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SPECIES DIFFERENTIATION OF FISH SAMPLES BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF CYTOCHROME *B* GENE

[1]Haryani Y, [2]Tunung R, [3]Zuraini M I, and [2]Son R

[1] Department of Chemistry, Faculty of Mathematics and Sciences, The University of Riau, Pekanbaru, Riau, Sumatera, Indonesia

[2]National Food Safety Research Centre, Faculty of Food Science and Technology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[3]Faculty of Hotel and Tourism Management, Universiti Teknologi MARA, Kampus Dungun, Kompleks Dura, 23000 Dungun, Terengganu, Malaysia

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ABSTRAK

Metode pengukuran polimorfisme fragmen hasil pemotongan produk reaksi polimorfik berantai oleh enzim restriksi spesifik (polymerase chain reaction-restriction fragment length polymorphism, RFLP-PCR) telah digunakan untuk membedakan beberapa jenis ikan mentah. Situs cytochrome b mitokondria, yang diamplifikasi oleh primer universal, dipotong menggunakan empat enzim restriksi (*Bfa* I, *Hinf* I, *Msp* I, *Mbo* II) sehingga dapat dianalisa fragment-fragment pendeknya. Hasil yang diperolah dari pemotongan oleh enzim restriksi tersebut ternyata dapat digunakan untuk membedakan tiap jenis ikan sampel. Hasil penelitian ini menunjukkan bahwa PCR dan RFLP-PCR merupakan metode yang sensitif dan dapat dilakukan dalam waktu singkat untuk membedakan berbagai jenis ikan mentah.

Key Word* :Polimorfisme fragmen, cytocrome b gene*

1.INTRODUCTION

Mitochondrial DNA, or sometimes abbreviated as mtDNA, was discovered in the 1960s, initially through electron micrographs that revealed DNA-like fibers within the mitochondria. The size of mitochondrial DNA molecules varies enormously, from about 16 to 17 kb in vertebrate animals to 2500 kb in some of the flowering plants. Each mitochondrion appears to contain several copies of the DNA. Each cell usually has many mitochondria; therefore the number of mtDNA per cell can be very large. Thus, mtDNA is the genetic system of choice in cases where tissue samples are very old, very small, or badly degraded by heat and humidity (Bravi *et al*, 2004).

The occurrence of conserved regions, for example cytochrome *b* gene, is the prerequisite for the high sensitivity of Polymerase Chain Reaction (PCR). The mitochondrial

cytochrome *b* gene has been used to study the evolution and phylogenetic relationships of many animals (Ferreira *et al.,* 2005; Hsieh *et al.* 2007). The cytochrome b gene located in the mitochondrial genome has been found to be a powerful marker for identification of species using DNA analytical techniques. The amino acid sequence of cytochrome b is a highly conserved region, but due to the degeneracy of the genetic code, the cytochrome b genes differ by at least a few nucleotides, even in very closely related species (Kocher *et al.*, 1989). The mitochondrial cytochrome b gene has been used in phylogenetic as well as in forensic investigations and has been shown in a variety of studies to be a very useful DNA-region for species determination (Pfeiffer *et al.,* 2004).

Usually PCR is coupled with other techniques able to detect differences in the sequence of the products obtained by PCR amplification. So far, restriction fragment length polymorphism (RFLP) has been one of the techniques most frequently used for this purpose (Cocolin *et al.*, 2000). RFLP is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species from one another.

The main objective of this study is to develop a method for differentiation of fish species. Restriction site analysis of Polymerase Chain Reaction (PCR) products of cytochrome b (cyt *b*) mitochondrial DNA was applied to differentiate seven species of fish. Base on the standard data established, determination of the species origin of processed food samples could be obtained for future work.

2. MATERIALS AND METHODS

2.1. Samples collection and preparation

Different species of fish samples were purchased from Pasar Borong Selangor, Malaysia, and collected on an ice box for transportation. Research was done on microbiology laboratory of National Food Safety Research Centre, Universiti Putra Malaysia during the period of June 2005 to January 2006. The seven types of fishes were *Parastromateus niger* (“bawal hitam”), *Pampus argenteus* (“bawal putih”), *Rastrelliger kanagurta* (“kembung”), *Trachinotus baillonii* (“selar”), *Polydactylus plebeius* (“senangin”), *Nemipterus bipunctatus* (“kerisi”), *Megalaspis cordyla* (“cencaru”).

