Tissue Kit from Qiagen. The amount of isolated DNA and also its purity was quantified using NanoDrop® spectrophotometer (Thermo Fisher Scientific) by measuring the absorbance at 260 and 280 nm. The DNA purity was calculated by dividing the absorbance of 260 nm by the absorbance of 280 nm, which was classified as pure DNA if the result is between 1.8–2.0.

DNA amplification and visualization

DNA amplifications were conducted using the Eppendorf Matercycler Nexus PCR Cycler with the cocktail PCR in Table 2. The PCR reaction was performed under the conditions: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C, and annealing for 1 min (annealing temperatures for primer pair number 1, 2, and 3 were 52, 56, and 56 °C, respectively), extension at 72 °C for 1 min, this process was repeated for 40 cycles. For the last cycle, the extension was prolonged for another 10 min.

Qualitative analysis of the DNA band was carried out using the MUPID-eXU electrophoresis machine. Separation of DNA fragments was performed using 1% agarose containing 0.01% (v/v) EtBr at 50 V for 60 min. The DNA band was then visualized using UV transilluminator Uvitec Firereader V10 Plus.

Data analysis

All the selected primers were analyzed using Nucleotide BLAST or BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the similarity of the primer sequence with all the databases stored in NCBI. This analysis helps to ensure the specificity of the primers towards the targeted organism. To gather information regarding primer's characteristics such as GC content, melting temperature (T_m) value, and hairpin formation, the online software Sequence Manipulation Suite: PCR Primer Stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) was used. These primer characteristics are useful to determine the condition for the amplification process.

Table 1. The Sequence of Frog Primer Pairs

Primer	Sequence (5'→3')
Fk1	GCAGCCCTATCAACCTTCTC
Rk1	TAAGGGAGCGAAGTTTGGAG
Fk2	TCTCTCTTCTTTGTTCCCTGAGC
Rk2	CCTTGAGAGGGGCAAGTAAGGC
Fk3	AGCGCCATGCTTATGCTGAG
Rk3	TTGATATTTGCTTGCGGGGC

