PCR-RFLP PROFILING OF SEVERAL CYPRINID FISHES

Siti Mardhiah Saharuddin

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JUDUL: PER_RFLP profing vs Several Cypripid Fitches

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Telah: 25/5/2011

(TANDATANGAN PENYELIA)

Dr Yuzaine bni Esm
Ketua Jabatan Zoologi
Fakulti Sains dan Teknologi Sumber
UNIVERSITI MALAYSIA SARAWAK
01000 KOTA SAMARAHAN

Tanggal: 26/5/2011

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PCR-REL P PROFILING OF SEVERAL CYPRINID FISHES

SITI MAR DHIAH SAHARUDDIN

This project is submitted to partial fulfillment of the requirement for the degree of Bachelor of Science with Honours (Animal Resource Science and Management)

Department of Zoology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2011
DECLARATION

I hereby declare that the thesis is based on my original work except for citation which has been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or any other institutions of higher learning.

Siti Mardhiah binti Saharuddin
Animal Resource Science and Management Programme
Department of Zoology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
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# TABLE OF CONTENTS

Acknowledgement 1  
Table of Contents II  
List of Abbreviations V  
List of Tables and Figures VI  
Abstract 1  

1.0 Introduction 2  

2.0 Literature Review 4  
2.1 Cyprinidac 4  
2.2 PCR-RFLP 5  
2.3 Molecular study related to PCR-RFLP 6  
2.4 Mitochondrial DNA 7  
2.5 Cytochrome Oxidase 1 (COI) 9  

3.0 Materials and Method 10  
3.1 Sample acquisition 10  
3.2 Laboratory work 10  
3.2.1 DNA Extraction 10  
3.2.2 Visualisation of DNA Products 11  
3.2.3 Polymerase Chain Reaction (PCR) 12  
3.2.4 Purification Process 13  
3.2.5 PCR-RFLP of mtDNA gene 14  

4.0 Results 15  
4.1 DNA Extraction of Tissue Samples 16  
4.2 Polymerase Chain Reaction (PCR) 17  
4.3 RFLP profiling 17  
4.4 Sequencing Data Analysis 20
5.0 Discussion  23
5.1 DNA Extraction  23
5.2 Polymerase Chain Reaction (PCR)  24
5.3 Purification  25
5.4 RFLP profiling  26

6.1 Conclusion and Recommendation  29

REFERENCES  33

APPENDIX  36
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAB</td>
<td>Cetyl-trimethyl Ammonium Bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>MgCl</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>Polymerase Chain Reaction-Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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<td>mM</td>
<td>Millimeter</td>
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<td>V</td>
<td>Volume</td>
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<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>The master mix for PCR reaction mixture</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Amplification cycle for PCR process</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Primer for partial cytochrome oxidase 1 (COI) gene sequences</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>The master mix for PCR-RFLP reaction mixture</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>The restriction enzymes digesting specificity</td>
</tr>
<tr>
<td>Table 4.1</td>
<td><em>In silico</em> restriction analysis of <em>H. macrolepidota, B. schwangenfeldii</em> and <em>T. douronensis</em></td>
</tr>
<tr>
<td>Table 4.2</td>
<td><em>In silico</em> restriction analysis</td>
</tr>
<tr>
<td>Table 4.3</td>
<td><em>In silico</em> restriction analysis</td>
</tr>
<tr>
<td>Table 4.4</td>
<td><em>In silico</em> restriction analysis</td>
</tr>
<tr>
<td>Table 4.5</td>
<td><em>In silico</em> restriction analysis</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Gel electrophoresis of DNA extraction products using three species of Cyprinids.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Gel electrophoresis of PCR products.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Gel electrophoresis of PCR-RFLP products by using <em>AhuI</em></td>
<td>17</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Gel electrophoresis of PCR-RFLP products by using <em>HaeIII</em></td>
<td>18</td>
</tr>
</tbody>
</table>
PCR-RFLP Profiling of Several Cyprinid Fishes

Siti Mardhiah bte Saharuddin

Animal Resource Science and Management Program
Department of Zoology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

