Specific Primer Design of COI Gene and Its Potential Application for Species Identification of Meats

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Abstract. Cytochrome oxidase I gene, COI, is known as a mitochondrial gene encoding cytochrome oxidase I protein. The aim of this study was to design specific primers for bovine, chicken, and porcine and its application for species identification containing in raw meats using PCR. A set of specific primers has been designed using primer3. A universal forward primer and three specific reverse primers have been synthesized and the PCR technique has been carried out to test their specificity using a DNA template extracted from three meat species, i.e. bovine, chicken, and porcine. The PCR condition was optimized with similar annealing temperatures at 64°C. The PCR product was then visualized using a 2% agarose gel under the UV light and sequenced to know its nucleotides. The results showed that primer pairs were perfectly working to amplify specific target sites for bovine, chicken, and porcine. They were indicated by 263 bp for bovine, 596 bp for chicken, and 168 bp for porcine. Besides, sequence analyses showed that they were a hundred percent similar to reference species. This result suggested that the primer set of COI genes designed in this study may be a powerful tool to identify species in meats using multiplex-PCR.

1. Introduction
Meat is one of the important animal products to fulfill human protein needs. The high demand for meat is not balanced by meat production, which can increase the price of meat, especially beef. It is an opportunity for individuals who are not responsible for counterfeiting meat [1]. Counterfeiting of meat can occur in processed meat products and fresh meat [2].

The identification of counterfeiting in meat can be conducted through Deoxy Nucleic Acid (DNA) using polymerase chain reaction (PCR). PCR is used to amplify specific DNA sequences, and this specific area is bounded by two oligonucleotide primers, which are assisted by the polymerase enzyme [3]. The primer is a short oligonucleotide, which is the opposite of the complement of the DNA template area [4]. A good primer will influence the success of PCR, primers for amplification using the PCR method can be obtained through the primer design. The things that must be considered in primer design are primer length, melting and annealing temperature, percentage of GC and primer secondary structure [5].

Animal genetic information is stored in nucleus DNA and organelle DNA, namely mitochondria and chloroplasts [6]. Mitochondrial DNA (mtDNA) is widely used in species detection compared to nucleus DNA which has a smaller amount of DNA [7]. Mitochondrial DNA has a small and simple size and also has a different arrangement with the nucleus DNA [8]. Mitochondrial DNA has a high diversity,
maternally inherited, and mostly used as an approach of a developmental model. Therefore, it can be an essential means of understanding the origin of a nation [9]. One of the mitochondrial DNA that can be used for species identification is Cytochrome Oxidase I (COI). Primers derived from the COI gene were chosen because the COI gene functions for DNA barcoding [10].

The COI gene can be used for phylogenetic correction at the species level. The COI gene has a slight substitution so that the COI gene is more stable and can be used as a marker of phylogeny analysis. Short fragments of COI can be used as markers to identify various kinds of animals to species level accurately [11]. COI has the characteristic of being a genetic marker to determine species in almost all higher animals because of the slight deletion and insertion of sequences, as well as many parts that conserve [12]. The purpose of this study is to design a COI primer and test it to identify beef, pork, and chicken in raw meat.

2. Materials and methods

2.1. Primer Design

Primer design was started by tracing COI gene sequences from bovine, chicken, and porcine through the genebank database on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Alignment analysis can be used to compare two or more sequences to get primers that are unique and different from other primers. The primers obtained are not attached to other organisms. The program used for alignment analysis is the Basic Local Alignment Search Tools (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) then chose the BLAST nucleotide (nblast). After the nblast appeared, a comparison was made between two or more sequences that have been selected to compare the two nucleotide sequences. The results from BLAST would display a scale that shows the level of similarity of compared sequences. The BLAST results were also analyzed to show conserved regions of COI gene sequences.

Primer candidates were obtained using a program accessed through the PRIMER3 version 0.4.0 website (http://bioinfo.ut.ee/primer3-0.4.0/). Then, COI gene sequence of each species in FASTA format was entered into the column without spaces and then clicked the pick primer. The results of the pick primer will display several primer candidates composed of 20 nucleotide bases with their respective properties. Primer candidates compiled were then analyzed in each sequence based on the order of the codons [5]. Primer candidates obtained with the provisions of the GC percentage of each primer must be above 50%, the difference in melting temperature between the two primers was set no more than 4°C. The length of each primer ranges from 15 bases to 25 bases.

