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Development of mitochondrial 12S rRNA gene for identification of dog and rat in beef using multiplex PCR

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ABSTRAK

Gen 12S rRNA merupakan salah satu daerah unik yang terdapat pada genom mitokondria dan umumnya digunakan untuk studi filogenetik dan identifikasi spesies. Penelitian ini bertujuan untuk mengembangkan primer spesifik dari DNA mitokondria 12S rRNA untuk mengidentifikasi keberadaan spesies anjing dan tikus pada daging sapi menggunakan multiplex PCR. Tiga pasang primer dari DNA mitokondria gen 12S rRNA telah dirancang dan dipilih untuk mengevaluasi spesifisitasnya. Sebanyak dua belas sampel DNA dari jaringan daging telah disiapkan untuk menguji pasangan primer tersebut menggunakan simplex dan multiplex PCR. Produk PCR selanjutnya divisualisasi menggunakan gel agarose 2% di bawah sinar UV dan tiga sampel masing-masing untuk sapi, anjing dan tikus dianalisis untuk mengetahui urutan nukleotidanya. Data urutan nukleotida tersebut dianalisis dengan software Clustal Omega dan BLAST. Analisis simplex PCR menunjukkan bahwa semua pasangan primer berhasil mengamplifikasi target DNA yang diindikasikan dengan pita DNA sebesar 155 bp (sapi), 244 bp (anjing), dan 491 bp (tikus) dan ukuran tersebut sesuai dengan hasil analisis sekuen masing-masing spesies. Analisis simplex dan multiplex PCR mengindikasikan bahwa primer yang digunakan dalam penelitian ini secara spesifik mengamplifikasi target DNA masing-masing pada sampel yang mengandung berbagai spesies. Hasil penelitian ini membuktikan bahwa primer yang dirancang dalam penelitian ini dapat digunakan untuk mengidentifikasi adanya campuran daging anjing dan tikus pada daging sapi. Penelitian lanjutan harus dilakukan untuk menguji sampel produk olahan daging dan produk komersial.

Kata kunci: anjing, daging sapi, identifikasi spesies, tikus, 12S rRNA

ABSTRACT

The 12S rRNA gene is one of unique regions in mitochondrial genome usually used for phylogenetic studies and species identification. The objective of present study was to develop species specific primers from mitochondrial 12S rRNA gene for identification of dog and rat in beef by using multiplex PCR assay. Three primer pairs of mitochondrial 12S rRNA gene specific for bovine, dog and rat were designed and selected to evaluate their specificity and fidelity. Moreover, a total of twelve DNA samples extracted from meat tissue were also prepared to test those primers using simplex and multiplex PCR. The PCR products were then visualized using 2% of agarose gel under the UV light and three of them were sequenced. In addition, sequence data were analyzed using Clustal Omega software and BLAST. The result showed that simplex PCR assay successfully amplified DNA targets which are respectively indicated by 155 bp (bovine), 244 bp (dog), and 491 bp (rat) of DNA bands. Furthermore,

DNA sample sequences were identically similar to reference sequence used in this study. Multiplex and simplex PCR analyses also indicated that these primer pairs specifically amplified DNA target for each species in the samples containing various species. The results suggested that designed primers in this study could be used to identify dog and rat in raw beef containing these species meat. Further experiment should be conducted using meat-processed products and commercial meat products as samples.

Keywords: beef, dog, rat, species identification, 12S rRNA

INTRODUCTION

Beef is one favorite animal product commodity which is able to be processed into various products such as sausage, meatball, and cornet. Increasing of beef consumption in recent years directly have an impact to its price creates an opportunity to get more benefits without considering of food safety and halal aspects. One of deceptions may be occurred in the beef markets is beef adulteration by mixing with cheaper meats form other species (Liyana *et al*., 2009). Meats adulteration is prohibited due to violation of consumer rights and rule of food safety, therefore, the effort to identify meat adulteration accurately is needed (Mahajan *et al*., 2010). In addition, Hertanto *et al*. (2017) reported that contamination undesired species in meat products do not only happen intentionally but also accidentally. By using same meat grinder for different species without strict control will automatically contaminate meat.

