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Determination of Cattle and Buffalo Skin Crackers Using Polymerase Chain Reaction Restriction Fragment Length Polymorphism

Uji Penentuan Bahan Baku Kulit Rambak dari Sapi dan Kerbau Menggunakan Polymerase Chain Reaction Restriction Fragmnet Length Polymorphism

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Abstrak

Penelitian ini bertujuan mengidentifikasi spesies sapi dan kerbau melalui metode PCR dengan mengamplifikasi gen *cytochrome b mitokondria* dan dilanjutkan pemotongan amplikon dengan ensim restriksi. Bahan kulit sapi dan kerbau didapatkan dari Rumah Potong Hewan di daerah Yogyakarta dan Kudus. Untuk mengetahui tingkat validitas dan efisiensi konfirmasi disiapkan campuran DNA kerbau dalam campuran dengan DNA sapi dengan level 10, 20, 40, 60 dan 80 %. Hasil isolasi DNA diamplifikasi menggunakan primer universal gen *cythochrome b*, hasil amplifikasi kemudian didigesti dengan ensim restriksi *RsaI*. Hasil penelitian menunjukkan bahwa DNA hasil isolasi dapat teramplifikasi dengan primer *cytochrome b* dan menghasilkann fragment DNA 359 bp. Ensim restriksi *RsaI* dapat memotong amplikon DNA pada gen *cytochrome b* dari kerbau menjadi dua fragment yaitu 326 dan 23 bp, sedangkan amplikon dari DNA *cytochrome b* sapi tidak terpotong oleh ensim tersebut. Kesimpulan penelitian ini adalah adanya kult kerbau dalam campuran matrix kulit sapi dapat dideteksi menggunakan metode PCR-RFLP menggunakan ensim *RsaI* dan kemampuan deteksi kontaminasi tersebut pada penelitian ini sampai pada level 10%. Hasil penelitian ini juga menyimpulkan bahwa *RsaI* dapat digunakan untuk menentukan ada tidaknya kontaminasi kerbau pada produk kulit sapi. PCR-RFLP adalah metode yang akurat dan berpotensi untuk digunakan sebagai analisis rutin kontaminasi spesies pada produk campuran pangan atau produk yang lain.

Kata kunci : Kulit sapi dan kerbau, *Gen cytochrome b*, Kulit Rambak, *Polymerase chain reaction.*

Abstract

The aim of this study was to determine of cattle and buffalo species based on cytochrome b gene using PCR-RFLP. Cattle and buffalo hides were obtained from a slaughterhouse in Yogyakarta and Kudus Regency. To confirm the effectiveness and specificity of this fragment, there are seven of DNA mixture samples in various levels. Isolate DNA samples were amplified using universal primer of cytochrome b gene, then PCR amplicon was digested by *Rsa*I restriction enzyme. The result showed that mitochondrial cytochrome b gene successfully amplified fragments of 359 bp. *Rsa*I restriction enzyme was able to cleave buffalo cytochrome b gene into two fragment (326 and 23 bp), while the cytochrome b gene of the skin cattle DNA was uncleaved. In conclusion, this study indicated that mixture DNA of cattle and buffalo hides could be digested by *Rsa*I restriction enzyme and determination of the buffalo hides in mixture samples could be detected into 10% level. Furthermore, RsaI enzyme could be used to specific identification buffalo species. PCR-RFLP technology has a potential and reliable method to identify of the existence of buffalo hides in the mixture with other hides.

Key words: Cattle and buffalo hides, Cytochrome b, Crackers, Polymerase chain reaction.

Introduction

The hide is a product of slaughterhouses that had high sales value. Hides used to tanning processing, and a little part of the hide was not used will be discarded because it can not be made in tanning. The hide not used to tanning will make “rambak”. Rambak is an hide crackers which are made from animal by product of hide. Rambak or hide cracker a traditional

food that is favored by most people of Indonesia (Muttaqien *et al.,* 2016). The verification of labeled components in any food products is necessary in order to prevent the adulteration practice. For this purpose, some countries make regulation for assuring that food products available are safe and authentic (Doosti *et al*., 2011). Especially if food products contain some prohibited component such as pig derivatives for Moslem community and cattle derivatives for hinduishm community are compulsory to show the ingredients label.

