

## Comparison of DNA Profiling between Fishes and Pork Meat using Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) Analysis

(Perbandingan Profil DNA antara Ikan dan Daging Babi menggunakan Analisis Tindak Balas Rangkaian Polimerase-Polimorfisme Panjang Cebisan Pematangan (PCR-RFLP))

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### ABSTRACT

*Genomic DNA of 13 fish (n=13) species consist of four freshwater which were catfish (Clarias gariepinus), shark catfish (Pangasius larnaudii), tilapia (Oreochromis mossambicus), perch (Lates calcarifer) and nine marine species which were black pomfret (Parastromateus niger), anchovy (Stolephorus commersonii), mabong (Rastrelliger kanagurta), red snapper (Lutjanus erythropterus), herring (Chirocentrus dorab), ray fish (Himantura gerrardii), sardine (Decapterus macrosoma), mackerel (Euthynnus affinis) and tuna (Thunnus tuna) were differentiated using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Seven endonucleases of AluI, BsaII, HaeIII, HindIII, HinfI, MboI and MboII were examined for the ability to digest cyt b amplicon from each species. Genomic DNA of pork (Sus scrofa domestica) were differentiated from fishes by comparing the digestion patterns produced by similar amplified region and enzymes used. In the present study, it was demonstrated that fishes and pork DNA genome were successfully differentiated using all endonucleases except for HindIII. Thus, PCR-RFLP analysis was found useful for future pork DNA detection in fish products.*

*Keywords: DNA profiling; fish; PCR-RFLP; pork (Sus scrofa domestica)*

### ABSTRAK

*DNA genomik 13 spesies ikan (n=13) terdiri daripada empat ikan air tawar iaitu keli (Clarias gariepinus), patin (Pangasius larnaudii), tilapia (Oreochromis mossambicus), siakap (Lates calcarifer) dan sembilan ikan air masin iaitu bawal hitam (Parastromateus niger), ikan bilis (Stolephorus commersonii), mabong (Rastrelliger kanagurta), ikan merah (Lutjanus erythropterus), ikan parang (Chirocentrus dorab), pari (Himantura gerrardii), sardin (Decapterus macrosoma), tenggiri (Euthynnus affinis) dan tuna (Thunnus tuna) telah dibezakan menggunakan tindak balas rangkaian polimerase-polimorfisme panjang cebisan pematangan (PCR-RFLP). Tujuh endonuklease iaitu AluI, BsaII, HaeIII, HindIII, HinfI, MboI dan MboII telah dinilai kebolehan mereka bagi mencernakan amplicon cyt b daripada setiap spesies. DNA genomik babi (Sus scrofa domestica) telah dibezakan daripada ikan dengan membandingkan corak pencernaan yang terhasil ke atas kawasan amplifikasi yang serupa menggunakan enzim yang digunakan. Dalam kajian ini, DNA genomik ikan dan babi telah berjaya dibezakan oleh kesemua endonuklease kecuali enzim HindIII.*

*Kata kunci: Babi (Sus scrofa domestica); ikan; PCR-RFLP; pemprofilan DNA*

### INTRODUCTION

The interests of *Halal* concept keep expanding over the world especially in Muslim majority countries. One of the common issues in *Halal* industry is *Halal* meat authentication. Authenticity, such as adulteration, improper description of the products and origin designation (Monin 1998) is an important aspect in meat quality evaluation. Due to health concern and awareness of fish meat nutrition, there are increasing trends in fish consumption nowadays. Fish consumption may lead to allergy in some group of people such as diet control for women in confinement, other than religious constriction. Thus, meat comparison between species is important as a fresh whole-fish can be easily identified by consumers, but identification has been a big issue after its transformation into processed products. Fish-based products like fish ball, fish fillet and ready-

to-eat canned product has been widely commercialized nowadays. There are potential adulterations of fish products such as surimi were added with pork gelatin to improve its texture and juiciness of the product. Both fish and pork gelatin are impossible to be differentiated by naked eyes especially when the product had undergone heat treatment (Koh et al. 1998).