Identification of species in meats is physically difficult to be carried out. An accurate technique, a DNA based approach such as PCR, was widely used for species identification in the animal-based products. Previous study conducted by Rahman *et al*. (2009) reported that PCR is simple, stable, and highly sensitive method for identification of dog species in processed food. In addition, PCR technique is mostly applied to identify species in the food due to its sensitivity (Dieffenbach *et al*., 1993). Principally, PCR technique is a method used to amplify specific DNA target across several orders of magnitude, producing million copies of a particular DNA sequence (Lima and Garces, 2006). The polymerase chain reaction contains deoxynucleotide triphoshates (dNTPs), buffer, *Taq* DNA polymerase, primer pairs and DNA template. Primer is short oligonucleotides that have a function to initiate polymerization of double strand DNA that provide 3'-OH^- as a site to anneal the first nucleotide of DNA molecule to a new of DNA strand (Yuwono, 2006). Primer plays a very important role in PCR. A good primer should be designed by following criteria such as the length of primer, melting and annealing temperatures, GC contents, and primer secondary structure. Primer design should be carefully performed to get specific primer which is able to amplify DNA target in PCR (Dieffenbach *et al*., 1993; Pradnyaniti *et al*., 2013). For example, primers which are designed for mitochondrial genome should not anneal in the region of nuclear genome and vice versa (Cahyadi *et al*., 2018).

Mitochondrial genome was well studied and widely used for species identification due to uniqueness of its nucleotide sequence. Previous studies successfully identified various species in the meats with and without temperature treatments by using multiplex PCR mitochondrial DNA cytochrome b gene (Matsunaga *et al*., 1999; Shin *et al*., 2006; Ni'mah *et al*., 2016; Hertanto *et al*., 2017). Irine *et al*. (2013) also used cytochrome b gene to identify species of tiger, cat, and dog. In addition to cytochrome b gene, another unique region contained in animal mitochondrial genome is 12S rRNA gene. This gene is commonly used and very useful in phylogenetic studies (Anderson *et al*., 1981). Sequence analysis of mitochondrial 12S rRNA has previously been used to identify species on the samples originated from animal tissue (Girish *et al*., 2004; Yang *et al*., 2014). Moreover, a current report revealed that 12S rRNA gene could be used for identification of chicken and pig in beef using multiplex PCR (Cahyadi *et al.*, 2018). In the present work, specific primers from mitochondrial 12S rRNA were designed and tested for identification of dog and rat meat in beef using multiplex PCR assay.

MATERIALS AND METHODS

Isolation of Total DNA Genome

The DNA genome was extracted from meat samples of cattle, dog and pig by following protocol of Genomic DNA Mini Kit for animal tissue (Geneaid Biotech Ltd., Taiwan). A total of

twelve DNA genomes, three samples for each species and three DNA mixtures were used in this study. A total of 30 mg muscle tissues was prepared and put into a 1.5 ml microtube, and then sample was pulped using micropestle. The mixture was added with 200 µL of GT buffer and 20 µL of proteinase K. This was shaken until homogeny and then incubated at 60^{o}C for 30 minutes. Moreover, the mixture in the microtube was added with 200 µL of GBT buffer. This was incubated at 60^{o}C for 20 minutes to produce clear lysate. The second step of DNA extraction process was precipitation. The clear lysate was added with 200 µL of absolute ethanol and then it was vigorously shaken for 10 seconds, immediately. It was transferred to the GD column which is inserted into 2 ml collection tube for centrifugation at 14.000 rpm for 2 minutes. The GD column was inserted into new 2 ml collection tube after centrifugation and added with 400 µL of W1 buffer for second centrifugation at 14.000 rpm for 30 seconds. The third centrifugation at 14.000 rpm for 30 seconds was performed after the 600 µL of wash buffer addition. Flow-through into 2 ml collection tube during first to third centrifugation was all discarded. The GD column was inserted back into the collection tube and the fourth centrifugation was carried out to dry the column matrix at 14.000 rpm for 3 minutes. For DNA harvesting, a 1.5 mL mictrotube was prepared to collect DNA solution by putting of GD column into the tube and adding 100 µL of pre-heated elution buffer to the center of the GD column for perfect absorption. Finally, the final centrifugation at 14.000 rpm for 30 seconds was conducted to harvest pure DNA genome. To evaluate the quality of isolated DNA, 1% agarose gel electrophoresis was performed and the photograph was documented using Gel Document (Bio-Rad Gel Doc^{TM} XR+, United State of America). Good quality of DNA genome was indicated by bright and clear DNA bands under UV light.