In the past years, many publications about identification species animals or animals production by analysis of DNA was fastly increase. Analysis of mitochondrial genome (mtDNA) has been extensively used as mtDNA has a greater abundance (higher copy number) in sample extracts than nuclear genome, and the rate of base substitution of mtDNA is higher than that of genomic DNA because a rapid evolution (Xu *et al.,* 2015). Furthermore, whereas nuclear DNA is stable during development, mtDNA is degraded and turns over in animals and plants (Kumar *et al*., 2014). The cytochrome b (cyt b) gene is one of the 37 genes within the circular mitochondrial genome (Caine *et al.,* 2006). However, few studies which examined animal by-product species identification based on molecular biology marker.

Polymerase chain reaction (PCR) are currently the method of choice for species identification (Erwanto *et al.,* 2014). A single molecule of a nucleic acid, DNA, can be amplified from 1 to 10 million copies (Bromley and Maher-Sturm, 2005). Restriction fragment length polymorphism (RFLP) is method relies on the highly specific recognition of base sequences by endonuclease within DNA fragment (Wu *et al.,* 1999). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis can be used to evaluate the genetic variability in buffalo population (Srisakwattana *et al.,* 2016). Therefore this study was used to analyze DNA fragment of cyt b gene to discriminate different species between cattle and buffalo.

Materials and Methods

Sample preparation and DNA extraction

Hide samples of cattle and buffalo were obtained from a slaughterhouse on Yogyakarta and Kudus respectively. Samples were removed further with different knives to avoid cross contamination and stored at -20°C until ready to used research. Different levels of concentrations of samples buffalo-hides were used to measure the level of detection capability, such as 10, 20, 40, 60, and 80%. DNA was extracted from 30-50 mg of hides. Samples were placed in a microcentrifuge tube 1.5 ml and added 500 µl TEN/STE *buffer* then mixed well by vortexing. Twenty microliters proteinase-k and 50 µl 10% SDS were added and mixed well by vortexing. The mixture was incubated at 42°C for 18 hours. Fifty microliters natrium clorida, 400 µl phenol, 400 µl CIAA were added in the mixture and incubated at 37°C for 1 hour. All tubes were centrifuged at 3.000 rpm for 5 min. Supernatant was moved in new tubes, added 50 µl NaCl 5 M and 1 ml absolute ethanol. Afterward, mixed with hand until looked like fiber. Incubated at -20°C in the freezer for 1 hour. The mixture was centrifuged at 8.000 rpm for 5 min in temperature 4°C.

PCR amplification of cytochrome b of mitochondrial gene

A pair of universal primers was employed in PCR reaction. The PCR primers used were L14841 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and H15149 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'), as reported by Kocher *et al.* (1989). Amplification of the cyt b gene was performed in a final volume of 25 µl contained 20 µl PCR master mix, 2 µl ddH_2O, 2 µl DNA template, and 20 pmol of each

primer (Thermo Scientific) in 0,2 ml tube. Amplification was performed with a thermal cycler according to the following PCR step cycle program: predenaturation 94°C for 2 min, denaturation 95°C for 36 sec, *annealing* 51°C for 73 sec, extension 72°C for 84 sec and followed by 35 cycles. A final extension at 72°C was conducted for 3 min and temperature down until at 4°C. The result of PCR was served at -20°C until ready to did for the next analysis, as reported by Erwanto *et al.* (2014). Ten microliters of PCR products were electrophoresed at constant voltage 100 V on 3% agarose gel for about an hour in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp (Thermo Scientific) was used as a size reference. The gel photo was taken using the Syngene gel documentation system.

Restriction fragment length polymorphism

The enzyme was used RsaI for digestion. The reaction of digestion enzyme was contained 18 μl ddH_2O, 2 μl tango buffer, 10 μl amplicon DNA, 2 μl enzymes. The mixtures were incubated at 37°C for 5 hours for optimal RsaI enzyme. Twenty microliters of the digested samples were electrophoresed at constant voltage 100 V on 3% agarose gel for about an hour in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp (Thermo Scientific) was used as a size reference. The gel photo was taken using the Syngene gel documentation system

Results and Discussion

Isolation of DNA was the first step in analysis DNA before doing the next step. Isolation of DNA did to separate from other raw. The result of the isolation of DNA has used Sambrook method with cattle, and buffalo hides in various levels were shown in Figure 1.

