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Comparison Between Pork and Wild Boar Meat (*Sus scrofa*) by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Perbandingan di antara Daging Babi dan Babi Hutan (*Sus scrofa*) dengan Menggunakan Analisis Tindak Balas Rantaian Polymerase-Polimorfisme Panjang Cebisan Pemotongan (PCR-RFLP)

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ABSTRACT

*A method of PCR-restriction fragment length polymorphism (RFLP) has been utilized to differentiate the mitochondrial genes of pork and wild boar meat (*Sus scrofa*). The amplification PCR products of 359 bp and 531 bp were successfully amplified from the cyt b gene of these two meats. The amplification product of pork and wild boar using mt-12S rRNA gene successfully produced a single band with molecular size of 456 bp. Three restriction endonucleases (*Alu*I,* Hind*III and* Bsa*JI) were used to restrict the amplification products of the mitochondrial genes. The restriction enzymes of* Alu*I and* Bsa*JI were identified as potential restriction endonucleases to differentiate those meats.* Hind*III enzyme was unable to restrict the PCR product of both meats. The genetic differences within the cyt b gene among the two meats were successfully confirmed by PCR-RFLP analysis.*

*Keywords: Polymerase chain reaction-restriction fragment length polymorphism; pork; wild boar meat (*Sus scrofa*)*

ABSTRAK

*Kaedah tindak balas rantaian polymerase-polimorfisme panjang cebisan pemotongan (PCR-RFLP) telah digunakan untuk membezakan gen mitokondria daging babi dan babi hutan (*Sus scrofa*). Hasil amplifikasi PCR, 359 pb dan 531 pb telah berjaya diamplikasikan daripada gen cyt b oleh kedua-dua daging. Sementara itu, hasil amplifikasi daging babi dan babi hutan menggunakan gen mt-12S rRNA telah berjaya menghasilkan jalur tunggal dengan saiz molekul 456 pb. Tiga enzim endonuklease pemotongan (*Alu*I,* Hind*III dan* Bsa*JI) telah digunakan untuk memotong hasil amplifikasi gen mitokondria. Enzim pemotongan* Alu*I dan* Bsa*JI telah dikenalpasti sebagai endonuklease pemotongan yang berpotansi untuk membezakan kedua-dua daging. Enzim* Hind*III tidak boleh melakukan pemotongan terhadap hasil PCR kedua-dua daging. Perbezaan genetik di antara gen cyt b antara dua daging telah berjaya disahkan dengan analisis PCR-RFLP.*

*Kata kunci: Babi hutan (*Sus scrofa*); khinzir; tindak balas rantaian polimerase-polimorfisme panjang cebisan pemotongan*

Introduction

Pork and its derivative are *haram* (unlawful or prohibited) to be consumed by muslims. Pork is a typical meat in Malaysian market while, wild boar (*Sus scrofa*) or *babi hutan* is found in the Malaysian rain forest. Adulterations of pork meat in food or processed food are possible since substitution of high quality meat to cheaper materials is common in most countries (Al- Jowder et al. 1997). Similarly, pork meat is likely to be used as an alternative to beef, chicken and goat meat due to its cheaper price.

Scientific evidence against fraud is important in halal authentication. In Malaysia, food products are strictly monitored by the Malaysian authorities such as the Department of Islamic Development Malaysia (JAKIM) for halal certification applications which complied with halal standards and integrity of halal. There are a number of molecular methods in determining the residual DNA content of food materials that can be used to identify indisputably of the product.

Most early tehniques were based on hybridization to specific probes (Chikuni et al. 1990; Ebbehoj & Thomsen 1991) but the recent development in identification of animal DNA contents is given much attention to the use of polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome *b* and 12S rRNA gene and DNA identification based on genomic-specific interspersed repetitive elements (Gurdeep et al. 2004; Rehben 2005; Wolf et al. 1999). Montiel-Sosa et al. (2000) used mitochondrial DNA (mtDNA) approaches for the identification of pork species which the primer set targeting the pork D-loop mt-DNA. The mt-DNA genes are present in thousands of copies per cell, the large variability of mt-DNA allows identification of precise pork DNA.