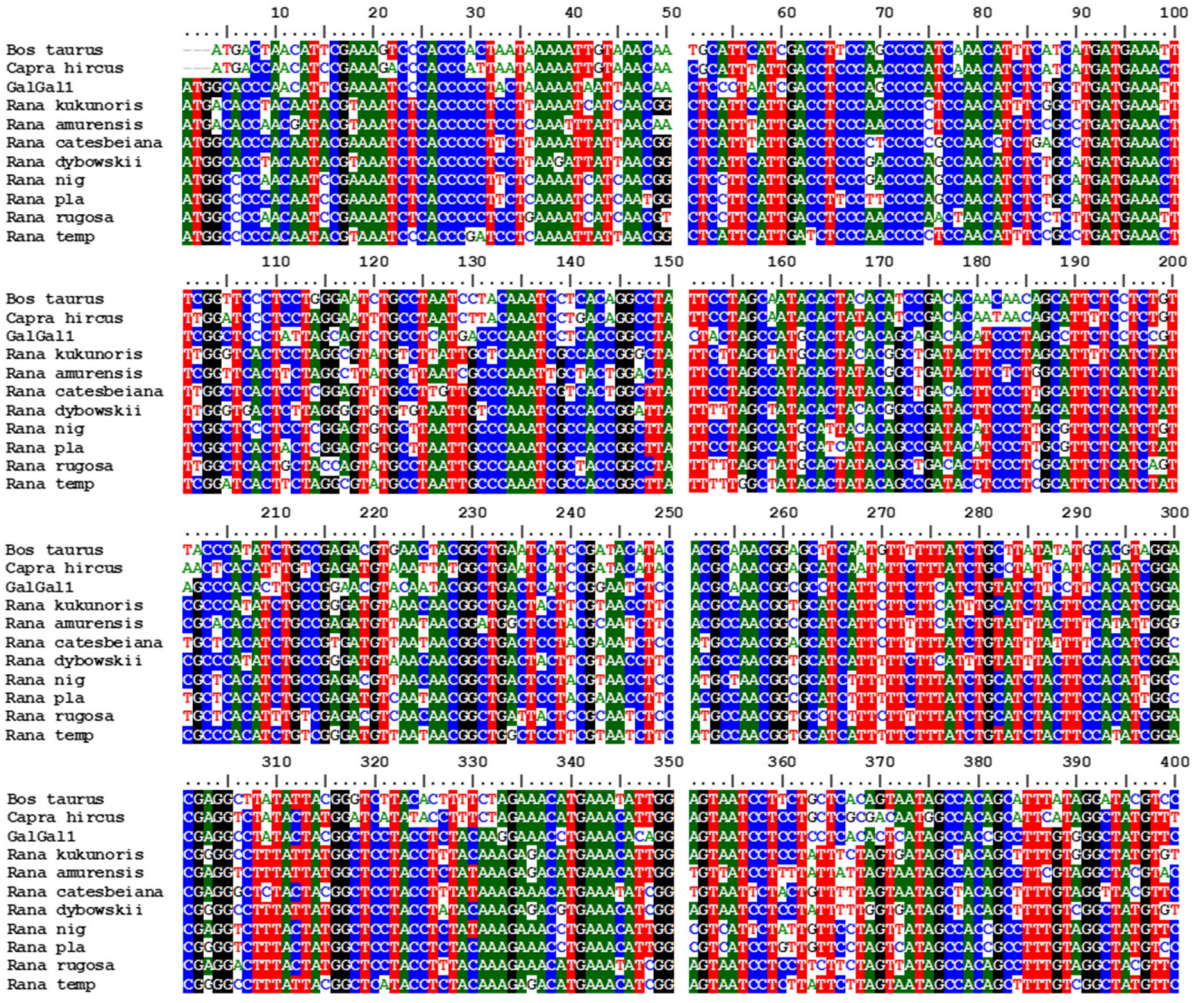
Table 2. Cocktail Mixture for PCR Reaction

