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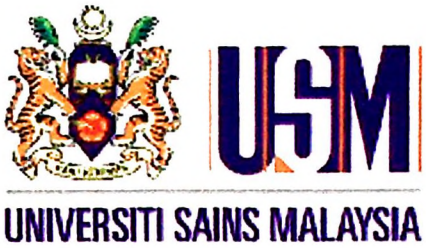

MOLECULAR CHARACTERIZATION OF ORNAMENTAL FOSH (*PEOCILIDAE*) SPECIES BY PCR-RFLP OF MITOCHONDRIAL DNA 12S rRNA GENE

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (HONS) IN FORENSIC SCIENCE

MOHAMAD NOR FATIHIN ABSHAR BIN MOHD NOR AZAM

SCHOOL OF HEALTH SCIENCES

UNIVERSITI SAINS MALAYSIA

HEALTH CAMPUS

16150 KUBANG KERIAN, KELANTAN,

MALAYSIA.

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Sincerely,
Mohamad Nor Fatihin Abshar Bin Mohd Nor Azam

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLE

Abstract

In this study four ornamental fish species from *Peociliidae* family namely *Xiphophorus maculatus, Xiphophorus hellerii, Poecilia reticulate, Limia Vitatta* and *Poecilia shenops* were used as a samples. Characterization of fish species was carried out by Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by digestion with endonucleases restriction enzymes. Three different enzymes were used such as *Alu* I, *Dde* I and *Mse* I. Based on result only *Xiphophorus maculatus* showed species specific profile after treated with *Alu* I enzyme. No species specific was seen in all fish species after treated with *Mse* I and *Dde* I.

Abstrak

Dalam kajian ini, empat ekor spesies ikan hiasan dari keluarga *Peociliidae* iaitu *Xiphophorus maculatus, Xiphophorus hellerii, Poecilia reticulata, shenops Limia Vitatta* dan *Poecilia* telah digunakan sebagai sampel. Pencirian spesies ikan telah dijalankan secara rantaian tindak balas polimerase– *restriction fragment length polymorphism* (*PCR-RFLP*) diikuti penghadaman dengan *endonucleases*.enzim. Tiga enzim yang berbeza telah digunakan seperti *Alu* I, *Dde* I dan *Mse* I. Berdasarkan keputusan, hanya *Xiphophorus maculatus* menunjukkan profil spesifik spesies tertentu selepas dirawat dengan enzim *Alu* I. Tiada spesifik spesis tertentu dapat dilihat dalam semua spesies ikan selepas dirawat dengan *Mse* I dan *Dde* I.

CHAPTER 1:INTRODUCTION

Several different approaches have been applied to distinguish fish species from processed food and canning food. In general, the specific morphological characters such as size, shape and appearance of fish species are sufficient to identify them unambiguously. However, efficient methods for species identification become increasingly important when specific characters of fishes are removed by industrial processing, especially involving fish fillets or canned fish (Di Finzio et al., 2007). In addition, Species identification of food products also an important for others reason including food safety management, avoiding fraud, allergic problem involving specific species and ethical or religious issues. There are various protein based method for fish species identification including isoelectric focusing, chromatography and immunological methods (Tepedino et al 2001; Ochiai et al 2001). All these methods become less useful when the proteins in the samples are irreversibly denatured due to cooking process. Moreover, differentiation of closely related fish species is difficult as they share a common set of protein profiles (Sylvia E. Bartlett, 1991)Therefore, the application of DNA technology to food identification is important since DNA is more thermostable than many proteins and present in almost all cells of the organism carrying the identical information, making all tissues suitable for the analysis (Mackie et al., 1999).