Polymerase Chain Reaction (PCR) technique was reported very precise to detect meat adulteration in meat species identification for routine analysis (Matsunaga et al. 2001). The use of seven restriction enzyme endonucleases in this PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis were expected to generate a unique genetic profiling for each meat species, based on the size and number of DNA fragments being produced after the enzymatic digestion of *cyt b* amplicons. The PCR-RFLP

analysis is useful for the detection of inter-species as well as intra-species genetic variations. Thus, the objective of this study was to compare the fish and pork DNA based on enzyme restriction patterns produced by the PCR-RFLP analysis.

## MATERIALS AND METHODS

### SAMPLE PREPARATION

Fresh samples of 13 fish species were chosen consist of freshwater and marine types. Four freshwater species namely catfish (*Clarias gariepinus*), shark catfish (*Pangasius larnaudii*), tilapia (*Oreochromis mossambicus*), perch (*Lates calcarifer*) and ten marine species which were black pomfret (*Parastromateus niger*), anchovy (*Stolephorus commersonii*), mabong (*Rastrelliger kanagurta*), red snapper (*Lutjanus erythropterus*), herring (*Chirocentrus dorab*), ray fish (*Himantura gerrardii*), sardine (*Decapterus macrosoma*), mackerel (*Euthynnus affinis*) and tuna (*Thunnus tuna*) were purchased from Carrefour supermarket, Alamanda, Putrajaya from March to December 2010. While, pork (*Sus scrofa domestica*) was purchased from a wet market in Seksyen 13, Petaling Jaya, Selangor in March 2009. All samples were kept frozen at -20°C before DNA extraction process to prevent enzymatic degradation.

### EXTRACTION OF TOTAL DNA

DNA of fish meats and pork samples were extracted using DNA Extraction Kit (DNeasy Blood and Tissue Kit Qiagen, USA). A total of 25 mg meat from each sample was minced and placed in a sterile 1.5 mL microcentrifuge. The extracted DNA were measured using ultra-violet (UV) spectrophotometer (Maestro-Nano, MY) at 260/280 nm wavelength (Ong et al. 2007).

### OLIGONUCLEOTIDE PRIMERS

The primers used were targeting the universal mitochondrial *cyt b* namely CYT b1: 5'-CCATCCAACATCTCAGCATGATGAAA-3' (forward) and CYT b2: 5'-GCCCTCAGAATGATATTTGTCCTCA-3' (reverse) as described by Kocher et al. (1989). The primers were supplied by First Base Laboratories (Selangor, MY).

### PCR AMPLIFICATION

PCR was done using PCR thermal cycler (Eppendorf, GY) to amplify mitochondrial *cyt b* region. PCR amplification was performed in 50 µL total reaction mixture consists of genomic DNA in 10 µL water (25-30 ng), 25 µL of PCR Dream Taq® master mix, 1 µL of forward primer (100 µM), 1 µL of reverse primer (100 µM) and 13 µL nuclease-free water. Positive control (pork DNA) and negative control were included in each PCR cycle.

The PCR step-cycle program used as described by Kocher et al. (1989): Early denaturation at 94°C for 2

min, followed by 35 cycle of denaturation at 94°C for 15 s, annealing at 50°C for 1 min and elongation at 72°C for 1 min. The final extension was at 72°C for 2 min to produce a complete PCR product. The amplification products were analyzed using 2% (w/v) agarose gel in 1 X TAE buffer at 100 V for 40 min. Nucleic acid staining was done using ethidium bromide solution. A 100 bp DNA ladder (Vivantis, MY) was used as size marker.

### ENZYMATIC DIGESTION OF AMPLIFIED DNA

A total of 10 µL amplicon, 10 µL enzyme reaction buffer (10X), 1 µL restriction endonuclease (RE) (10 u/µL) (Vivantis, MY) and 9 µL nuclease-free water were mixed to give a final volume of 30 µL in each reaction. The mixture was then incubated at 37°C to 55°C, depending on the type of enzyme used. The incubation period was set for 3 h. This step was applied to all samples using restriction enzyme *AluI*, *BsaI*, *HaeIII*, *HindIII*, *HinfI*, *MboI* and *MboII*.