Several other authors that used the mitochondrial DNA (mtDNA) in species idenfication of pork DNA and conducted RFLP analysis were as described by Aida et al. (2005); Chandrika et al. (2009); Lenstra et al. (2001), Kesmen, Yetim & Sahin (2007) & Partis et al. (2000). In this work, we examined and compared, pork and wild boar raw meat using 2 different mitochondrial DNA oligonucleotide primers for PCR amplification and conduct RFLP analysis that allow the identification of pork meat as a trusted tool for halal authentication. The vertebrate-specific primers as described by Pandey et al. (2007) to identified *Panthera pardus pusca* (leopard) using mt-12S rRNA gene will also be used in this study as a comparison.

Materials and Methods

Sample preparation

Frozen pork was purchased from retail market in Selangor area. Fresh wild boar meat (*Sus scrofa)* was frozen and obtained from *Perkampungan Orang Asli* (Orang Asli Village) Temerloh, Pahang. All samples were stored in ice container while transporting and kept in refrigerator before used.

Extraction of total DNA

The DNA of fresh raw pork and wild boar meat were extracted using DNA extraction kit (SureFood ® Animal ID, Congen Biotechnology GmbH, German) was provided by the manufacturer. A total of 40 mg meat from each sample was minced in a 1.5 mL sterile microfuge tubes. The DNA was stored at -20°C until used as PCR templates. The DNA of pork and DNA of wild boar were labeled as B and W (*Sus scrofa*), respectively.

Oligonucleotide primers

A total of 3 pair primers were utilized in each PCR reaction. Two pairs of mitochondria cyt *b* primers used in this work is tabulated in Table 1 which were described by Lenstra et al. (2001) and Monteil-Sosa et al. (2000). The mitochondrial 12S rRNA (mt-12S rRNA) gene using universal, vertebrate-specific primers as described by Pandey et al. (2007). The primers were supplied from First Base Laboratories (Selangor, Malaysia).

PCR amplification

Amplification of the mitochondria cyt *b* gene of both pork and wild boar meat was performed in a final volume of 50 µL containing 10.0 µL of 10X PCR buffer (100 mM of Tris–HCl, 500 mM KCl and 0.1% Triton™X-100), 4 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs (Promega, Madison, USA), 1.5 µL of 5 µM each primer (Forward and reverse), 0.5 µL of 2.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 29.0 µL of nucleas free water (NFW) and 3 µL of 100 ng DNA template. A negative-DNA control was performed by adding 3 µL of NFW, a positive control was performed by adding 3 µL of the DNA sample. PCR was carried out in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 2 min to complete denature the DNA template, followed by 35 cycles of denaturation at 94°C for 15 s, annealing for 1 min at 42°C, polymerization at 72°C for 1 min and final elongation at 72°C for 2 min. Negative controls (water) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination. The amplification products were analyzed by electrophoresis using 1.0% agarose gel in 1X TAE buffer (40 mM Tris-OH, 20 mM acetic acid and 1mM of EDTA; pH 7.6) at 90 V for 40 min and stained by ethidium bromide. A 100 bp DNA ladder (Vivantis, Malaysia) was used as size reference. The gels were visualized using UV transilluminator (AlphaImager™ Gel Documentation).

Enzymatic digestion of amplified DNA

The endonuclease digestion of the amplicon was conducted as describe by Lenstra et al. (2001). All PCR products of mitochondrial cyt *b* and 12S rRNA of pork and wild boar were digested by *Alu*I, *Bsa*JI and *Hind*III restriction enzymes (Vivantis, Malaysia). Digestions were performed in a total volume of 20 µL according to the manufacturer's instruction. All digests were incubated in 37°C except *Bsa*JI digest, which was incubated at 55°C. The digested samples were analyzed by electrophoresis using 2.5% agarose gel in 1X TAE buffer (containing 0.9M Tris-OH, 0.5 M acetic acid and 20mM EDTA; pH 7.6) at 90 V for 40 min and stained by ethidium bromide. A 100 bp DNA ladder (Vivantis, Malaysia) was used as size reference. The gels were visualized using UV transilluminator (AlphaImager™ Gel Documentation).