Primer Design

Primer design was started by tracing 12S rRNA gene sequence in the mitochondrial genomes for dog and rat through National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Two GenBank accession numbers (Acc. No), KF907307.1 and AY769440.1, were selected as reference sequences for dog (*Canis lupus familiaris*) and rat (*Rattus nervogicus*), respectively. Furthermore, sequence of 12S rRNA gene region was analyzed to obtain primer candidates using software of Primer3 which is online available. Primer annealing site of primers could be discovered by conducting alignment analysis using Clustal Omega software. Then, all of candidate primers produced by Primer3 were evaluated to know their secondary structures and their potentials to create heterodimer, miss-priming, and self-priming (Dieffenbach *et al*., 1993; Apte and Daniel, 2009). Selected primer candidates for dog and rat are presented in Table 1. Primer pair for bovine used in this study was previously reported by Cahyadi *et al*. (2018) while the new dog and rat reverse primers were synthesized by Integrated DNA Technologies, Singapore.

Evaluation of Primer using Polymerase Chain Reaction (PCR) and Sequencing Analysis

Evaluation of designed primers was performed by in vitro analysis using simplex and multiplex PCR. Simplex PCR is a technique to

Table 1. The Characteristics of Selected Primer Pairs for Bovine, Dog and Rat

Primer	Species	Sequence (5' to 3')/Primer Length	GC Content (%)	Product Size (bp)	Reference
Forward	Universal	ACCGCGGTCATACGATTAAC/20	50		Cahyadi *et al*. (2018)
Reverse	Bovine	AGTGCGTCGGCTATTGTAGG/20	55	155	Cahyadi *et al*. (2018)
	Dog	TCCTCTGGCGAATTATTTTGTTG/23	39.13	244	This paper
	Rat	TCTGGGAAAAGAAAATGTAGCC/22	40.91	491	This paper

amplify DNA fragment using a primer pair, on the other hand, multiplex PCR uses more than one primer pairs in its reaction. A total of 10 reactions containing different DNA sample and primer pair were tested to evaluate specificity of the primers in this study (Table 2). The PCR was carried out in total volume of 25 µL containing 12.5 µL of My *Taq* HS Red Mix solution (Bioline, United Kingdom), 1 µL (10 µM) of universal forward primer, 1 µL (10 µM) of reverse primers, 1 µL of DNA template, and 8.5 µL ddH_2O (Cahyadi *et al*., 2018). The PCR was performed by following these steps: initial denaturation at 95ºC for 3 minutes, 30 cycles of denaturation at 95ºC for 15 seconds, annealing temperature at 64ºC for 30 seconds, and extension at 72ºC for 30 seconds. The PCR was enhanced by final extension 72ºC for 3 minutes (GeneAmp® PCR System 9700, Singapore). The results of PCR were visualized using 2% agarose gels under UV light (Biorad Gel Doc XR+, United State of America). In addition, PCR products were sent to First BASE (Malaysia) to be sequenced for further confirmation whether the primers precisely amplified the right target regions. The sequencing was carried out using forward and reverse primers.