The fragments obtained after digestion of amplification products by restriction enzyme were analysed using agarose gel electrophoresis 3% (w/v) to estimate the size of each fragment produced. Gel was stained using ethidium bromide (EtBr) before viewed under a UV-transluminator imager (Alpha Innotech, USA). Sizes of the amplicons were estimated from the DNA marker. A table based on the size (bp) of bands appeared on the gel was tabulated to differentiate between samples (Table 1).

## RESULTS

### PCR AMPLIFICATION USING MITOCHONDRIAL *CYT B* OLIGOPRIMERS

Genomic DNA 13 species of raw fishes and pork (*Sus scrofa domestica*) were successfully extracted using extraction kit (DNeasy® Blood and Tissue Kit Qiagen, USA). PCR assay was performed using the same universal mitochondrial *CYTb* primers (Kocher et al. 1989) on both fish and pork samples. Gel electrophoresis on 2% (w/v) agarose for fish amplicon produced 359 bp in size except for tilapia (*Oreochromis mossambicus*) and anchovy (*Stolephorus commersonii*) (Figure 1). None amplicons were detected for the said fish samples though the experiment was repeated three times using the same universal primers (Primers *CYTb1/CYTb2*). While, amplification of pork DNA using the same universal primer (*CYTb1/CYTb2*) produced similar amplicon size of 359 bp (Figure 2).

### PCR-RFLP ANALYSIS

The patterns produced by each type of enzyme were recorded to tabulate a unique DNA profiles for each fish and pork, after enzymatic digestion by seven restriction endonucleases. Molecular weights of DNA fragments for 12 samples (fishes and pork) produced after enzymatic digestion using the seven restriction enzymes were listed in Table 1. Every restriction pattern produced by amplicon

TABLE 1. *Cyt b* DNA amplicon sizes of samples after digestion by restriction endonucleases

Types of samples	Size of DNA Band (bp)						
	<i>AluI</i>	<i>BsaII</i>	<i>HaeIII</i>	<i>HindIII</i>	<i>HinfI</i>	<i>MboI</i>	<i>MboII</i>
Pig ( <i>Sus scrofa domestica</i> )	*(1) 115, 244	*(2) 131, 228	*(3) 125, 134	*(4) 359	*(4) 359	*(1) 115, 244	*(4) 359
Catfish ( <i>Clarias gariepinus</i> )	*(5) 170, 130, 59	*(6) 225, 134	*(7) 229, 130	*(4) 359	*(8) 200, 159	*(4) 359	*(8) 200, 159
Shark catfish ( <i>Pangasius larnaudii</i> )	*(5) 170, 130, 59	*(4) 359	*(4) 359	*(4) 359	*(8) 200, 159	*(9) 300	*(4) 359
Tilapia ( <i>Oreochromis mossambicus</i> )	-	-	-	-	-	-	-
Perch ( <i>Lates calcarifer</i> )	*(10) 130, 59	*(4) 359	*(9) 300	*(4) 359	*(12) 270, 89	*(4) 359	*(14) 175, 130, 64
Black pomfret ( <i>Parastromateus niger</i> )	*(10) 130, 59	*(4) 359	*(11) 200, 120	*(4) 359	*(13) 149, 105, 105	*(4) 359	*(15) 170, 120, 79

Types of samples	Size of DNA Band (bp)						
	<i>AluI</i>	<i>BsaII</i>	<i>HaeIII</i>	<i>HindIII</i>	<i>HinfI</i>	<i>MboI</i>	<i>MboII</i>
Anchovy ( <i>Stolephorus commersonii</i> )	-	-	-	-	-	-	-
Mabong ( <i>Rastrelliger kanagurta</i> )	*(10) 130, 59	*(4) 359	*(16) 239, 120	*(4) 359	*(17) 200, 100	*(18) 250, 109	*(19) 200, 90
Red snapper ( <i>Lutjanus erythropterus</i> )	*(5) 170, 130, 59	-	*(11) 200, 120	*(4) 359	*(22) 220	*(21) 179, 180	*(20) 170, 130
Herring ( <i>Chirocentrus dorab</i> )	*(10) 130, 59	*(23) 249, 110	*(4) 359	*(4) 359	*(24) 149, 110, 100	*(9) 300	*(15) 170, 120, 79
Ray fish ( <i>Himantura gerrardii</i> )	*(10) 130, 59	*(6) 225, 134	*(11) 200, 120	*(4) 359	*(26) 170, 120	*(4) 359	*(30) 160, 110
Sardine ( <i>Decapterus macrosoma</i> )	*(10) 130, 59	*(4) 359	*(16) 239, 120	*(4) 359	*(8) 200, 159	*(4) 359	*(29) 160, 120
Mackerel ( <i>Euthynnus affinis</i> )	*(10) 130, 59	*(4) 359	*(11) 200, 120	*(4) 359	*(27) 259, 100	*(4) 359	*(31) 280, 79
Tuna ( <i>Thunnus tuna</i> )	*(5) 170, 130, 59	*(32) 210	*(25) 150, 100	*(4) 359	*(28) 200	*(4) 359	*(30) 160, 110

