Specific primer design of mitochondrial 12S rRNA for species identification in raw meats

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Abstract. Polymerase chain reaction (PCR) is a molecular technique that widely used in agriculture area including species identification in animal-based products for halalness and food safety reasons. Amplification of DNA using PCR needs a primer pair (forward and reverse primers) to isolate specific DNA fragment in the genome. This objective of this study was to design specific primer from mitochondrial 12S rRNA region for species identification in raw beef, pork and chicken meat. Three published sequences, HQ184045, JN601075, and KT626857, were downloaded from National Center for Biotechnology Information (NCBI) website. Furthermore, those reference sequences were used to design specific primer for bovine, pig, and chicken species using primer3 v.0.4.0. A total of 15 primer pairs were picked up from primer3 software. Of these, an universal forward primer and three reverse primers which are specific for bovine, pig, and chicken species were selected to be optimized using multiplex-PCR technique. The selected primers were namely UNI_F (5'-ACC GCG GTC ATA CGA TTA AC-3'), SP_R (5'-AGT GCG TCG GCT ATT GTA GG-3'), BB_R (5'-GAA TTG GCA AGG GTT GGT AA-3'), and A_R (5'-CGG TAT GTA CGT GCC TCA GA-3'). In addition, the PCR products were visualized using 2% agarose gels under the UV light and sequenced to be aligned with reference sequences using Clustal Omega. The result showed that those primers were specifically amplified mitochondrial 12S rRNA regions from bovine, pig, and chicken using PCR. It was indicated by the existence of 155, 357, and 611 bp of DNA bands for bovine, pig, and chicken species, respectively. Moreover, sequence analysis revealed that our sequences were identically similar with reference sequences. It can be concluded that mitochondrial 12S rRNA may be used as a genetic marker for species identification in meat products.

Keywords: Design specific primer, mitochondrial RNA, species identification, raw meats

1. Introduction

The high price of beef increases the rise of adulteration of beef with other meats. The main factor is because the quarrel of price between beef and pork or chicken relatively high. Adulteration of meat products is intended to produce the final product with relatively cheaper price [1]. However, adulteration of meat products interfere the consumers, thus detection of meat contamination to know the existence of fake meat is highly necessary. An effort to detect contamination in meat can be done by using multiplex polymerase chain reaction (multiplex-PCR). Previous study proved that the
multiplex-PCR method can point out the specific difference between animal species, because each animal has unique area on each of its DNA [2].

According to [3], PCR is used to amplify the specific sequence of DNA. PCR reaction components consist of template DNA, a pair of primers, deoxynucleotide triphosphates (dNTPs), buffers, and Taq DNA polymerase, which then proceed to a series of amplification cycles with a mixture of three different temperatures. Multiplex-PCR can amplify a small target DNA into millions of copies of DNA with more than one primers in a single reaction. PCR uses a pair of primers, which complement each of the two target DNA regions to be amplified. Primer candidate which is designed by the program based on the conserved area where the best attachment is on this area [4].

Primer is a short oligonucleotide molecule that serves as a limiting target DNA fragment which will be amplified [5]. A good primer influences the success of PCR. Primer for DNA amplification by PCR is obtained through primer design. This primer design is an important aspect for obtaining specific, effective and efficient PCR products [6]. Primer design can be complicated when a pair of primers is needed to amplify specific areas within different species [3]. Specific primers will attach on specific regions of DNA templates such as in the area of mitochondrial DNA (mt-DNA).

Mitochondrial DNA is derived maternally, so that there is limited variation within the same species while inter-species variation is very large [7]. Mitochondrial DNA has two rRNA encoding genes which are 12S and 16S rRNAs that have high sequence variations, uniqueness and high variation at the species level so that 12S rRNA areas can be used for specific detection of certain species using multiplex PCR technology [8]. Commonly, cytochrome b gene is used to identify species in raw, cooked and processed-meat products. However, mitochondrial 12S rRNA is widely used for forensic analyses in wide-range of species [9]. Previous studies reported that multiplex PCR of 12S rRNA, tRNA Val and 16S rRNA has been successfully identified ruminants, poultry, fish and porks [10] in feedstuffs. The use of mt DNA 12S rRNA as genetic marker in one tube PCR reaction for species identification in meat-based foods has not been reported. Hence, the objective of this study was to obtain a specific primer which used to amplify mt DNA 12S rRNA region of pig, chicken and bovine in one multiplex PCR reaction.

2. Materials and Methods

2.1. Primer design

The 12S rRNA sequences of cow, pig and chicken were obtained from the genebank database on NCBI website (National Center for Biotechnology Information). 12S rRNA sequence search was done by accessing NCBI website (http://www.ncbi.nlm.nih.gov/) which then chose nucleotide in search reference and typed keyword of section and species needed as an example (Bos Taurus complete mtDNA genome). Related sequences will appear on the screen and then selected the required sequence. Complete genome of the mitochondrial DNA will appear on the screen then selected the part to be taken which was 12S rRNA region and then clicked FASTA and copied.