This study examines the DNA profiling of three freshwater fish species which are Hampala macrolepida, Tor douromensis and Barbonymus schwanenfeldii by using PCR-RFLP method. The amplification of the mitochondrial gene, COI gene generated fragment size approximately 520 bp in size. The analysis using restriction enzymes Alul and HaeIII showed variation in the digestion profile while BamHI, HpaI, DraI and XbaI did not showed banding fragment in most of the fish sample analysed. Diagnostic digestion profiles were observed among three species, H. macrolepida produced one fragment while the other species (T. douromensis and B. schwanenfeldii) produce two fragments by using Alul. Variation in digestion profile was also detected between two samples of B. schwanenfeldii by using HaeIII. Moreover, by using HaeIII (T. douromensis and H. macrolepida) were produce unique digestion profiles, which can be used as genetic marker for discriminating the two species. Results in silico are different from results using agarose gel. Overall, RFLP analysis of the cytochrome oxidase I (COI) mtDNA segment have proven effective, fast and non-expensive technique to discriminate among freshwater fishes.

Keywords: B. schwanenfeldii, H. macrolepida, PCR-RFLP, T. douromensis; sequence data, restriction enzyme REs.

ABSTRAK

Kajian ini untuk mengkaji DNA profil daripada tiga jenis ikan air tawar iaitu Hampala macrolepida, Tor douromensis, dan Barbonymus schwanenfeldii dengan menggunakan kaedah PCR-RFLP. Daripada kajian ini, hasil proses amplifikasi untuk DNA daripada mitokondria, COI gen adalah lebih kurang 520 bp. Analisis menggunakan enzim Alul dan HaeIII menunjukkan perbezaan dalam profil pencernaan sementara, HpaI, BamHI, DraI, dan XbaI tidak menunjukkan jalur fragment dalam ikak yang dianalis. Diagnostik profil pencernaan yang diamati antara tiga spesies, H. macrolepida menghasilkan satu fragment manakala spesies lain (T. douromensis dan B. schwanenfeldii) menghasilkan dua serpihan dengan menggunakan Alul. Perbezaan dalam profil pencernaan juga dapat dikesan diantara dua sampel B. schwanenfeldii dengan menggunakan HaeIII. Tambah lagi, dengan menggunakan HaeIII H. macrolepida dan T. douromensis menghasilkan unik profil pencernaan, dimana boleh digunakan sebagai penunjuk genetik untuk membezakan dua spesies. Keputusan dalam in silico adalah berbeza daripada keputusan menggunakan gel agaros. Secara keseluruhannya, analisis RFLP cytochrome oxidase I (COI) mtDNA segmen teknik membuktikan efektif, cepat dan tidak mahal untuk membezakan diantara ikan air tawar.

Kata kunci: B. schwanenfeldii, H. macrolepida, PCR-RFLP, T. douromensis; fujukan data, enzim pembatas REs.
1.0 INTRODUCTION

The island of Borneo has one of the highest diversity of freshwater fishes in the world (Ismail, 1990) with more than 350 species have been recorded (Inger and Chin, 1962; Roberts, 1989; Kottlelat et al., 1993). Cyprinidae is the largest freshwater fish family which consists of 210 genera and more than 2010 species (Welson, 1994). It commonly found in the rivers and water body throughout the region (McConnel, 1975). In Western Borneo, about one third of all freshwater fishes belong to Family Cyprinidae (Roberts, 1989).

Cyprinids are important in aquaculture and provide basic protein for indigenous people and as income in terms of fish trading (Litls et al., 1997). But the natural population of Cyprinidae is rapidly decline due to environment degradation such as river pollution, deforestation, soil erosion, and others (Ng, 2004). Moreover, the impact of overfishing and illegal exploitation using chemicals and electric shock constantly added to their declined (William, 1997).