2.2. DNA extraction

Samples were cut to an average weight of 25 mg using sterile instruments under a laminar flow hood, placed in sterile 1.5 ml

micro centrifuge tube and labeled as F1 to F7. DNA was extracted from the meat samples using the DNeasy Protocol for Animal tissue provided with the DNeasy^{TM} Tissue Kit (Qiagen).

2.3. Polymerase Chain Reaction (PCR)

A pair of universal primers (cyt *b*1, 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3' and cyt *b*2, 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3') was employed in the PCR reaction for amplification of the cytochrome b gene region (Kocher *et al.* 1989). Reactants for amplification were combined in 0.2 ml polypropylene tubes to give a final volume of 25 µl and final concentration of: 1x PCR reaction buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$ (Finnzymes), 200 µM dNTP mix (Finnzymes), 10 pmol of each primer, 100-150 ng template DNA and 1.25 unit *Taq* DNA polymerase (Finnzymes).

The PCR was performed in a thermocycler Perkin Elmer (GeneAmp PCR system 2400), using the following conditions: a single initial denaturation at 94ºC for 2 min followed by 40 cycles of 94ºC for 5 s (denaturation), 52ºC for 30 s (annealing), 72ºC for 40 s (primer extension) and a final extension step at 72ºC for 2 min. Negative controls (water) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

2.4. Restriction Enzyme Digestion

Four types of restriction enzymes (*Bfa* I, *Hinf* I, *Msp* I, *Mbo* II) were chosen to cut the PCR products of mitochondrial cytochrome b gene. Digestions with 4 types of restriction enzymes were performed in a total volume of 20 µl containing 10 µl of amplified DNA, 5 U of enzyme and 1× digestion buffer (10 mM Tris-HCI, 50-100 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol). The digestion mixture was incubated for 16 hours at the specific incubation temperature according to the restriction enzymes used.

The digested samples were electrophoresed through 1.2% agarose gel in 1X Tris-Borate-EDTA buffer at 80V until the markers migrated to about two-thirds of the gel. The sizes of the bands produced were compared with the 100 bp ladder (Fermentas). The gels were stained with 0.5 µg/ml ethidium bromide for 5 minutes and destained for 10 minutes. The destained gels were viewed with Gel Doc 2000 (Bio-Rad), and printed using a Sony digital graphic printer (UP-D890).

3. RESULTS AND DISCUSSION

There have several protein analysis methods reported for identification of fish species, including electrophoresis (Etienne *et al*., 2001), isoelectric focusing (Chen *et al*., 2003), liquid chromatography (Knuutinen and Harjula, 1998), immunoassays (Carrera *et al*., 1996), and molecular biology techniques

(Cespedes *et al*., 1998; Hall and Nawrocki, 1995). However, the above protein analysis methods are often hard to conduct because proteins were easily denatured by heat-treated. Therefore, there have more and more DNA analysis methods reported for identification of fish species. DNA technique is more promising and reliable than protein technique, and is well suited to routine surveys due to robustness and easy application (Cocolin, D'Agaro, Manzano, Lanari, & Comi, 2000).

In our research, DNA was isolated from different species of fish sold for human consumption using DNeasy™ Tissue Kit (Qiagen) for animal tissue. DNA was successfully extracted from five out of seven fishes, namely *Pampus argenteus*, *Rastrelliger kanagurta*, *Trachinotus baillonii*, *Nemipterus bipunctatus*, and *Megalaspis cordyla* (Figure 1), and then used for PCR amplification of the mitochondrial cytochrome b region