Data Analisis

The data obtained in this study was analyzed by comparing estimated PCR product size (bp) with DNA bands visualized in the agarose gel wherein marker ladder was as a standard. Sequence data was also analyzed using Bioedit 7.2.6.1 software to obtain clear sequences. Furthermore, clear sequences were aligned using Clustal Omega software and Basic Local Alignment Search Tool (BLAST) analysis was also performed to check similarity of DNA sequences in this study with GenBank data (Lima and Garces, 2006).

RESULTS AND DISCUSSION

Primer Characteristics

Primer design is the most critical factor determining specificity for successful PCR. At least four major parameters should be taken into account when designing multiplex PCR primers i.e. primer length, annealing temperature, GC content and extension times. The characteristics of selected primer sets are presented in Table 1.

A total of 15 sets of primer were generated in this study (Supplementary Material 1). Of these, primer pair for bovine was designed in previous studies (Cahyadi *et al.*, 2018) while two sets of primer for dog and rat were novel finding and firstly reported in this work. Universal forward primer was a hundred percent similar for cattle, dog, and rat. When running multiplex PCR, either forward or reverse primer should conserve in every species used as a target region (Apte and Daniel, 2009). The length of primers in this study was around 20 to 23 nucleotides (Table 1). In general, oligonucleotides between 18 to 24 bases are extremely sequence specific and proportional to optimal annealing temperature (Apte and Daniel, 2009; Borah, 2011; Cahyadi *et al*., 2018). Longer primer is more inefficient to anneal, on the

Table 2. The DNA Template Designed for Primers Specificity Test

No.	Sample ID	DNA Template	Primer Pair	Reaction
1.	S1	Bovine	Bovine	Simplex PCR
2.	A1	Dog	Dog	Simplex PCR
3.	T1	Rat	Rat	Simplex PCR
4.	S2	Bovine, dog, rat	Bovine	Simplex PCR
5.	A2	Bovine, dog, rat	Dog	Simplex PCR
6.	T2	Bovine, dog, rat	Rat	Simplex PCR
7.	S3	Bovine	Bovine, dog, rat	Multiplex PCR
8.	A3	Dog	Bovine, dog, rat	Multiplex PCR
9.	T3	Rat	Bovine, dog, rat	Multiplex PCR
10.	SAT	Bovine, dog, rat	Bovine, dog, rat	Multiplex PCR

other hand, shorter primer is reducing primer specificity and miss-priming (Handoyo and Rudiretna, 2000). The length of primer affects temperature and time of annealing. The time and temperature of annealing in this study was set up for 30 seconds at 64^{o}C after optimization in serial temperatures. Lower annealing temperature produces unspecific PCR product because primers attach to more than one target sites, on the other hand, higher annealing temperature produces low PCR product yield even sometimes no PCR product is detected (Borah, 2011). In addition, GC content in a primer is the number of guanine (G) and cytosine (C) bases compared to the total nucleotides in a primer and it should be considered when designing primer for PCR since they will give appropriate hybridization stability. The GC content of the primers tested in this work was 39.13 to 55% (Table 1). You *et al*. (2008) reported that the ideal GC content of oligonucleotide primer for PCR reaction is around 30 to 70%. Lower GC content decreases the ability of primer to anneal in DNA template (Handoyo and Rudiretna, 2000).

Evaluation of Primer Specificity using PCR and Sequencing Analysis

The specificity of primers in this study was conducted. The results indicated that primers specifically and correctly amplified DNA target. The existences of bovine, dog, and rat in the samples were indicated with 155, 244, and 491 bp, respectively (Figure 1). Primer specificity is one of important aspects in multiplex PCR assay due to directly affecting to the developed marker in identification of target species (Ali *et al*., 2014). The use of a primer pairs in DNA pools containing those three species in one PCR tube and the use of multiple primer pairs containing only one species as a DNA template in the one reaction tube showed that primer pairs were still working extremely well and specific which is indicated by only one DNA band appeared in the agarose gel (Figure 1). This success PCR could not be separated by statement that mitochondrial 12S rRNA gene is able to be used as a marker for species identification due to the big variation among those three species (Springer and Douzery, 1996). Previously, 12S rRNA was mostly used for