The results of DNA isolation used the method of Sambrook with cattle and buffalo hides at various concentrations seen the emergence marked DNA bands

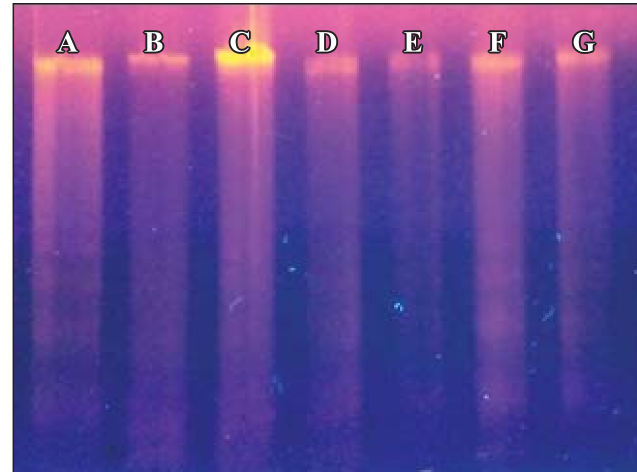

Figure 1. Electrophoretic of DNA isolation used sambrook method with 0,8% agarose gel from cattle, buffalo and mixture hides. A= cattle (100%), B= buffalo (100%), C= cattle : buffalo (20%:80%); D= cattle : buffalo (40%:60%); E= cattle : buffalo (60%:40%); F= cattle : buffalo (80%:20%); and G= cattle : buffalo (90%:10%).

used UV Trans-illuminator visualization of the electrophoresis process. Electrophoresis technique used a medium made of agarose gel with the movement of charged particles in an electric field. The principle of electrophoresis is moved from negative to positive. DNA bands were clear and indicate a successful DNA isolated. The principles of the isolation of DNA are centrifugation and precipitation.

The quality of isolated DNA was evaluated using two different manners. There are electrophoresis using agarose gel and spectrophotometry. The spectrophotometer was a method for measured quantity of DNA concentrations. The 260/280 absorbance ratio was calculated by quantifying the sample using spectrophotometry. The isolation of DNA concentrations and the absorbance ratios are displayed

Table 1. The Quantity of isolated DNA using spectrophotometry analysis

Sample	DNA concentration	260/280 ratio
A	380	0.869
B	352.5	0.876
C	355	0.910
D	342.5	0.878
E	310	1.068
F	335	1.117
G	302.5	1.080

in Table 1.

A 260/280 ratio between 1.8 and 2.0 is indicative of pure DNA while a ratio below 1.8 indicates contamination by proteins and a ratio above 2.0 indicates phenol contamination (Romano and Brasileiro, 1999; Couto *et al.,* 2013).

Amplification process was the next step after the isolation of DNA and measurement of DNA concentration. The results of such amplification of DNA fragments have been multiplied. Based on the research results can be obtained DNA fragment amplified on individual skin samples and composite skin in Figure 2.

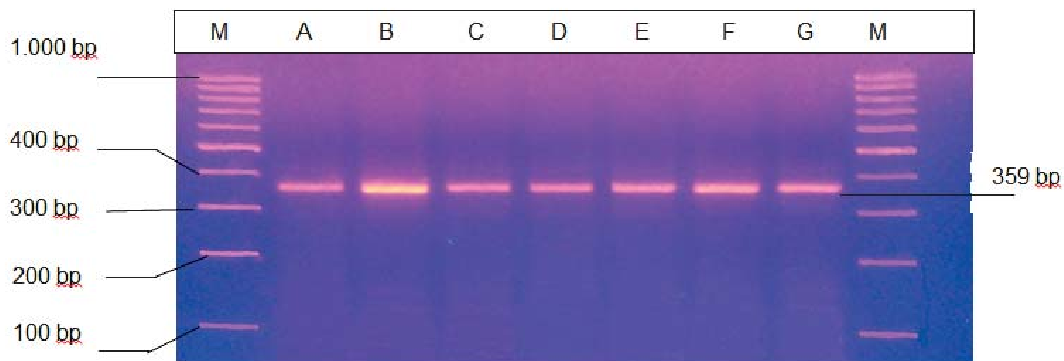

Figure 2. Electrophoretic of PCR gene *cyt* b used universal primer with 2% agarose gel from cattle,buffalo and mixture hides. A= cattle (100%), B= buffalo (100%), C= cattle : buffalo (20%:80%); D= cattle : buffalo (40%:60%); E= cattle : buffalo (60%:40%); F= cattle : buffalo (80%:20%); G= cattle : buffalo (90%:10%); and M= marker DNA ladder 100 bp.