\*(Profile number based on digestion pattern)

of fish species were compared to the restriction pattern of the pork amplicon.

Digestion of *AluI* towards amplicons of 11 fish samples has produced two digestion patterns. The first pattern resulted in three DNA fragments after digestion; 170 bp, 130 bp and 59 bp for catfish, shark catfish, red snapper and tuna. While the second pattern only two fragments produced; 130 bp and 59 bp for perch, black pomfret, mabong, herring, ray fish, sardine and mackerel. The use of restriction enzyme *BsaII* produced five digestion patterns towards the fish

sample amplicons. A 225 bp and a 134 bp fragments were produced from amplicon of catfish and rayfish while, no digestion was observed towards amplicon of shark catfish, perch, black pomfret, mabong, sardine and mackerel. None of DNA fragments were observed for red snapper amplicon. Digestion of herring gives two fragments of 249 bp and 110 bp while, only one fragment was observed from tuna amplicon after digestion by *BsaII*.

The third restriction enzyme used was *HaeIII* with six digestion patterns observed. This enzyme did not digest

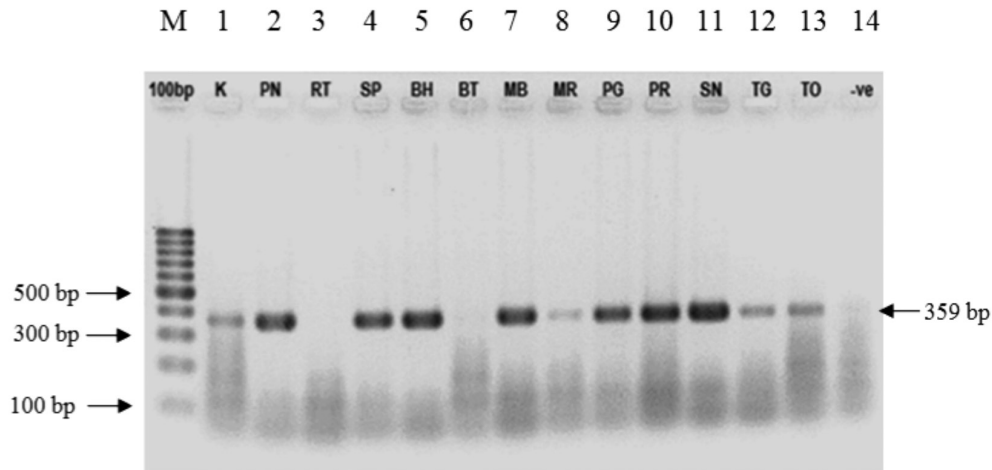


FIGURE 1. The PCR amplification product of oligonucleotide primers of CYTb1/CYTb2 for 14 species of raw fish. Lane M: 100 bp DNA ladder; 1: Catfish (*Clarias gariepinus*); 2: Shark catfish (*Pangasius larnaudii*); 3: Tilapia (*Oreochromis mossambicus*); 4: Perch (*Lates calcarifer*); 5: Black pomfret (*Parastromateus niger*); 6: Anchovy (*Stolephorus commersonii*); 7: Mabong (*Rastrelliger kanagurta*); 8: Red snapper (*Lutjanus erythropterus*); 9: Herring (*Chirocentrus dorab*); 10: Ray fish (*Himantura gerrardii*); 11: Sardine (*Decapterus macrosoma*); 12: Mackerel (*Euthynnus affinis*); 13: Tuna (*Thunnus tuna*); 14: Negative control (Nuclease-free water)