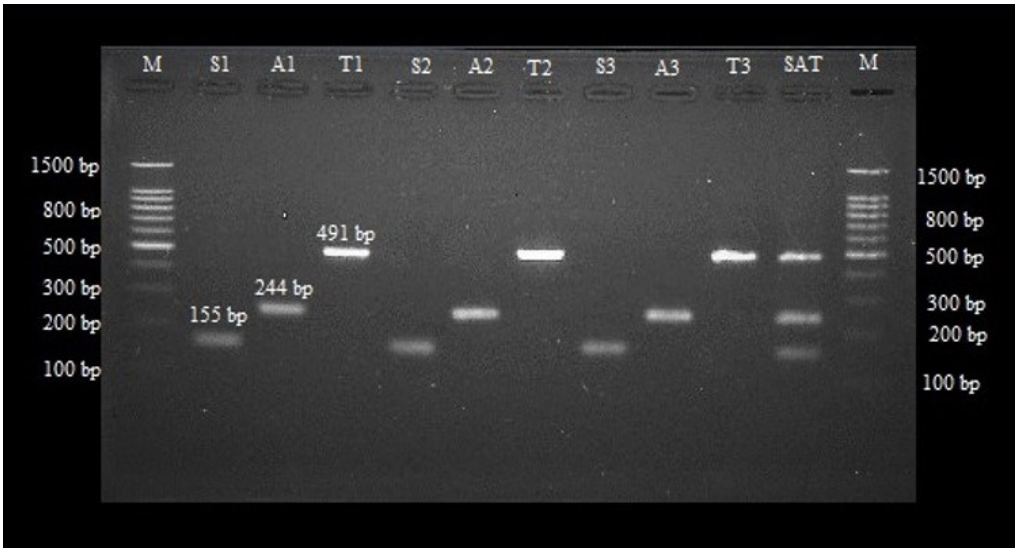

Figure 1. Simplex and multiplex PCR product sizes. M is 100 bp marker ladder 100 bp; S1 is simplex PCR product for beef sample (155 bp); A1 is simplex PCR product for dog sample (244 bp); T1 is simplex PCR product for rat sample (491 bp); S2 is simplex PCR product using bovine primer and mix DNA template containing bovine, dog, and rat; A2 is simplex PCR product using dog primer and mix DNA template containing bovine, dog, and rat; T2 is simplex PCR product using rat primer and mix DNA template containing bovine, dog, and rat; S3 is multiplex PCR product using bovine DNA as a template; A3 is multiplex PCR product using dog DNA as a template; T3 is multiplex PCR product using rat DNA as a template; and SAT is multiplex PCR product using mix DNA template containing bovine, dog, and rat.

phylogenetic studies intra- and inter-species (Allard and Honeycutt, 1992; Tougard *et al*., 2001). In addition, Girish *et al*. (2004) proved that 12S rRNA could be used for species identification in feedstuffs. Recently, Cahyadi *et al*. (2018) revealed that 12S rRNA gene was able to detect pork and chicken in bovine samples.

The sequence analysis was also conducted to confirm whether the primers designed in this study precisely annealed in target regions. Multiple alignment analysis of 12S rRNA sequences from those three species indicated the perfection of reverse primers to attach to species specific DNA template (Figure 2). Those reverse primers were designed in such way to ensure that they correctly annealed in the right site of DNA target and to avoid primer attachment to undesired DNA template from other species. The numbers of nucleotide in sequence data were also exactly similar to the expected PCR product sizes, i.e. 155, 244, and 491 bp for bovine, dog, and rat, respectively (Figure 2). This result indicated that forward and reverse primers were complement with specific DNA template and they successfully attached to 5' end and 3' end of DNA target (Carson and Robertson, 2005). Additionally, multiple alignment of sequences presented that reverse primer for each species was very unique. This uniqueness made them working incredibly specific. The extension of DNA template will not perform in the polymerase chain reaction when there are two or more different nucleotides at the