Fish identification can be achieved by various methods such as morphological characteristic, protein profiles and DNA study. From Sibley (1986), classification of fish depends on morphologies like tail, and fin length. Standard measurement that being used by fish researches all over the world, so the result that they obtained could be compared with others, even from different region (Inger and Chin, 1962).
Molecular technique based on polymerase chain reaction (PCR) and restriction enzymes (RE) have proven to be more credible for species identification (Comi et al., 2005). Mitochondrial DNA is suitable target DNA, due to the faster evolutionary rate of mitochondrial DNA than nuclear DNA and thus contains more sequence variation thereby facilitating identification of closely related species (Donaldson and Wilson, 1999). Other than that, high copy number of the mitochondrial DNA which around 1600–6000 circular mitochondrial DNA molecules per animal cell (Girish et al., 2004). In general, mitochondrial DNA (mtDNA) genetic markers have been widely used as a tool to distinguish within and among species (Patarnello et al., 1994; Christan et al., 2000; Klossa et al., 2002).

Commonly, fish identification based on morphological techniques to classification in taxonomy (Stepien and Kocher, 1997). The classical method to determine the genetic diversity by using morphological identification and differentiation is challenging and not accurate because the feature of fish quitey similar, although different family can easily differentiate but within species in same family are difficult.

In this study, the PCR-RFLP method was used to examine the mtDNA profiling of three Cyprinids species using COI gene. The objectives are:

i) To optimise the DNA extraction of Cyprinids

ii) To optimise the PCR amplification of Cyprinids

iii) To develop genetic marker of Cyprinids using PCR-RFLP
2.0 LITERATURE REVIEW

2.1 Cyprinidae

Cypriniformes occur all over the world (Saitoh et al., 2006). This family has a relatively diverse fauna in Africa, Europe and North America, over 1200 species are recorded from Asia with the centre of diversity being China and South East Asia (Liu and Chen, 2003). They are commonly distributed in Borneo (Inger and Chin, 1990) and dominated in fresh and brackish water. The distribution in Borneo are now limited to the upper streams and lower altitude (Ng, 2004). They also are well being adapted with high flowing water and mostly graze on the algae growing on the rocks (Inger and Chin, 1990).

The movement activity of cyprinids is high at dawn, dusk and night in free flowing stream sections (Baade and Fedrich, 1998) whereas low activity occurs during daylight (Allouche et al., 1999). Diurnal behaviour in cyprinids can also be influenced by local habitat characteristics (Baade and Fedrich, 1998). A large number of well known fish species belong to the Cyprinidae including the barbels, the common carp, goldfish, chubbs and roach (Thai et al., 2007).

Previously, taxonomic classification based on morphology characteristic, including the presence and number of barbells and the pharyngeal dentition, without investigating the intersubfamily relationships (Howes, 1991). Howes (1991) conducted a cladistic analysis of numerous morphological characters and since many character conflicts were found. By having molecular technique could resolve the problem in classify species.
2.2 Restriction Fragment Length Polymorphism (RFLP)

In 1983, Kary Mullis had invented an important, widely used technique in molecular biology known as PCR. As its name addressed, one of the key components of this technique is a DNA polymerase that used to amplify a piece of DNA by in vitro enzymatic replication. This technique is a rapid, inexpensive and simple means of producing more than 10 million copies of a target DNA sequence initiated by reaction of a few molecules (Thain and Hieckman, 2000). Each PCR reaction solutions contains a double stranded DNA template, short single stranded DNA template, short single stranded DNA template, short single stranded oligonucleotide primers, thermostable DNA polymerase, enzyme cofactors and four deoxynucleotides (dNTPs).

RFLP is a genetic marker that can be used to verify pedigree, screen wild population to maximize diversity in founder animal, and monitor inbreeding levels in breeding population (Moore et al., 1999). According from Fernandez et al. (2002), PCR-RFLP can give detailed comparison of individual sequences, especially when large numbers of samples need to be analyzed. It also inexpensive due to screening of the result only used agarose gel as compared to the standard PCR-RFLP technique which needs to screen using hybridization and autoradiography (Soltis et al., 1998). Moreover, RFLP is useful in forensic science as RFLP is applied for analyzing DNA fingerprinting. RFLP is also important in paternity case, disease status, restriction mapping and gene tracking (Anon, 2002).
RFLP is a technique where a polymorphism in an individual, population or species is based on restriction fragments of a distinctive length that resulted from a certain restriction enzyme (Hillis et al., 1996). RE are enzymes that used to cut DNA into several fragments and it has own restriction sequence. Thus, the action of a restriction enzyme is specific. Different individuals will have different cleavage sites for restriction enzyme in the DNA even they are of the same species (Hine and Martin, 2004). Therefore, different individuals that treated with same restriction enzyme will produce different sets of restriction fragments (Hine and Martin, 2004).