2.2. Polymerase chain reaction (PCR)

The polymerase chain reaction is carried out with a total volume of 25 µl contained 12.5 µl 2X KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems, Inc., USA), each primer as much as 1 µl, DNA of bovine, chicken, and porcine each 1 µl and 7.5 µl ddH2O. PCR reaction begins with the initial denaturation at 95°C for 3 minutes, followed by 30 cycles with changes in denaturation at 95°C for 15 seconds, annealing with a temperature of 64°C for 30 seconds and an extension of 72°C for 30 seconds, then the cycle ends with a final extension at 72°C for 3 minutes. The results of the PCR reaction were electrophoresed with agarose gel 2% stained with Ethidium Bromide (EtBr) and visualized using gel documentation.

2.3. Analysis of COI gene sequence

PCR product sequencing from the COI gene region amplification was carried out at PT. Genetika Science Indonesia. The results of the sequencing were processed using the Bioedit 7.2.6.1 program, and an alignment analysis was carried out using the Clustal Omega program (http://ebi.ac.uk/Tools/msa/clustalo/) and BLAST on the NCBI program. Data analysis was performed by comparing the results of PCR products using the specific primer design of the COI gene with the
sequencing results of the primer design. The results were used to determine the similarity of the nucleotide sequence between species used in this study and published sequence in Genbank.

3. Results and Discussion

3.1. Primer Design
The tracing for the COI gene sequence for bovine, chicken and porcine species was carried out at the NCBI GeneBank. The COI gene sequence is then inputted into the PRIMER3 program to obtain several candidates primer [5]. Selected forward (F) and reverse (R) primer are shown in Table 1.

Table 1. Primer candidates selected from COI gene

| Species | Primer | Nucleotide Length | Product Length | GC (%) | Tm (°C) |
|---------|--------|-------------------|----------------|--------|---------|
| Chicken | F      | GATTCTTCGGACACCCCGAAG | 21 | 596 bp | 57, 14 | 60,74 |
|         | R      | AGCTAGGCCCCAGGAATGT | 20 | 50 | 59 |
| Bovine  | F      | GATTCTTCGGACACCCCGAAG | 21 | 263 bp | 57,14 | 60,74 |
|         | R      | CCCGGTTGGAATAGCAATAA | 20 | 45 | 55, 49 |
| Porcine | F      | GATTCTTCGGACACCCCGAAG | 21 | 168 bp | 57,14 | 60,74 |
|         | R      | TGTGGTGAGCCCATACGATA | 20 | 50 | 58,22 |

The selected forward primer can amplify each COI gene region from chicken, bovine, and porcine species. Primers of each species (Table 1) were selected based on an analysis of excellent primer characteristics for amplification using PCR, namely the primary length between 17-28 nucleotides, the primary GC composition (%) between 40 to 60% and the length of the amplicon obtained through the PRIMER3 program [5,13,14]. Too long nucleotide primers may cause annealing temperatures to be less efficient. On the other hand, too short nucleotide primer may cause mispriming (priming the primers in other places that are not desired) [13,15].

Based on the results of the study, the nucleotide length of the selected forward and reverse primer pairs ranges between 20-21 bp. The selected primer pairs have GC content for bovine, chicken, and porcine species, which are 45%, 50%, and 50%, respectively. The right percentage of GC elements (%) in the primer design can produce more specific and stable bonds [13,16]. The GC percentage of primers designed in this study did not exceed than 5 GC for both forward and reverse primers. It is needed in order to maintain bond stability between the primers designed for each species with the DNA template to perform the PCR method (Table 2). The results of the primer that have been designed do not have a sequence of GC base repeats more than four times (for example, AGCTGGGGGATCGGG) to prevent mispriming [14].

The results of the primer design using the PRIMER3 program produces melting temperature (Tm) for chicken, bovine, and porcine species at 59°C, 55.49°C, and 58.22°C, respectively. In addition to using the PRIMER3 program, some methods can be used to estimate Tm calculations. According to Nakano et al. [17], the estimated Tm value can be obtained by the equation $Tm = 100.5+(41*(yG+zC)/(wA+xT+yG+zC)-(820/(wA+xT+yG+zC))+16.6*\log10([0.050])$ where $w$, $x$, $y$ and $z$ are the sum of the nucleotide bases A, T, G and C. The equation is accurate for a base with a length of 18-25 bp [18]. The Tm in the primer should be the same or have a difference that is not too significant. Melting temperature (Tm) is used as a reference for determining or optimizing annealing temperature (Ta) [19]. High Ta produces in insufficient DNA primer-mold hybridization resulting in low PCR products. On the other hand, low Ta lead to nonspecific DNA amplification caused by primer attachment errors in the DNA template [16].