DNA tire size in 7 samples with various concentrations of raw material hide were shown on an agarose gel (2%) after the amplification process, fragment DNA size of 359 bp obtained using a universal primer that is L14841 and H15194. The primer should have a DNA sequence corresponding to the GenBank database to be amplified. Band DNA PCR results clearly visible in Figure 2. The advantage of using a universal primer was used as a control to see the success of DNA amplification. Based on the visualization of the PCR technique was able to double the number of DNA molecules at specific targets with the advent of DNA band light.

The goal of PCR is double-stranded nucleic acids extracted from the cells and denaturation into single-stranded nucleic acids. PCR target length ranges are from tens to thousands nucleotide position between a pair of primers. PCR reaction components consist of a form of specific oligonucleotide primers for the target gene is selected. Primer located before the target area called the forward primer and after the target area called the reverse primer. Cytochrome b gene amplification produces approximately 359 bp fragments. These results indicate that of the samples for DNA amplification mix enough with the lowest concentration of 10%. This result was to Erwanto *et al.* (2011) reported that nugget and sausage can be early detected by PCR-RFLP analysis of cytochrome b gene. Cytochrome b gene of several vertebrates, including mammals, had been used to investigate the evolutionary and genetic diversity and molecular phylogenetic studies (Hartatik *et al.,* 2013).

The universal primer detection method shows positive results in cows and buffaloes but does not amplify at the same point. Sequence DNA of cyt b gene cattle and buffalo obtained from database of NCBI was further employed for sequence alignment using software of nebcutter. As a result of nebcutter software

analysis for detection of specific restriction sites on cattle and buffalo, a site recognized by RsaI enzyme was cleaved into two fragments, namely 326 bp and 33 bp (Figure 3) but RsaI enzyme restriction only cleaved on buffalo. The positive result of DNA cleabed by nebcutter can be an indicator in the visualization of electrophoresis results with agarose.

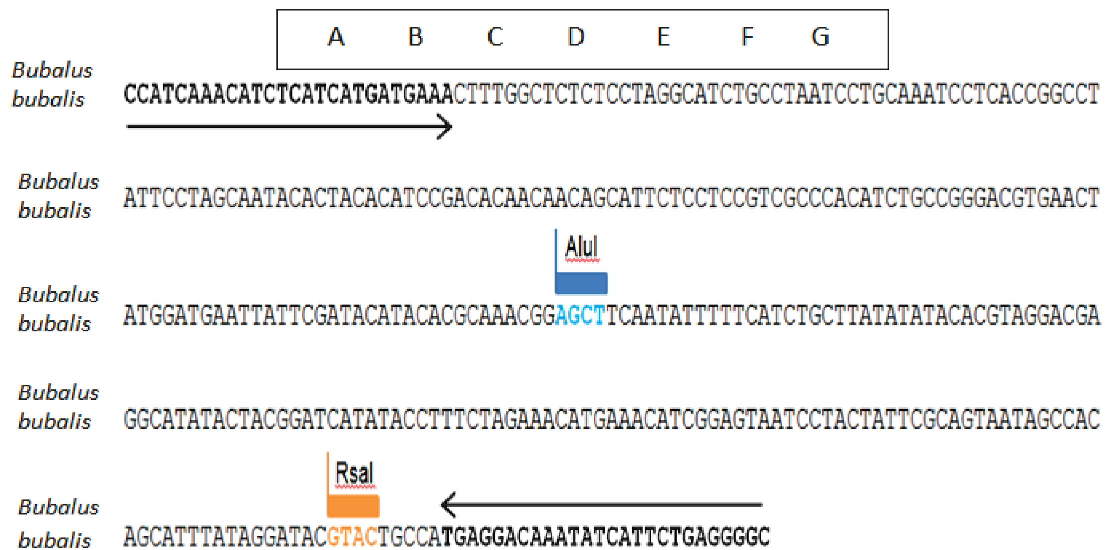

Figure 3. Restriction side of the cyt b gen of Bubalus bubalis using AluI and RsaI enzyme.