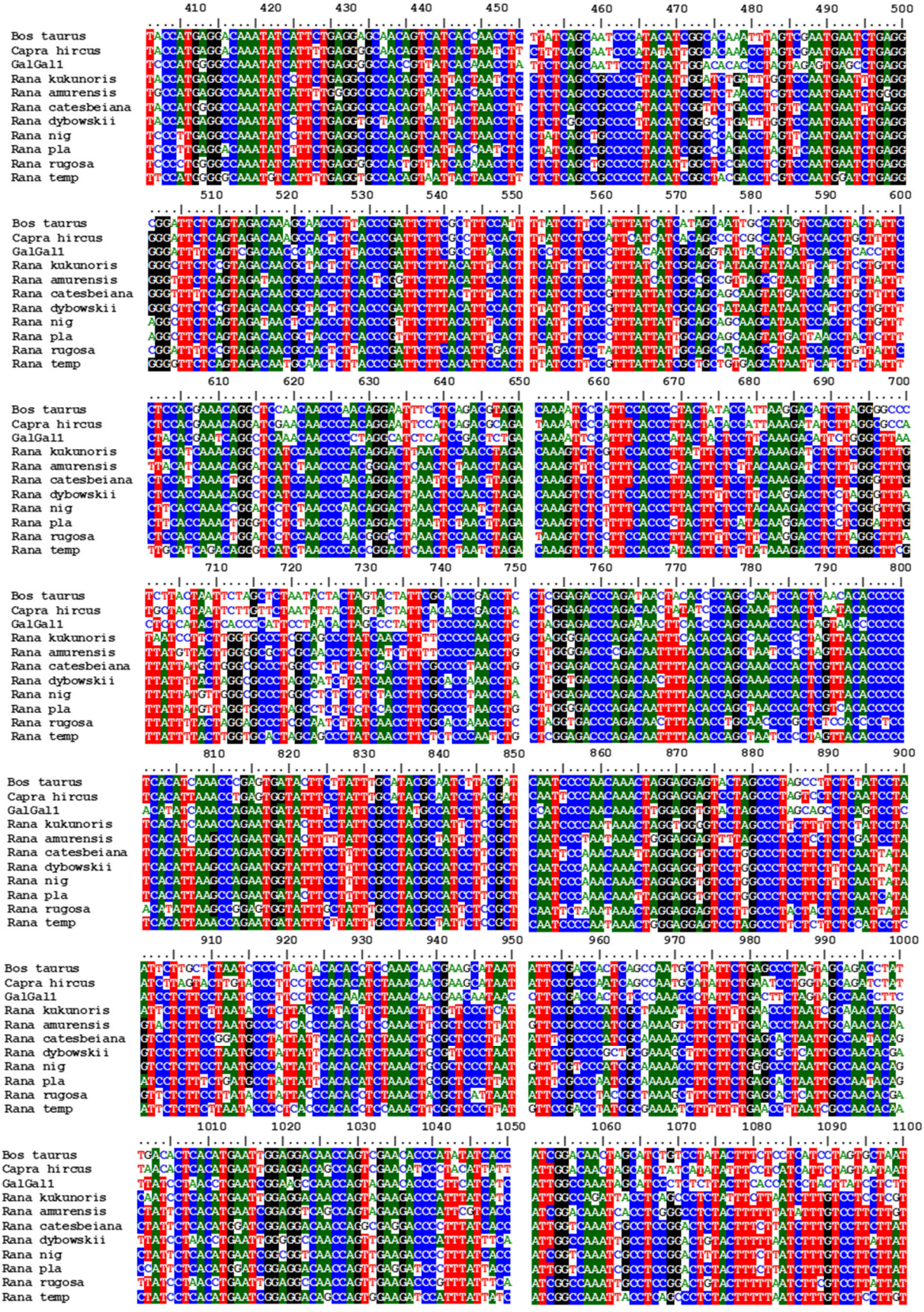
Mixture component	Volume (μL)
Dreamtaq Green Master Mix 2x	12.5
Forward primer (10 μM)	1.00
Reverse primer (10 μM)	1.00
DNA template	According to the amount needed, which is 100 ng
Nuclease Free Water	Until total volume reaches 25 μL

■ RESULTS AND DISCUSSION

The primer design was based on eight organisms' cyt-*b* genes. Cyt-*b* was chosen due to its characteristic of having species-specific mutation sites indicating its usefulness as a marker for species identification [16]. In addition to that, the cyt-*b* gene was reported to have a short DNA fragment that acts as the universal DNA that can be used as a barcode region to differentiate many animal species [17]. Mitochondrial DNA, which encodes the cyt-*b* gene, possesses distinct characteristics being the second genetic information system of eukaryotic cells [18], and has a closed circular double-stranded structure that is able to do self-replication semi-conservatively [19]. All of those characteristics make mitochondrial DNA (cyt-*b* gene) a beneficial resource for understanding the evolution as well as genetic relationship between species [20], therefore suitable for developing specific primers for the PCR method. Many studies reported that the use of mitochondrial DNA, especially cyt-*b*, has delivered accurate identification for organism identification, such as the detection of pork contamination [21], characterization of tropical fishes [20] and even phylogenetic analysis for some insects [22]. Eleven mitochondrial cyt-*b* genes obtained from NCBI were used for designing primer number 1 (later known as primer Fk1-Rk1), including 3 genes from non-*Rana* species and 8 *Rana* species. The alignment is shown in Fig. 1.