Alignment analysis can be used to compare two or more sequences to know that the region used is truly unique and the primers obtained are not attached to other organisms. The program used for alignment analysis was Basic Local Alignment Search Tools (BLAST) program. Alignment with BLAST analysis done by accessing NCBI BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) then chose the link nucleotide BLAST (nblast). After the nblast page appeared, then Allign two or more sequences columns were selected to compare 2 nucleotide sequences. Then entered the sequences which expected to compare in the form of FASTA on available column and BLAST was chosen at the bottom of the page. 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 12S rRNA gene sequences.

Primer candidates obtained by using PRIMER3 program which could be accessed on PRIMER3 website with 0.4.0 version (http://bioinfo.ut.ee/primer3-0.4.0/) and entered the 12S rRNA sequences of each species which will be used in the form of FASTA into provided columns without spaces and then
clicked pick primer. The results of pick primer will feature a number of primer candidates and each primer was composed by 20 nucleotide bases together with their each properties. The primer candidates that have been compiled were then analyzed on its each sequence based on the order of codons. The primer candidates obtained from the data processing of 12S rRNA region consist of forward and reverse primers.

2.2. The DNA extraction

The DNA of cattle, pigs and chickens were extracted according to the gsyncTM DNA extraction kit procedure for animal tissues. A total of 25 mg samples of each species of cattle, pigs and chickens were inserted into 1.5 ml microtube and added 200 μl of GST buffer then homogenized. A 20 μl Proteinase K was added to the microtube and then homogenized. Then the mixture was incubated at 60 °C overnight until the mixture becomes clear.

After overnight incubation, the supernatant was transferred to a new microtube and added 200 μl of GSB buffer, homogenized with vortex for 10 seconds. A total of 200 μl of absolute ethanol was added and homogenized. Furthermore, the mixture was moved to GS column which had been put into 2 ml collection tube. The removed mixture was centrifuged at a rate of 14,000-16,000 rpm for 1 minute. Then, 2 ml collection tube was replaced with a new one.

A total of 400 μl W1 buffer was added to the GS column and purged for 30 seconds. Then GS column was put into new collection tube and added 600 μl wash buffer, centrifugation was occurred in 3 minutes. In addition, the GS column was then transferred to 1.5 ml new microtube and added elution buffer which had previously been incubated and let stand for 3 minutes and it was centrifuged for 30 seconds for DNA to elute. The extracted DNA obtained was electrophoresis on 1% agarose gel and then it was visualized using gel document (Vilber Lourmat Infinity 1100126M, France).

2.3. Polymerase chain reaction and DNA sequencing

PCR was performed on a PCR machine with a total volume of 25 μl consisted by 12.5 μl 2X KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems. Inc., USA), 0.5 μl (10 μM) for each primer, 1 μl template DNA of cow, pork and chicken, respectively and 8.5 μl aquabidest. The PCR reaction was performed by denaturation initiation using 95°C for 3 minutes and followed by 30 cycles of denaturation using 95°C for 15 seconds, annealing temperature at 60°C for 30 seconds, extension at 72°C for 30 seconds and final extension 72°C for 3 minutes. The result of the reaction was then electrophoresis on 2% agarose gel and visualized by using gel document.

2.4. Data analyses

Data obtained from the gel document visualization and sequence analysis using Chromas 2.6.2 and alignment (clustal omega) were then analyzed by using qualitative descriptive analysis. Qualitative descriptive analysis is an analysis by combining all the data obtained from the research results then compared between obtained data, sorted and then descriptively analyzed [11].

3. Results and discussion

3.1. Primer design

The primary candidate was obtained by using PRIMER3 program by accessing PRIMER3 website at 0.4.0 version (http://bioinfo.ut.ee/primer3-0.4.0/). The mt-DNA of 12S rRNA fragment was present in the genetic data bank NCBI with accession number HQ184045 (cow), JN601075 (pig) and KT626857 (chicken). The mt-DNA fragment inputted in the PRIMER3 program will provide several pairs of primer candidates. Forward (F) and reverse (R) primer of 12S rRNA gene from species of cattle, pigs and chickens can be seen in Table 1.

Each primer was analyzed to obtain a primer with the best criteria which was analyzed based on the primer characteristics including position, length, composition (GC content) and amplicon length of primer obtained from PRIMER3 [12]. The primer candidate has a length of 20 bp. This length was expected to be enough to bind templates at annealing temperatures and obtain a specific sequence [13].
The best pair of primers of 12S rRNA gene from cow, pig and chicken of primer design results after confirmed with the NCBI primer BLAST is presented in Table 1. The NCBI primer BLAST analysis was used to determine the area used was completely unique and the primers obtained did not stick to any other organisms [3].