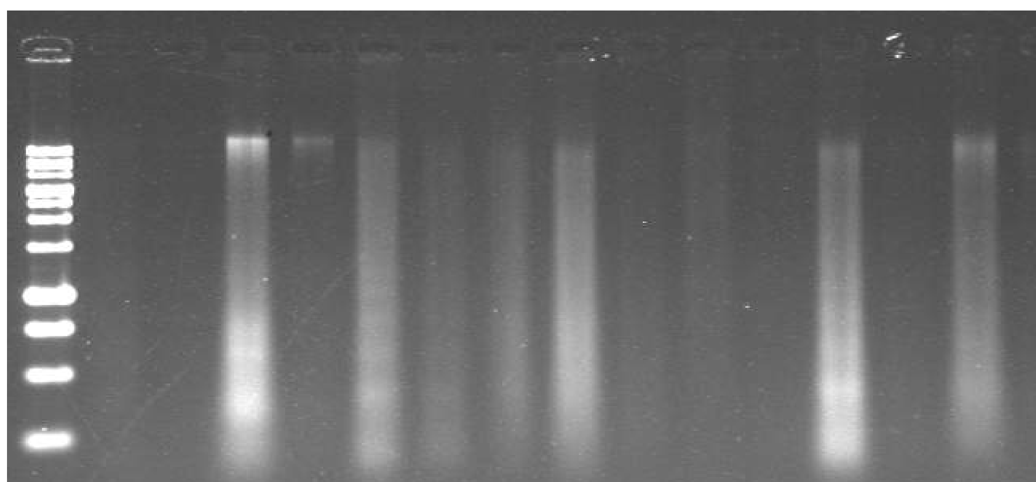

Figure 1. Agarose gel electrophoresis showing the extracted genomic DNA from all samples. Lane M: 1kb molecular marker; 1-7: Fish1 – Fish7; a: primary dna solution; b: secondary dna solution(1. *Parastromateus niger*; 2. *Pampus argenteus*; 3. *Rastrelliger kanagurta*;4. *Trachinotus baillonii*; 5. *Polydactylus plebeius*; 6. *Nemipterus bipunctatus*; 7. *Megalaspis cordyla*;).

The cytochrome b loci are extensively used in intra and inter specific molecular systematic studies. As each cell may contain up to 1000 copies of the cytochrome b locus, PCR assays based on its

amplification should offer the advantage of increase sensitivity in comparison to single or low copy nuclear DNA targets (Partis *et al.* 2000). Abdulmawjood and Bulte (2002) reported that primers cyt *b1* and cyt *b2* were designed by Kocher *et al.* (1989) for the amplification of a conserved region of the cytochrome b gene.

These universal primers consistently amplified a fragment of 359 bp of the cytochrome b gene. An advantage in using universal primers is that it obviates the requirement for an internal control, which is otherwise used to monitor the success of DNA amplification (Partis *et al.*, 2000). From all investigated samples, the 359 bp long PCR product was amplified and no unspecific PCR amplicons were detected (Figure 2). The successful amplification of amplicons had proven that PCR is a highly sensitive method.

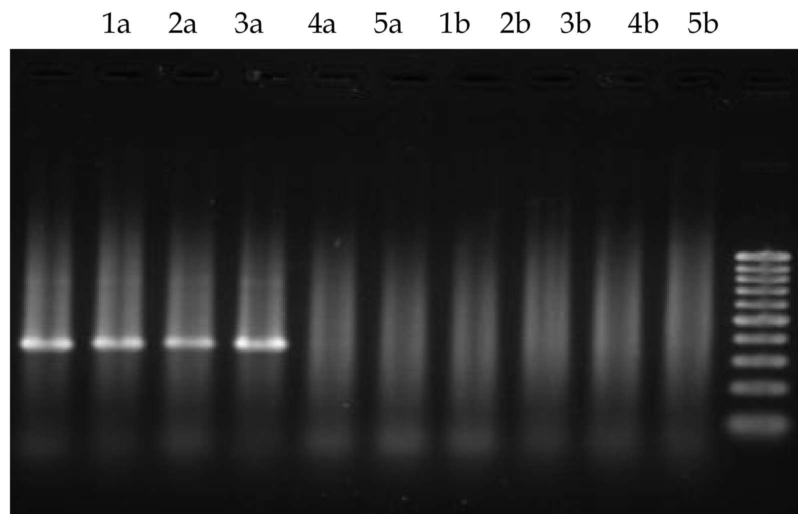

Figure 2: Agarose gel electrophoresis showing PCR products of five different fishes.Lane M: 100 bb molecular marker; 1. *Pampus argenteus*; 2. *Rastrelliger kanagurta*; 3. *Trachinotus baillonii*; 4. *Nemipterus bipunctatus*; 5. *Megalaspis cordyla* (a: primary dna solution; b: secondary dna solution).