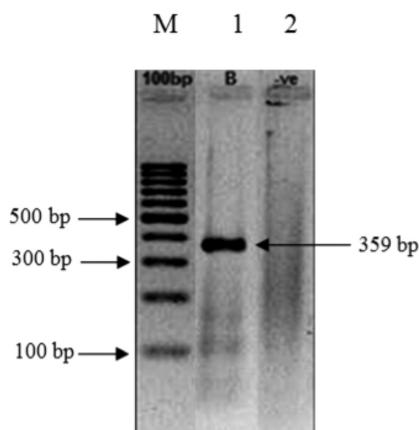


FIGURE 2. The PCR amplification product of oligonucleotide primers of CYTb1/CYTb2 for *Sus scrofa domestica*. Lane M: 100 bp DNA Ladder; Lane 1: *Sus scrofa domestica*; 2: Negative control (Nuclease-free water)

amplicon of shark catfish and herring. It digested catfish amplicon into two fragments of 229 bp and 130 bp while, only one fragment of 300 bp was observed after digestion of perch amplicon. On the other hand, digestion by *HaeIII* restriction enzyme towards amplicon of black pomfret, red snapper, ray fish and mackerel has produced two fragments of 200 bp and 120 bp. Mabong and sardine amplicon were also digested into two fragments but of different sizes which were 239 bp and 120 bp. Tuna amplicon were digested into two other fragments of 150 and 100 by *HaeIII*. All DNA amplicon bands showed no digestion by restriction enzyme *HindIII*.

Nine digestion patterns were observed after amplicon digestion by *HinfI*. This enzyme produced the same digestion patterns toward catfish, shark catfish and sardine

with 200 bp and 159 bp DNA fragments. Perch amplicon was digested into 270 bp and 89 bp fragments while black pomfret amplicon was also digested into 149 bp and two fragments of 105 bp. Mabong, ray fish and mackerel amplicon were digested into two fragments which are 200 bp and 100 bp, 170 bp and 120 bp and 259 bp and 100 bp fragments. For red snapper and tuna, only one fragment of 220 bp and 200 bp was detected after digestion by *HinfI*, respectively. Meanwhile, herring amplicon was digested into three fragments of 149 bp, 110 bp and 100 bp.

The sixth enzyme used in this study was restriction enzyme *MboI*. There were four digestion patterns identified after its enzymatic digestion towards PCR products. No digestion by *MboI* was detected towards catfish, perch, black pomfret, ray fish, sardine and mackerel amplicons. Digestion towards shark catfish and herring amplicon produced only one fragment of 300 bp. *MboI* digested amplicon of mabong into 250 bp and 109 bp while, amplicon of red snapper were digested into 179 bp and 180 bp.

The last endonuclease used in the study was *MboII*. There were nine digestion patterns produced after digestion of sample amplicons by *MboII*. Two fragments of 200 bp and 159 bp were produced after digestion of catfish amplicons by *MboII* but this enzyme did not digest shark catfish amplicon. Meanwhile, three fragments with different sizes were identified after digestion of perch, black pomfret and herring amplicons. Perch amplicon was digested into 175 bp, 130 bp and 64 bp fragments. Amplicon of black pomfret and herring were digested into the same fragments of 170 bp, 120 bp and 79 bp. The other fish sample amplicons were digested into two fragments of different sizes. Amplicon of mabong was digested into 200 bp and 90 bp fragments while red snapper was digested into 170 bp and 130 bp fragments. Ray fish and tuna amplicon



were digested into same fragments of 160 bp and 110 bp. Amplicon of sardine was digested into 160 bp and 120 bp fragments while, digestion of mackerel amplicon by *Mbo*II produced two fragments of 280 bp and 79 bp in molecular weight.

Comparison of fish PCR product digestion patterns to pork PCR product digestion pattern was shown in Figure 3. After digestion with all enzymes, amplicon restriction of *Alu*I and *Mbo*I produced the same two DNA fragments of 115 and 244 bp molecular sizes from the pork amplicon. *Bsa*JI also produced two DNA fragments but with different molecular sizes which were 131 and 228 bp. Enzymatic digestion process towards *cyt* b amplicon of pork using *Hae*III endonuclease produced three DNA fragments with molecular weight of 125 bp and 134 bp. Meanwhile, the use of *Hin*fI, *Hin*dIII and *Mbo*II showed no restriction reaction.