The chosen primer was based on the conserved region between 8 cyt-*b* genes of *Rana* species and also has the most difference of the other 3 cyt-*b* genes from non-*Rana* species. Therefore, the designed primer pair of Fk1 is located in region 721–741 bp, while Rk1 is located in region 930–949 bp. Primer pair numbers 2 and 3 were microsatellite primers constructed from *R. temporaria*




Bos taurus
Capra hircus
GalGal1
Rana kukunoris
Rana amurensis
Rana catesbeiana
Rana dybowskii
Rana nig
Rana pla
Rana rugosa
Rana temp

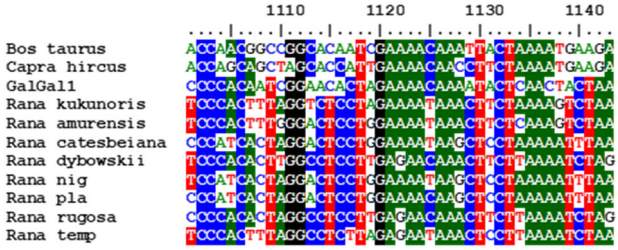


Fig 1. Alignment of cyt-*b* genes from 11 species

Table 3. Summary of primer characteristics

Primer	Sequence (5'-3')	Length (bp)	T_m (°C)	GC (%)	Primer dimer
Fk1	GCAGCCCTATCAACCTTCTC	20	59.3	55.0	No
Rk1	TAAGGGAGCGAAGTTTGGAG	20	57.3	50.0	No
Fk2	TCTCTCTTCTTTGTTCCCTGAGC	23	60.6	47.8	No
Rk2	CCTTGAGAGGGGCAAGTAAGGC	22	64.0	59.1	No
Fk3	AGCGCCATGCTTATGCTGAG	20	59.3	55.0	No
Rk3	TTGATATTTGCTTGCGGGGC	20	57.3	50.0	No

that showed characteristics of 10 polymorphic microsatellite loci and a bi-allelic marker [16]. Hence, primer pairs Fk2-Rk2 and Fk3-Rk3 are expected to give different DNA amplification patterns. Meaning instead of a single DNA band amplified by the primer, microsatellite primer can amplify many DNA fragments from many loci. This is because microsatellite belongs to subcategories of tandem repeats (TRs) distributed to make up genomic repetitive regions [23].

The characteristic of the six primers was tabulated in Table 3, and the difference in T_m value for each primer pair was rather significant (especially primer pair Fk2-Rk2). The downfall of having a significant difference between T_m is their annealing temperature (T_a) could be too low for one primer or too high for the other. This could lead to either the occurrence of an unspecific DNA band or no amplification was not carried out. Thus, determining the optimal T_a for each primer pair that has a different T_m value is crucial. On the other hand, the GC content of each primer met the requirement of a good primer for PCR which is 40–60%, because a low GC content could reduce the efficiency of the PCR while high GC content can cause hairpin formation, which can hinder the primers from annealing themselves to the template [24].

The specificity of each primer pair was determined by the DNA amplicon resulting from the amplification process. Primer pair is said to be specific if only the targeted DNA fragment was produced for the investigated species and no amplification for other species. Amplification using primer pair Fk1-Rk1 did not produce any DNA fragment for all the meat tested (data not shown). This phenomenon indicates that primer pair Fk1-Rk1 does not have any specificity toward all the meat samples that had been tested. Although primer pair Fk1-Rk1 was said not to have the possibility to form primer dimer based on the data from Table 1, some proposed self-dimer structures are actually existing (Table 4). It would suggest that the non-specificity of primer Fk1-Rk1 for not having any amplification is not merely because it is not specifically against the frog DNA template but due to the high possibility of forming a primer dimer.

Amplification using primer Fk2-Rk2 and Fk3-Rk3 is shown in Fig. 2. From the visualization of DNA amplification using both pairs of primers, DNA fragments of frog meat can be seen slightly above the 100 bp region, whereas in other samples (T2, A2, C2, U2, and S2), there was no DNA amplification using Fk2-Rk2 primer pairs and primary dimers occurred using Fk3-Rk3 primer pairs. The primer pair Fk2-Rk2 might be used as a candidate primer for frog meat identification.