2.3 Molecular study related to PCR- RFLP

Based on Mitsutoshi et al. (2009) the authentication of flying fish meal content of processed food using PCR-RFLP were successfully determine by combining amplified DNA fragment with universally designed primers and digesting products with restriction endonuclease (Rn). According to Esa and Ryan (2005), RFLP analysis using Alul, BamHI and HpaII found variation in digestion profile among Cyprinids analysed. Diagnostic digestion profile was also detected between two same species (*H. binaculata*). Type A and Type B (using Alul and BamHI) supporting their status as distinct species. Additionally, unique digestion profile was observed in other species such as *H. macrolepidota* and *T. dourensis* which Alul could be used as genetic markers for discriminating species where produced two fragments lengths.
According to a research done by Zhang et al. (2005) the identification fish by using restriction enzyme likes HaeIII, ScaI, SmaI, MaeII could authentication species by the comparison of RFLP profiles. The most important requirement for species identification is that the intraspecific variation exhibit by the targeted DNA sequence should not obscure the interspecific variation for the question of interest (Gharrett et al., 2001). Based on Jantrarotai et al. (2007), PCR-RFLP was used to identify three closely related Hypsibarbus spp. The sequencing result of PCR products in these species showed very low interspecific variation. The RFLP analysis could be used to discriminant these species with Bsp143I and BclI.

Moreover, research done by Nebola et al. (2009) the identification of fish by using PCR-RFLP could perform the authentication in commercial fish product. The PCR products are digested with restriction enzymes AulI, HinfI, HaeIII, NlaIII, HincII and MboII. The DNA molecules have been chose in species identification due to their high stability compared with protein.

2.4 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is the circular DNA strand that can be found in mitochondrial and widely used in population genetics and evolutionary studies due its characteristics which give information on maternal and non recombining mode of inheritance (Jones, 2001; Irwin et al., 1991). Moreover, mtDNA has a number of specific biological properties which make it as a marker of molecular biodiversity (Galtier et al., 2009). It is used in the understanding of
evolutionary relationships among individuals, populations and species (Hartl and Jones, 2001; Szalanski Owens, 2003).

Moreover, mitochondrial genome is known as a control region or displacement loop region and can become a useful genetic marker in research on species identification (Hwang and Kim, 1999). The ability of control region also is the same as another gene region in mitochondria such as COI gene (Lam and Morton, 2004).

2.5 Cytochrome Oxidase I (COI)

COI is a catalytic enzyme Cytochrome Oxidase C that located in mtDNA genome. Its amino acid sequences are highly conserved across phyla, making it easy to align sequences to one another and the substitutions are rare within species which results in the possibility to design useful universal primers (Hillis et al., 1996). Barth et al. (2006) states that COI gene in mitochondrial DNA is a good genetic marker for both intraspecific and population genetics studies due to high variation (ranged from 7 % to 9.5 %) inferred in Paramecium species.

Moreover, COI could be fast and accurate marker to the resolution of the diversity of animals and has been used successfully for species-level identification in several animal groups (Hebert et al., 2003). It has been acknowledged that molecular and phylogenetics studies is essential for better understanding towards the evolution involving molecular, morphological, behavioral, of a particular species (Mindel et al., 1997).
Based on Esa et al., (2008) carried out molecular systematics of Mahseers (Cyprinidae) in Malaysia inferred from sequencing of a mitochondrial cytochrome c oxidase I (COI) gene, the size is about 466 bp. From the study, showed that the *T. tambroides* samples from Peninsular Malaysia and those from Sarawak also supported their classification as belonging to the same species while Sabah samples forming its own cluster. Thus, this study showed that only a single mtDNA gene managed to provide useful insights into the systematics status of the Mahseers found in Malaysia.