TABLE 1. Oligonucleotide primers of mitochondrial DNA (cty *b*) and 12S rRNA with their sequences

Gene target	Primer	Sequence (5'-3')	Source
Cyt *b*	CYT b1	CCA TCC AAC ATC TCA GCA TGA TGA AA	Lenstra et al. (2001)
	CYT b2	GCC CCT CAG AAT GAT ATT TGT CCT CA	
Cyt *b*	Pork F	AAC CCT ATG TAC GTC GTG CAT (15592)	Monteil-Sosa et al. (2000)
	Pork R	ACC ATT GAC TGA ATA GCA CCT (16124)	
12S rRNA	12SL	AAA CTG GGA TTA GAT ACC CCA CTA	Pandey et al. (2007)
	12SH	GAG GGT GAC GGG CGG TGT GT	

RESULTS

PCR AMPLIFICATION USING DIFFERENT MITOCHONDRIA DNA OLIGOPRIMERS

The PCR amplification analysis was conducted using 3 types of mitochondria oligonucleotide primers on pork (*Sus scrofa domestica*) and wild boar pork meat (*Sus scrofa linneus*). The amplification products of each reaction were electrophoresed on 1% of agarose gel. Figure 1 shows the PCR amplification products of pork and wild boar meat by mt DNA oligonucleotide primers as described by Lenstra et al. (2001). Using these primers the amplification products of both, pork and wild boar meat, produced a single band, respectively with molecular size of 359 bp. Whereas, the species-specific mt cyt *b* primers using primers as described by Monteil-Sosa et al. (2000), pork and wild boar meat also produced a single band with molecular size of 531 bp (Figure 2). As shown in Figure 3, the amplification product of pork and wild boar using mt-12S rRNA gene (Pandey et al. 2007), produced a single band with molecular size of 456 bp. No band was observed for negative control (Lane C) in each experiment.

PCR-RFLP ANALYSIS

The PCR amplification product using different mitochondrial DNA oligonucleotide primers were then examined for PCR-RFLP analysis using three different restriction endonucleases of *Alu*I, *Hind*III and *Bsa*JI. Table 2 shows the band sizes of mt cyt *b* and 12S rRNA PCR amplification

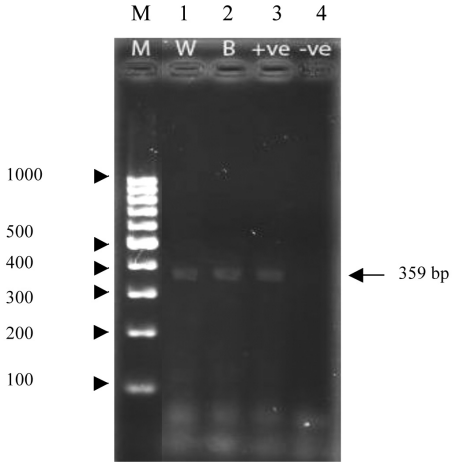

FIGURE 1. Specific PCR amplification of pork and wild boar meat (*Sus scrofa*) by cytochrome *b* primers of mitochondrial DNA (Lenstra et al. 2001). Lane M, 100 bp DNA ladder (molecular weight in base pair, bp); Lane 1, wild boar; and Lane 2, pork; Lane 3-4, positive control and negative control

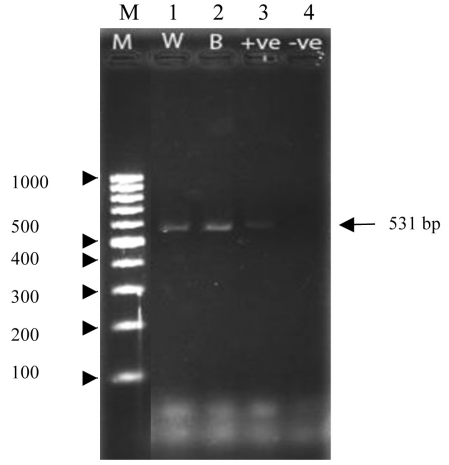

FIGURE 2. Specific PCR amplification of pork and wild boar meat (*Sus scrofa*) by cytochrome *b* primers of mitochondrial DNA (Montiel et al. 2000). Lane M, 100 bp DNA ladder (molecular weight in base pair, bp); Lane 1, wild boar; and Lane 2, pork; Lane 3-4, positive control and negative control