In contrast, the primer pair Fk3-Rk3 showed a different pattern of amplification. The targeted sample,

Table 4. Proposed primer dimer structure

Primer	Proposed structure of primer dimer
Fk1	5' GCAGCCCTATCAACCTTCTC || || 3' CTCTTCCAACTATCCCGACG
	5' GCAGCCCTATCAACCTTCTC || 3' CTCTTCCAACTATCCCGACG
	5' GCAGCCCTATCAACCTTCTC || 3' CTCTTCCAACTATCCCGACG
Rk1	5' TAAGGGAGCGAAGTTTGGAG | ||::|| | 3' GAGGTTTGAAGCGAGGGAAT

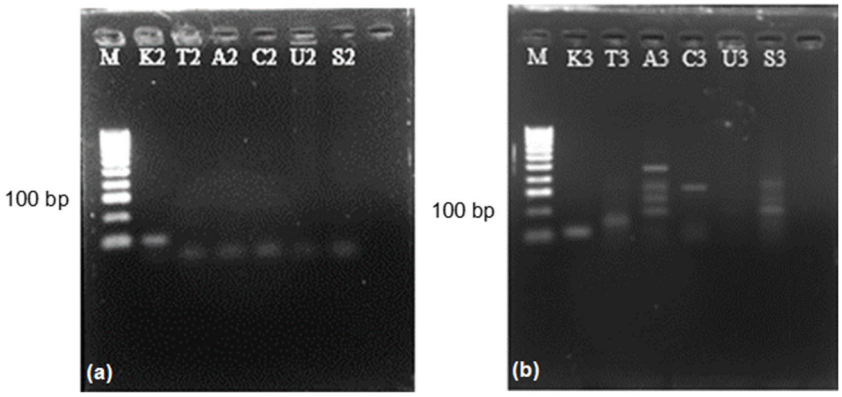


Fig 2. Visualization of DNA amplification using (a) primer Fk2-Rk2 and (b) primer Fk3-Rk3 (M: DNA marker K: frog, T: mackerel, A: chicken, C: squid, U: shrimp, and S: beef)

which is frog meat (K3), also had DNA fragments slightly above the 100 bp region. The amplification from mackerel meat (T3) resulted in DNA fragments at ~150 bp, and amplification from chicken, squid, and beef meat (A3, C3, and S3, respectively) resulted in multiple bands with different patterns from each other. This occurrence was due to the microsatellite primer characteristic that is able to amplify many DNA fragments from the different locus. The last sample, which was shrimp meat (U3), showed no amplification happening. Based on this visualization, primer Fk3-Rk3 could also be a candidate primer for frog meat identification to further improve the development of halal food assurance; that is, the unique pattern of multiple bands could be used to identify species of interest but would probably suffer from the level of accuracy and reproducibility [25-26]. However, due to the ability to amplify many other meat samples other than frog meat, primer Fk3-Rk3 could not be used for analysis using RT-PCR, as it would generate misleading results [27].

In this study, the identification of various types of meats was successfully carried out using the PCR technique. Since the objective is to develop a method for checking the presence of frog meat as a non-halal food source in food products, therefore only a specificity test is needed, and the primer pair Fk2-Rk2 and Fk3-Rk3 have already shown their potential to do so. Nevertheless, the information derived from this study might complement the technology implemented in the

food supply chain, especially in the halal food industry [28].

■ CONCLUSION

The best primer pair for frog meat identification using the PCR technique in this study was primer pair Fk2-Rk2 and Fk3-Rk3. The primer pairs had distinguished amplification patterns against other common meat for food, while primer pair Fk1-Rk1 did not exhibit the ability to amplify the frog meat DNA. However, it is highly recommended to conduct a further investigation regarding the determination of a limit of detection (sensitivity analysis) and implementation towards food products as well as developing more specific primers, such as multiplex primers for frog meat.

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■ AUTHOR CONTRIBUTIONS

Norman Yoshi Haryono conceived the original idea. Rizqi Layli Khusufi and Delia Wahyu Pangesti carried out the experiment. Norman Yoshi Haryono with the support from Rina Rifqie Mariana and Hartati Eko Wardani wrote the manuscript. Evi Susanti and Norazlinaliza Salim contributed to the final version of the manuscript.

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