The study done by Fong, (2010) provided information related to genetic study of cyprinids. Based on the study, the size of COI gene generated about approximately 550 bp using the same primer, COI-f and COI-c designed by (Palumbi, 1996). The study was done because to determine molecular data of cyprinids in Malaysia either it is concordant with the current classical classification using morphology and meristic data. From the neighbourhood joining (NJ), the tree produced did not show clear grouping between cyprinids samples. But, the same species clustered together in their own group with high bootstrap value of 100%.
3.0 MATERIALS AND METHODS

3.1 Sample acquisition

The fish samples were collected from Sungai Rompin, Pahang. Additional samples were bought from wet market, Sarawak. The cyprinid were caught by using scoop net and cast net. The cyprinids were identified through their morphological characteristics and measurements using the keys provided by Inger and Chin (1962) and Mohsin and Ambak (1983). The tissues are stored in freezer at -20 °C for long term storage.

3.2 Laboratory work

3.2.1 DNA Extraction

DNA of specimens was extracted using muscle tissue were collected from museum specimens. The laboratory apparatus needed was prepared and sterilized using ethanol and lighter.

Amount of 70 mg of selected tissue samples was minced and placed into 1.5 ml eppendorf tube which contains 700 μL of 2X CTAB (Cetyl-trimethyl Ammonium Bromide) buffer (Grewe et al., 1993). Then, 5 μL-10 μL of Proteinase K was added into the tube. The specimens were incubated at 65 °C in the waterbath until the tissue lysed.

Total amount 600 μL of chloroform-isoamyl alcohol was added into the tube that containing lysed sample. The content of each tube were shaken for about two to three minutes. Then, tube was centrifuged at 13000 rounds per minute (rpm) for 20 minutes. As a result of this process, three layers of mixture will be visible. The upper aqueous phase was pipette for about 400 μL.
and transferred into a new microcentrifuge tube. The same amount of absolute ethanol as the pipetted product was added into the same tube. The tube was sat on the bench for a few minutes before proceed centrifuge step.

Then, the tube was centrifuged at 13,000 rpm for 15 minutes. After centrifuged, the supernatant from each tube was discarded. Amount of 400 μl of 70 % cold ethanol, EtOH, 25 μl of 3M NaCl, were added into the tubes and mixed by slowly invering it. The tube was then centrifuged at 13,000 rounds per minute (rpm) for 15 minutes. Again, the supernatant from the tube was discarded and checked for the presence DNA pellet. The tube was left at room temperature to let the pellet dry. The pellet was dissolved by adding 50-100 μL of distilled water, ddH2O, before storing it into the freezer at -20 °C.

3.2.2 Visualisation of DNA Products

The DNA yield from the extraction was visualised using 1 % and 2 % agarose gel, which was mixed with one μL of ethidium bromide. The ethidium bromide is often used to stain the DNA molecules for subsequent visualization under UV light. The gel was then immersed in 1X TAE buffer. This solution contains a minimal amount of ionic strength to prevent denaturation of DNA. Amount of one μL of DNA product and one μL of loading dye were mixed and loaded into the wells of the solidified immersed gel. Amount of one kb DNA ladder was also loaded as an indicator of the products size. Electrophoresis was conducted at 90 volts for approximately half an hour and viewed was by using UV transluminator.
3.2.3 Polymerase Chain Reaction, PCR

Before the PCR process took place, the master mix was prepared based on pre-configuration of the reagents which consists of 5X reaction buffer, 10 mM deoxynucleotide (dNTP mix), magnesium chlorides (MgCl₂), 10 mM forward primer, 10 mM reverse primer and sterilize distilled water (ddH₂O). PCR chemical reagents used were obtained from Promega kits. The reagents were mixed briefly and quick spin for one minute to bring all reaction components to the bottom of the tube. After that, 1.0 µl of template DNA was added into the PCR tube. Then, 0.2 µl of Taq polymerase was also added into the PCR tube to make the volume into 25 µl. Amplification was carried out in PCR machine for about three hours. The components needed for the PCR mixture is shown Table 3.1. Table 3.2 shows the steps of PCR cycles.