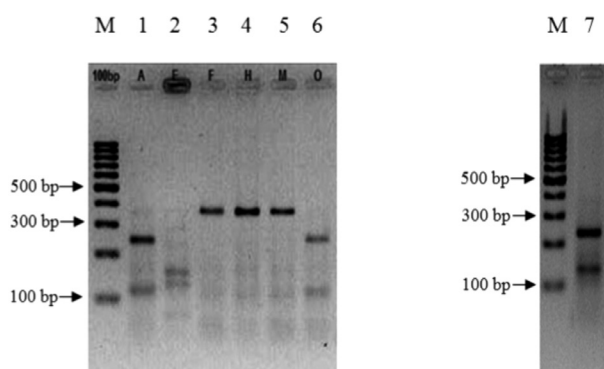


FIGURE 3. Pork (*Sus scrofa domestica*) DNA bands after digestion using seven restriction enzymes (RE). Lane M: 100 bp DNA Ladder; 1: RE *Alu*I; 2: RE *Hae*III; 3: RE *Hin*fI; 4: RE *Hin*dIII; 5: RE *Mbo*II; 6: RE *Mbo*I; 7: RE *Bsa*JI

#### DISCUSSION AND CONCLUSION

In this study, universal *cyt* b primer CYTb1/CYTb2 (Kocher et al. 1989) was used to amplify *cyt* b mitochondrial DNA from all 13 fish species and pork due to mitochondria DNA endurance to degradation at high temperature and extreme processing condition. Partis et al. (2000) also reported that PCR analysis using *cyt* b as primer got higher sensitivity compared to nuclear DNA, due to the number of *cyt* b locus copies exist in each cell. Discriminatory ability of the selected primer was also crucial for identification purposes (Sahilah et al. 2010). This primer targeted the 359 bp sequence of *cyt* b to be amplified. The size of amplification products obtained for each sample was 359 bp except for tilapia (*Oreochromis mossambicus*) and anchovy (*Stolephorus commersonii*) samples. The reason no amplicon was detected for both fish samples were probably due to no specific site on the genomic DNA for the annealing of the *cyt* b primer. No specific site on both fishes were cause by sequence variation of *cyt* b region binding site in different fish species as reported by Ivanova et al. (2007). In their report, a mixture of several complicated

primers usually is required to ensure the annealing process occurs.

In PCR-RFLP, the unique sizes of fragments produced were due to the variation of restriction sites detected by the endonucleases on the gene sequences by species. Every species may have their own specific sequences even for the same region. Molecular weights for each fragment is differ from each other, allowed them to be compared between species. Each restriction endonuclease has the ability to identify a specific site based on DNA base recognition (Perbal 1988). All PCR products showed no digestion towards *cyt* b amplicon by *Hin*dIII may be due to the absence of the targeted nucleotide sequence. This observation was similar as reported by Murugaiah et al. (2009) which *Hin*dIII could not be used to differentiate between pork, beef, buffalo, chicken, mutton, puyuh and rabbit samples. Thus, *Hin*dIII is not advisable to be used in meat differentiation in future.

In the present study, the restriction of fish DNA genome showed variation of lengths produced after the enzymatic digestion, since different species consist of different genome sequences as in many genome sequencing results (Salih et al. 2017; Yoon et al. 2017). The results indicated in Table 1 supports this statement, both in genomic fishes and pork DNA patterns. These findings were in agreement in the previous studies by Ong et al. (2007) and Sahilah et al. (2012) who reported the ability of *Alu*I and *Bsa*JI to digest *cyt* b DNA amplicon of pork as well as fish in this study. In this study, analysis of the endonuclease restriction patterns from fish and pork samples tabulated in Table 1 indicates the differentiation of fishes and pork DNA genome with different fragments size produced. It showed a specific fragment patterns for both DNA genome. Thus, PCR-RFLP analysis is useful for future pork DNA detection in fish products.

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