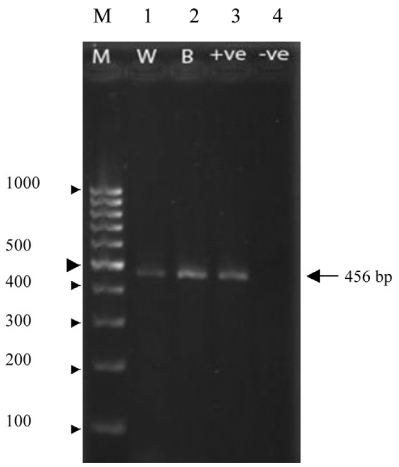

FIGURE 3. Specific PCR amplification of pork and wild boar meat (*Sus scrofa*) by 12S rRNA primers of mitochondrial DNA (Pandey et al. 2007). Lane M, 100 bp DNA ladder (molecular weight in base pair, bp); Lane 1, wild boar; and Lane 2, pork; Lane 3-4, positive control and negative control

products of pork and wild boar when digested by *Alu*I, *Bsa*JI and *Hind*III restriction endonuclease. As shown in Table 2, using DNA oligoprimers as described by Lenstra et al. (2001) in wild boar meat, all endonucleases failed to digest the DNA fragments in repeated experiments which produced a single band of 359 bp in size. While in pork meat, two bands were observed when PCR amplification product was digested with *Alu*I and *Bsa*JI restriction endonucleases. The PCR-RFLP analysis of pork meat using *Alu*I restriction endonuclease produced bands with molecular sizes of 224 bp and 115 bp. The restriction of *Bsa*JI endonuclease showed 2 bands with molecular sizes of 228 bp and 131 bp. While, using the *Hind*III endonuclease was undigested DNA which indicated by a single band with the molecular size of 359 bp.

The PCR amplification products produced by DNA oligoprimers as described by Monteil-Sosa et al. (2000) were also undigested for wild boar meat which indicated by a single band with the molecular size of 531 bp. Whereas, in pork meat the restriction endonuclease of *Alu*I, produced a single band with molecular size of 415 bp. While, the *Bsa*JI endonuclease produced a single band with the molecular size of 470 bp. Similar observation as mentioned above, the *Hind*III endonuclease failed to digest the DNA fragments which showed by a single band of 531 bp in size.

The oligoprimers of mitochondrial 12S rRNA vertebrate-specific (Pandey et al. 2007) has not been used to identified the pork species. Those primers were used to identify the vertebrate of *Panthera pardus fusca* (leopard). In this work, the undigested DNA fragments produced from PCR amplification were 456 bp in size. As shown in Table 2, in wild boar meat all DNA fragments produced were undigested using all restriction endonucleases. While, in pork the PCR amplification product was digested with *Alu*I and *Bsa*JI restriction endonuclease in which they produced 322 bp and 410 bp, respectively.

Discussions and Conclusion

In this work we compared between pork and wild boar meat (*Sus scrofa*) by polymerase chain reaction-restriction fragment length polymorhism (PCR-RFLP) analysis for halal authentication. Three pair of mitochondrial DNA (mtDNA) primers were used in differentition of pork and wild boar meat. Two pairs of mitochondria cyt *b* primers used in this work were described by Lenstra et al.(2001) and Monteil-Sosa et al. (2000). While, the mitochondrial 12S rRNA (mt-12S rRNA) gene using universal, vertebrate-specific primers as described by Pandey et al. (2007) was also studied. The halal authentication needs easy detection using a very simple method such as PCR amplification using mitochondrial DNA. Though the halal status of foods does not requiring RFLP, the analysis is still needed if the species of mixed meats in the foods to be known.