Table 3.1: the master mix for PCR reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock solution</th>
<th>1x reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>-</td>
<td>13.3</td>
</tr>
<tr>
<td>5x reaction buffer</td>
<td>-</td>
<td>5.00</td>
</tr>
<tr>
<td>0.2 mM dNTPs</td>
<td>2 mM</td>
<td>0.50</td>
</tr>
<tr>
<td>1.5 mM MgCl₂</td>
<td>25 mM</td>
<td>1.50</td>
</tr>
<tr>
<td>10 mM COI-f</td>
<td>10 mM</td>
<td>1.25</td>
</tr>
<tr>
<td>10 mM COI-c</td>
<td>10 mM</td>
<td>1.25</td>
</tr>
<tr>
<td>80 ng DNA template</td>
<td>40 ng/µl</td>
<td>2.00</td>
</tr>
<tr>
<td>1 U Taq DNA polymerase</td>
<td>5 U/µl</td>
<td>0.20</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>
Table 3.2: Amplification cycle for PCR process

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preheating</td>
<td>94</td>
<td>2 mins</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1 mins</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>47</td>
<td>45 secs</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>2 mins</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>5 mins</td>
<td></td>
</tr>
<tr>
<td>Soak</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Primers for partial cytochrome oxidase I (COI) gene and sequences (Palumbi et al., 1991)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>COI-f</td>
<td>5'-CCTGCAGGAGGAGGAYCC-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>COI-e</td>
<td>5'-CCAGAGATTAGAGGAATCAGTG-3'</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

3.2.4 Purification Process

The purification of PCR product was carried out using PROMEGA Purification Kit following the protocols provided by the manufacturer. Purification was used to obtain pure DNA and eliminate any wastes that exist in the PCR product. DNA purification is essential in avoiding contaminants such as reagents and primer-dimer in the PCR product. Purification process involved three parts, which are removal of the protein, RNA and fragmented DNA. The PCR products that were undergone purification produced single band when visualized under the UV transilluminator. The purified PCR products were sent to private laboratory for DNA sequencing.
3.2.5 PCR-RFLP of mtDNA gene

PCR-RFLP of mtDNA gene was carried out using six different restriction enzymes (REs) namely \textit{XhoI}, \textit{BamHI}, \textit{DraI}, \textit{AluI}, \textit{HaeIII} and \textit{HpaII}. The digestion specificity of each restriction enzyme is as shown in Table 2. The restriction enzymes acted with the presence of 5.9 $\mu$l distilled water, 1.0 $\mu$l of buffer specific for each enzyme and 0.1 $\mu$l BSA upon digestion of 2.5 $\mu$l PCR products. The preparation PCR mixtures are shown in Table 3. Each digestion reaction was incubated at 37 °C for 3 hours before loading it onto 2 % agarose gel. The gels are then visualized under UV light and photographed.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Cocktail & Stock Solution & 1x reaction ($\mu$l) \\
\hline
10X Buffer Tango with BSA & - & 1.1 \\
10 U \textit{AluI} (Restriction enzyme) & 10 U/$\mu$l & 0.5 \\
Sterile distilled water & - & 5.9 \\
\textbf{Total} & - & 7.4 \\
PCR product & - & 2.5 \\
\textbf{Total} & & 10.0 \\
\hline
\end{tabular}
\caption{The master mix for PCR-RFLP reaction mixture}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Type & Restriction enzyme & Specificity 5'-3' \\
\hline
Six base & \textit{BamHI} & \textit{G\downarrow GATCC} \\
& \textit{DraI} (\textit{AluII}) & \textit{T\downarrow TAAA} \\
& \textit{XhoI} & \textit{C\downarrow TCGAG} \\
\hline
Four base & \textit{AluI} & \textit{A\downarrow GCT} \\
& \textit{HaeIII} & \textit{G\downarrow GCC} \\
& \textit{HpaII} & \textit{C\downarrow CGG} \\
\hline
\end{tabular}
\caption{The table shows the types of Restriction Enzymes (Manufactured by Fermentas Company) and their restriction sites digestion specificity}
\end{table}

\textit{(Adapted from Carvalho et al., 2004)}