DNA fingerprinting is a technique that is used to identify patterns that occur in DNA. No two organisms have identical DNA so this procedure can be used to identify if a sample of DNA came from a particular individual. This technique requires that the DNA be cut up into small fragments. In RFLP analysis, the DNA of an organism is cut up into fragments using restriction enzymes and a large number of short fragments of DNA will be produced. Restriction enzymes (RE) for RFLP analysis were selected by the following criteria: they had to be reasonably active in the PCR-mix.

In this investigation, four REs (*Bfa* I, *Hinf* I, *Msp* I, *Mbo* II) were used to perform the digestion of PCR product and generated different sizes of DNA fragments which are unique to the fish types (Figure 3 and 4). All of the samples were successfully digested with *Mbo* II, but none with *Bfa* I. *Hinf* I produced two bands of 150 bp and 280 bp for *Pampus argenteus*, and two bands of 190 bp and 210 bp for *Polydactylus plebeius*. Cyt *b* of *Rastrelliger kanagurta* and *Trachinotus baillonii* was digested into two fragments of 100 bp and 200 bp with *Mbo* II. Two fragments in size of 100 bp and 300 bp were also produced using *Mbo* II for *Polydactylus plebeius*. Digestion using *Msp* I was produced two bands of 100 bp and 300 bp for *Pampus argenteus* and *Trachinotus baillonii*. Unfortunately, only one band was detected after cutting by *Mbo* II (200 bp for *Pampus argenteus*), and *Hinf* I (150 bp for *Rastrelliger kanagurta*). This could be due to incomplete digestion as we can see a faint band upper those thick bands (in the area of 300 bp).

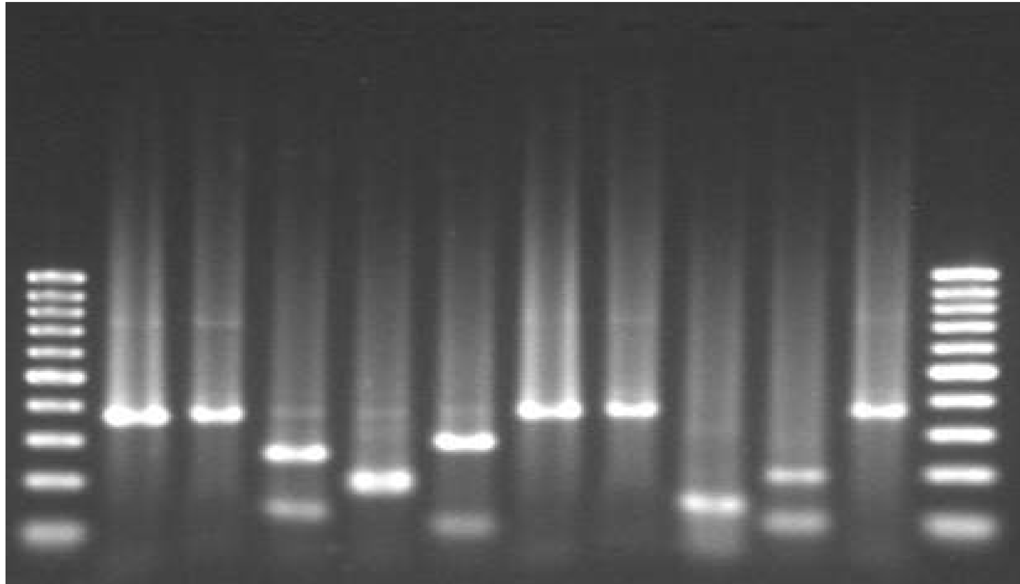

Figure 3: PCR-RFLP analysis 360 bp PCR amplicon with the four types of RE.Lane M: 100 bp molecular marker; 1. *Pampus argenteus*; 2. *Rastrelliger kanagurta* (a: undigested; b-c-d-e: digested using *Bfa* I-*Hinf* I- *Mbo* II-*Msp* I).