The use of mtDNA in halal (lawful of permitted) authentication to identify indisputably of food product with pork is given much attention by several workers (Aida et al. 2005; Chandrika et al. 2009; Kesmen, Yetim & Sahin 2007). The species identification using PCR-RFLP of mitochondria cytochrome *b* (cyt *b*) segment has been reported by Lenstra et al. (2001) in cheese samples of water buffalo, cattle, goat and sheep. In this work, using the similar primers above, both pork and wild boar meat produced amplified fragment of 359 bp in size (Figure 1). However, when the PCR-RFLP analysis was conducted the RFLP profiles of both meats were differentiated by two different restriction endonucleases of *Alu*I and *Bsa*JI (Table 2). The DNA fragments produced by pork meat through PCR analysis were restricted by these endonucleases. However, the DNA fragments produced by wild boar failed to be restricted by all enzymes. The *Hind*III endonuclease was not able to differentiate between pork and wild boar meat which indicated a single band with molecular sizes of 359 bp. Although the enzymes used were limited here, the PCR-RFLP analysis results indicated here was an effective tool

TABLE 2. The band sizes of mt cyt *b* and 12S rRNA PCR products (pork and wild boar) digested by *Alu*I, *Bsa*JI and *Hind*III restriction enzymes

Primers and type of raw meat samples	Band sizes (bp)		
	*Alu*I	*Hind*III	*Bsa*JI
Cytochrome b (Lenstra et al. 2001)			
Wild boar	359	359	359
Pork	244, 115	359	228, 131
Cytochrome ***b*** (Monteil et al. 2000)			
Wild boar	531	531	531
Pork	415	531	470
12S rRNA (Pandey et al. 2007)			
Wild boar	456	456	456
Pork	322	456	410

to differ the genetic differences among pork and wild boar within the cyt *b* gene at the restriction sites of the restriction endonuclease. More restriction endonucleases are tested more mutation regions could be identified.

We conducted further experiment using the cyt *b* primers described by Monteil-Sosa et al. (2000) which produced amplified fragment of 531 bp in size (Figure 2). They reported that those primers were highly species-specific for pork and wild boar which are phylogenetically close subspecies. Those primers were also reported to produce amplified fragment of a very similar size in wild boar and pork. Similar finding has also been observed by these primers which wild boar and pork meat produced amplified fragments of 531 bp in size (Figure 2). Further analysis using PCR-RFLP, pork meat was distinguished by two restriction endonucleases of *Alu*I (415 bp) and *Bsa*JI (470 bp) (Table 2). However, both enzymes produced one band with the smallest bands were not able to detect. The endonuclease of *Hind*III was not able to digest both of wild boar and pork meats DNA amplified fragments. The present finding showed the genetic differences of both wild boar and pork showed the differences in its point mutation at the cyt *b* gene using different enzymes. For example, in pork meat the restriction endonuclease of *Alu*I, produced a single band with molecular size of 415 bp. The *Bsa*JI endonuclease produced a single band with the molecular size of 470 bp. Only high molecular band in size can be observed while the smallest bands were not be able to detect on the agarose gel which suggested the used of capillary electrophoresis (CE) in future work due to its better resolution rather than the agarose gel. Our findings are in agreement as described by Chandrika et al. (2009) who reported the smallest bands resulted from the restriction endonualeases were not observed on the agarose gel.

The use of oligoprimers of mitochondrial 12S rRNA vertebrate-specific (Pandey et al. 2007) was studied in this work. Through PCR amplification, those primes produced the DNA fragments of 456 bp in size for both wild boar and pork meat. The wild boar meats were undigested using all restriction endonucleases (Table 2). Whereas, in pork the PCR amplification products was digested with *Alu*I and *Bsa*JI restriction endonucleases in which they produced 322 bp and 410 bp, respectively. Similar observation as above, the PCR-RFLP analysis only detected the high molecular band while the smallest bands were not detected on the agarose gel.

In the present study, in all experiments conducted the wild boar meat (*Sus scrofa*) showed high degree of its cyt *b* conserve region rather than the pork meat. This showed that all enzymes used was not be able to digest the wild boar PCR amplification products (Table 2). This finding showed that the level of point mutation between the two species was difference within the cyt *b* gene.

In conclusion, this study showed that the PCR-RFLP analysis is an effective tool in differentiation of pork and wild boar meat. The limitation of the method may be due to the limited enzymes used and it could be over come by increasing the number of enzymes used. Thus, more restriction sites could be identified.

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