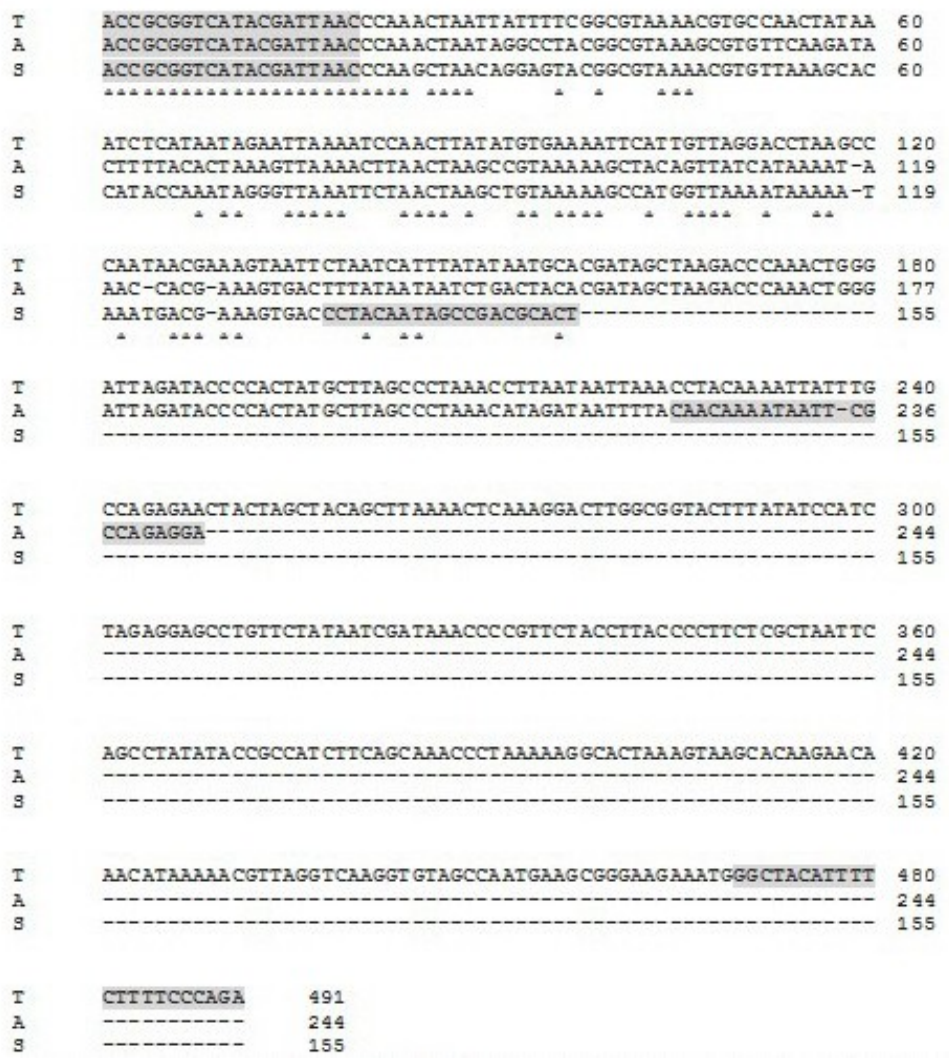

Figure 2. Multiple Alignment of 12S rRNA Gene Sequence Originated from Bovine, Dog and Rat Samples. S is 12S rRNA gene sequence for bovine; A is 12S rRNA gene sequence for dog; T is 12S rRNA gene sequence for rat.

end of 3' end of the primer.