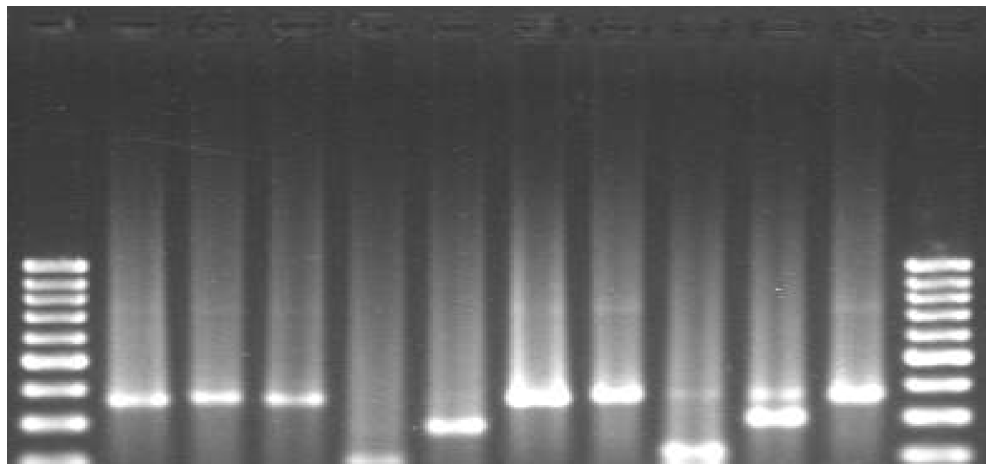

Figure 4: PCR-RFLP analysis 360 bp PCR amplicon with the four types of RE. Lane M: 100 bp molecular marker; 1. *Trachinotus baillonii*; 2. *Polydactylus plebeius* (a: undigested; b-c-d-e: digested using *Bfa* I-*Hinf* I-*Mbo* II- *Msp* I).

or some samples studied, RFLP profiles from combined results of four REs (*Bfa* I, *Hinf* I, *Msp* I, *Mbo* II) allowed differentiation of all four investigated species keeping the effort in an acceptable scope. According to Wolf *et al.* (2000), it is important to note that in most cases the use of only two or three REs will be sufficient for identification depending on the number of species among which to distinguish.

Not only for raw materials, PCR-RFLP is also suitable for processed and heated products as described earlier by Meyer *et al.* (1995) that use the example of processed meat and meat products and by Quinteiro *et al.* (1998) for canned tuna and thus will allow identification of other canned fish such as sardine or mackerel.

In our experiments, a brief PCR-RFLP protocol with a set of selected enzymes allowed us to discriminate the different fish species. This work describes a simple and promising method for differentiation of fish species, allowing detection of falsely declared fish or fish products made up of a single species. Three different restriction enzymes (*Hinf* I, *Msp* I, *Mbo* II) were found to be sufficient for identification of all investigated species and the genomic DNA was found to be suitable as PCR templates using the DNeasy Tissue Kit (Qiagen). In the future, the specific DNA fragments can even be used as species-specific probes to be used for other diagnostic applications. As PCR-RFLP is also suitable for processed and heated products (Meyer *et al.* 1995; Quinteiro *et al.,* 1998), it is possible to identify species origin of canned fish such as sardine or mackerel for future study.

4. CONCLUSION

PCR–RFLP technique could be used to distinguish different raw fish species. The specific primer cyt *b1* and cyt *b2* used in this study could successfully amplify a 359 bp of the cytochrome b gene. Furthermore, PCR amplified products and restriction digestion using the restriction enzymes *Hinf* I, *Msp* I, *Mbo* II could successfully differentiate the fishes. The RFLP patterns generated by *Hinf* I, *Msp* I, *Mbo* II for 359 bp DNA amplified fragment were easy to distinguish all raw fishes.

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