Restriction analysis of cyt b amplified products from 7 samples showed various polymorphism with *Rsa*I (Figure 4). RsaI restriction enzyme was applied to differentiate species of cattle and buffalo in the hide mixture samples. The assay developed during this work was able to detect 10% of their respective target species. The digestion of PCR amplicon of 359 by cyt b gene resulted in different fragment size of 326 bp and 33 bp in buffalo species (figure 3 and 4) but DNA of cyt b gen of cattle could not be digested using *Rsa*I enzyme. Fragment DNA of 326 bp from the cytochrome b of buffalo was clearly showed by electrophoresis visualization but the small fragment of 33 bp did not appear by electrophoresis visualization.

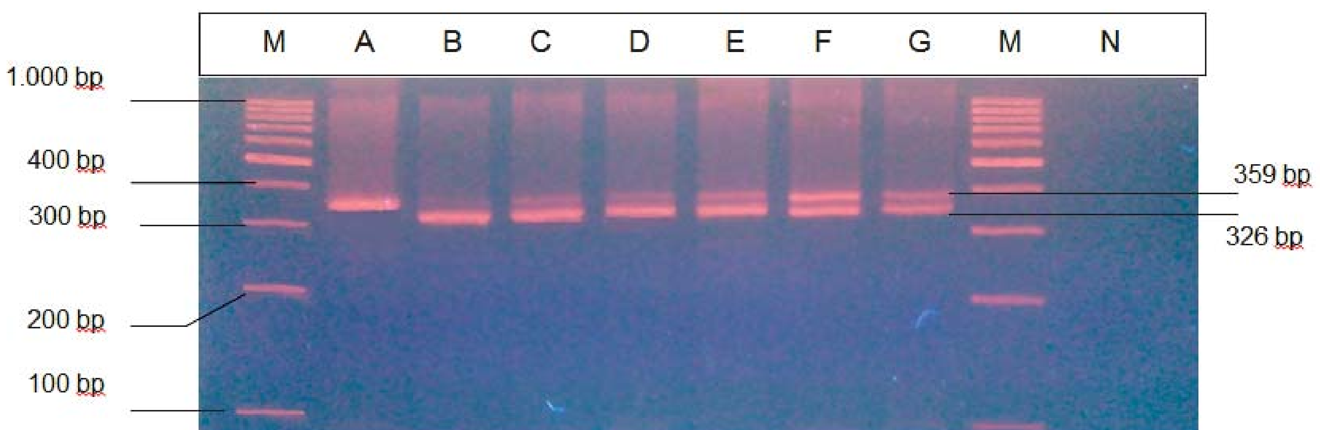

Figure 4. *RsaI* restriction of cyt b PCR product amplified from sample. M = marker DNA ladder 100 bp; A = cattle (100%); B = buffalo (100%); C = cattle : buffalo (20%:80%); D = cattle : buffalo (40%:60%); E = cattle: buffalo (60%:40%); F = cattle : buffalo (80%:20%); G = cattle : buffalo (90%:10%); dan N = negative control.

The cleave of enzyme restriction used to nebcutter shows that buffalo can be detected by AluI and RsaI enzyme, and the locations were different. AluI enzyme cleaved at 190 bp and RsaI at 326 bp.

The result on cty b gen DNA of cattle indicates that using the AluI enzyme cleaved one side AGCT located at 190 bp. Li *et al* (2003) reported that Alul enzyme can catalyze the bonding of phosphodiester bonds on AGCT base. The estimated used to nebcutter show the location of the same cleave side on cattle and buffalo by the AluI enzyme. The positive result of DNA cleaved using nebcutter can be an indicator in the visualization of electrophoresis results with agarose.

Figure 5. Restriction side of the cyt b gen of *Bos indicuss* using AluI enzyme.

Conclusion

The restriction endonucleases would be able to use for study the polymorphism among cattle and buffalo. The polymorphism in cyt b region of cattle and buffalo mtDNA can be revealed by PCR and restriction enzyme analysis. Using RsaI enzyme of mitochondrial cyt b DNA gene is a suitable alternative that can be applied to buffalo detection species in hide mixture.

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