3.2. PCR assay validation
Primer specificity testing was done by the PCR method on the DNA of chicken, bovine, and porcine isolated from raw meat with three primer pairs. The PCR method uses a PCR machine (Gene Amp PCR
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System 9700, Singapore) with PCR stages, initial denaturation at 95°C for 3 minutes, followed by 30 cycles with changes in denaturation at 95°C for 15 seconds, annealing with a temperature of 64°C for 30 seconds and an extension of 72°C for 30 seconds, then the cycle ends with a final extension at 72°C for 3 minutes. In vitro species detection test results using PCR are shown in Figure 1.

![PCR Products](image_url)

**Figure 1.** PCR products. M is a marker benchtop ladder 100 bp; A is chicken; S is bovine; B is a porcine.

Optimization of primer annealing temperature (Ta) has been carried out at 58, 60, 62, and 64°C to get clear DNA band amplification. The use of annealing temperature of 64°C showed the formation of clear and bright DNA bands. According to Rahman *et al.* [20], the higher annealing temperature will produce a more specific primer attachment but reduce the resulting product size. A specific primer pair will only amplify one size fragment from the target area [21]. The results showed that primer pairs were perfectly working to amplify specific target sites for bovine, chicken, and porcine. They were indicated by 263 bp for bovine, 596 bp for chicken, and 168 bp for porcine, although the thickness of the DNA band in each sample was different.

| Specific Primer | Bovine | Chicken | Porcine | Sheep | Goat | Rat | Dog |
|-----------------|--------|---------|---------|-------|------|-----|-----|
| Forward (21 bp) | 100    | 95      | 100     | 90    | -    | 90  | 95  |
| Bovine (20 bp)  | 100    | -       | -       | -     | -    | -   | -   |
| Chicken (20 bp) | -      | 100     | -       | -     | -    | -   | -   |
| Porcine (20 bp) | -      | -       | 100     | -     | -    | -   | 94  |

**Table 2.** The level of primer similarity is specific to seven species

3.3. Sequencing Validation

The sequencing results were advanced with the Bioedit 7.2.6.1 program, and then an alignment analysis was performed using the Clustal Omega program. Primers designed in this study were specifically limit the fragment to be amplified in the expected area (Figure 2) [22]. COI regions from a bovine, chicken and porcine were cut at 263 bp, 596 bp, and 168 bp, respectively. Forward primers are marked with a red square, while for reverse primers are marked with green, blue, and pink boxes for each species. Meanwhile, asterisks showed the same nucleotide bases in the fragments which are compared. The homology test in this study was conducted to determine the level of primer specificity through the similarity of nucleotide sequences in each species. This homology test was carried out using the BLAST program on the NCBI website. The results of the specific primer homology test are shown in Table 2.
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Figure 2. Alignment of COI gene primer sequences from three different species (Bovine, Porcine, and Chicken). SequenceS is a sequence for bovine; SequenceB is a sequence for Porcine; SequenceG is a sequence for chicken. Red, green, blue, and pink boxes are primer regions for each species.

The results showed that the COI gene forward primer had high similarity in chicken and porcine species, which was between 95-100% (Table 3). The level of similarity of specific primers is also done by comparing forward primer and reverse primer in 4 other species, namely sheep, goat, rat, and dog. The forward primer of COI genes has a high similarity in sheep, rat, and dog species, which is between 90-95% and not at all in goat species. According to Matsunaga et al. [21], reverse primer is said to be specific, if they have a high value of similarity in one species and low in other species. Reverse primer of bovine was 100% similar with published bovine COI gene sequences. On the other hand, bovine sequences have lower similarity with other species. This result was also applicable for reverse primers of chicken and porcine species. The low level of similarity in bovine reverse primer to other species is due to a mismatch of base sequences at the 3’ end of the reverse primer and differences in the mismatch between reverse primer in each DNA sequence sample (9-45%) yielding different Tm [21,23].
4. Conclusions
The new specific primer designed from the mtDNA COI gene successfully identified bovine, chicken, and porcine. Besides, specific primers were indicated by high levels of homology of the reverse primers for each species. Further study may be needed to test primer sets designed in this study for the identification of species in meat and its products using multiplex-PCR.

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