**Table 1.** Selected primers from Mitochondrial DNA 12S rRNA region for bovine, pig and chicken

| Species | Forward Primer (5' to 3') | Reverse Primer (5' to 3') | Product size (bp) |
|---------|--------------------------|---------------------------|------------------|
| Bovine  | ACCGCCGGTACATCGATTAAC    | AGTCCGTCGGCTATTGTAGG      | 155              |
| Pig     | GAATTGGCAAGGGTTGGTAAG    |                           | 357              |
| Chicken | CGGTATGTACGTGCCTCAGA     |                           | 611              |

3.2. **Optimation of PCR**

The use of 12S rRNA gene has been widely used in phylogenetic studies [8], [14], [15]. 12S rRNA was one of the genes in mitochondrial DNA. 12S rRNAs which can be used as biomarkers to identify species in meat because they have a higher sequence variation between different organisms than the order within a species [8]. Mitochondrial DNA of 12S rRNA gene from species of cattle, pigs and chickens was amplified by PCR method through three stages of temperature which were denaturation, annealing and extension [16].

Primer specificity of the target species was checked by PCR using a pair of primer which were universal forward primer pair for all three species and each species-specific reverse primer. Based on a 2% agarose gel electrophoresis visualization of PCR results showed that the PCR reaction produced a single band according to the target size of one meat species and no fragments produced by non-specific amplification. The result of PCR reaction visualized with gel document using 2% of agarose gel is presented in Figure 1.

Primer designed of 12S rRNA amplifies the target DNA of the 12S rRNA gene in cattle, pigs and chickens species. Amplification of the 12S rRNA gene resulted in 155 (cattle), 357 (pigs) and 611 bp (pigs), respectively. This study has successfully detected species in meat using a DNA target of 12S rRNA gene.

![PCR products](image)

**Figure 1.** The PCR products. M is DNA ladder; 1, 2, 3 are simplex-PCR for bovine, pig and chicken; 4, 5, 6 are multiplex-PCR containing one DNA template (bovine, pig, and chicken, respectively); 7, 8, 9 are simplex-PCR containing three DNA templates (mixture of bovine, pig, and chicken DNA genomes); 10 is multiplex-PCR containing DNA mixture of bovine, pig, and chicken.

The results showed that the optimum annealing temperature in this study was 60°C. One of important parameter for successful PCR was PCR program optimization. One of them was annealing temperature optimization that aims to avoid miss-priming. Annealing temperatures which are too high
can produce a low PCR product and annealing temperatures which are too low tent to stick elsewhere and produce non-specific products [13]. Annealing temperature can be determined by calculating Tm and 5°C annealing temperature under the actual primary Tm. However, sometimes annealing temperatures with Tm calculations did not show optimal results, resulting in PCR annealing temperature gradients [17].

### 3.3. Sequence analysis

Based on the results of 12S rRNA primer sequence analysis from cows, pigs and chickens that have been processed into alignment, it was found that the primers were attached in accordance region with the targeted primer which designed on 12S rRNA. Sequencing is the method used to determine the sequence of nucleotide bases (adenine (A), guanine (G), cytosine (C) and thymine (T)). DNA sequencing can be used to determine the identity and function of genes or other DNA fragments by comparing target sequences with other known DNA sequences [18]. The PCR DNA sequencing results were exposed using Chromas 2.6.2 program copied in FASTA as shown in Figure 2.

![Alignment of mt-DNA 12S rRNA sequences from three different species (Bovine, Pig, and Chicken). AYAM is sequence for chicken; BABI is sequence for Pig; SAPI is sequence for bovine. Red and blue boxes are primer regions for each species.](image)

The sequence analysis results were processed in the form of alignment (Figure 2.) which shows the primer as the boundary of the amplified target DNA area. Primary forwards marked with red and primary reverse boxes are marked with a complementary blue box against the target DNA sequence.
The forward and reverse primers were complementary DNA sequences against the amplified sequences and attached to both ends of the target DNA in opposite directions [13].

The successful of DNA amplification depends on the primer accuracy used [19]. The primers used in the PCR process should be able to limit the area to be amplified and to limit the target DNA fragments to be amplified [5]. The primer that resides before the target area is called the forward primer and which resides after the target area is called the reverse primer.

Sequence alignment analysis results show that primer of cattle, pigs and chicken species were well known. The length of the product produced corresponds to the length of the product that has been designed. The length of PCR product by using the primary design result of mt-DNA 12S rRNA was 155 bp for beef, 357 bp for pork and 611 bp for chicken meat, respectively. The primer used as limiter of the amplified region called UNL_F was the universal forward primer that used for all three species and each species-specific primary reverse. PCR reactions produced specific PCR products because they use specific primers of species of cattle, pigs and chickens. Specific primers are primers that do not amplify other target DNA [20].

4. Conclusions
The new primers designed from mt DNA 12S rRNA of bovine, pig and chicken have been successfully obtained and applied to amplify DNA target in vitro by using multiplex PCR technique.

Acknowledgments
This research was supported and funded by the Research Scheme of the “Pusat Keunggulan–Universitas Sebelas Maret (PK-UNS)” and Mandatory research group scheme (MRG-UNS) with contract number: 623/UN27.21/PP/2017.

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