Analysis of BLAST was conducted to evaluate similarity index of the sequence obtained from this study with reference sequences in the GenBank through NCBI website. The sequences data of present study were submitted to NCBI database with following accession numbers: MH753651 for bovine, MH753649 for dog, and MH753650 for rat, respectively. The analysis revealed that the sequences of bovine, dog, and rat were identically similar with GenBank database. Bovine sample was 100% similar with *Bos taurus*, dog sample was 100% identical with *Canis lupus* and *Canis lupus familiaris,* respectively. Moreover, rat sample was 99% similar with *Rattus norvegicus*. These results suggested that primer pairs precisely amplified the right DNA target. The sequence similarity percentage is presented in Table 3.

Species Identification using Multiplex PCR 12S rRNA Gene

Application of multiplex PCR mitochondrial 12S rRNA gene was conducted in this study. The result of multiplex PCR was indicated with S3, A3, T3, SAT in the Figure 1. Three meat samples, namely S3, A3, and T3, represented the results of multiplex PCR with only one DNA template inside the reactions. They were bovine, dog, and rat, respectively. This result proved that primer pairs for each species specifically worked only for DNA template originated from its self. In addition, the SAT sample represented multiplex PCR result containing multiple primer pairs and multiple DNA templates in the reaction. According to the electrophoresis agarose gel, oligonucleotide primer pairs for bovine, dog, and rat specifically and sensitively attached to the desired regions which are clearly indicated with 155, 244, and 491 bp of DNA bands in SAT sample (Figure 1). This analysis performed to ensure that oligonucleotide primers designed in this study and other components of PCR were working together and they could be used as a marker for identification of dog and rat in food products containing beef and bovine derivatives.

Table 3. The Results of BLAST Analysis of DNA Sequence of Bovine, Dog, and Rat Obtained in This Study

No.	Species used in this study	Sequence Acc. No	Species in NCBI Database	GenBank Acc. No	Similarity (%)
1.	Bovine	MH753651	*Bos taurus isolate* Rom498	HQ184040.1	100
			Bos taurus isolate Cin19	FJ971086.1	100
			Bos taurus isolate Per12	FJ971084.1	100
2.	Dog	MH753649	*Canis lupus familiaris* isolate 1381T1A2b Clade 1 China	KY659812.1	100
			Canis lupus isolate Bul729 Bulgaria	KU696388.2	100
			Canis lupus isolate Mongolia4	KU696395.1	100
3.	Rat	MH753650	*Rattus norvegicus* strain YiZ	KP241960.1	99
			Rattus norvegicus strain LiN	KP233827.1	99
			Rattus norvegicus isolate 78	KM820837.1	99

The studies in food authentication using PCR technology were reported using duplex PCR mitochondrial Cytochrome b gene to detect pork and chicken contamination in raw meats and meatballs using published primers (Ni'mah *et al*., 2016; Hertanto *et al*., 2017; Novianty *et al*., 2017). The use of mitochondrial 12S rRNA gene as a marker for species identification had been successfully conducted using sequence analysis of processed meats samples. The effect of high temperatures and methods when processing the meats showed no significant effect to the amplicons (Girish *et al*., 2004). The success multiplex PCR of 12S rRNA regions were also described able to identify various species in feedstuffs (Dalmasso *et al*., 2004; Safdar and Junejo, 2015). Cahyadi *et al*. (2018) currently published the use of multiplex PCR 12S rRNA gene to differentiate beef with pork and chicken in raw meats. Linacre (2006) explained 12S rRNA gene of mitochondrial genome has possibility to be developed as a marker for species identification due to its small variation within species while big variation was found among species. Thus, this study tried to develop new primer sets for dog and rat in accordance to prior study conducted by Cahyadi *et al*. (2018) and this study succeeded to create novel oligonucleotide primers in exploring of 12S rRNA gene as a marker for identification of dog and rat meat in beef.

CONCLUSION

Novel primer sets were designed from mitochondrial 12S rRNA gene and they were precisely and specifically working to detect species of rat and dog in beef by simplex and multiplex PCR assays. Further study should be conducted to test the sensitivity of those primer pairs in processed meat products.

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