The advents of recombinant DNA techniques generated more reliable genetic markers which are useful to address the problem of genetic identification of species with high sensitivity and specificity. Both mitochondrial and nuclear DNA is used as DNA markers for species identification (T D Kocher, 1989). As stated by (Fernandes, 2007), DNA

marker is developed in order to allow for identification of taxa especially at species level. In nuclear DNA, there are two main regions usually apply namely exon and intron. Exon is coding region in nuclear DNA provides information at the species level. The disadvantages of using nuclear DNA is low copy number, which easily affected by environmental condition, therefore typing of nuclear DNA is impossible due severely degraded samples.

Recently, many studies utilized mitochondrial DNA (mtDNA) markers for species identification. Mitochondrial DNA is a small portion of the DNA of eukaryotic cells (<%1), located within organelles in the cytoplasm. As mentioned by Billington (2003), the major features of mtDNA such as maternal inheritance, not subject to recombination, high copies in each cell and no introns present made mtDNA as a good marker for species identification compared to nuclear DNA. Mitochondrial DNA also possess extra-chromosomal DNA and features such as a high copy number (2-10 copies per cell and as many as 1,000 mitochondria per somatic cell) made mtDNA able to type from severely or degraded samples (Karabasanavar et al., 2010). Hence, mitochondrial DNA sequences are preferred for wildlife forensics, zoological and molecular analysis of plants (Budowle et al., 2003). Study by Wilson et al (1986), showed that the application of ribosomal RNA genes in solving the problems of food authentication as well as in phylogenetic reconstruction of fish species at different taxonomical levels.

Mitochondrial DNA markers include cytochrome B (cyt b), cytochrome oxidase I (COI), ribosomal RNA genes (12S rRNA and 16S rRNA) and hypervariable region. Cytochrome oxidase I also known as DNA barcoding is widely applied as a standardized molecular

tool for species identification (Hebert et al., 2003). DNA barcoding identifies a species from short standardized fragment that can be applied to different kinds of specimens from processed foodstuffs to fossil samples, as well as various stages of life for a particular species. The limitation of the DNA barcoding is might not be effective in phylogenetic tree reconstruction and unable to resolve the closely related species since it's recognized as universal identification for species (Wong, 2011). Cytochrome b gene as stated by Merrit et al. (1998), accumulate the changes in nucleotide at a sufficient rate which can be used to resolve the phylogenetic relationships among closely related species. Hence, Cyt b works well involving mixed samples in food processed. Mitochondrial DNA ribosomal RNA genes are highly conserved in sequence, therefore the application of these regions is suitable in closely related species. According to Parkash et al. (2000), mitochondrial 12S rRNA is proven as a good candidate marker for species identification.

Due to characteristics mtDNA, suggested that these genes are valuable for molecular phylogenetic studies. For example, mitochondrial 16S rRNA gene has been used to explore the phylogenetic relationships of fishes at different taxonomic levels mainly due to the fact that it is highly conserved and has a slow evolution (Moyer et al. 2004). Meanwhile, mitochondrial 12S rRNA gene is considered a promising tool for tracing the history of more recent evolutionary events (Hillis and Dixon, 1991) and it has been widely used to study the phylogenetic relationships among different levels of taxa such as families, genera and species (Wang et al., 2003). Palumbi (1996) and Di Finizio et al. (2007) have reported that Mitochondrial 12S rRNA is located between the $tRNA^{Phe}$ and $tRNA^{Val}$ genes, relatively conserved and evolving more slowly than the mitochondrial genome as a whole.

There are several DNA based methods that can be applied in species identification such as DNA sequencing, PCR- restriction fragment length polymorphism (RFLP), Single nucleotide polymorphism (SNP), PCR specific primer and PCR-Random amplified polymorphic DNA (RAPD) (Teletchea, 2009). Traditionally, DNA sequencing is time consuming, technically demanding and require good data handling capacity. Recently, both prices and time have significantly reduced by introduction of new generation of sequencer. Though, PCR-RAPD does not required previous knowledge of DNA sequences, but this method may not be practical for species identification involving samples of mixtures species as well as not suitable for degraded samples since its required good quality of DNA materials (Teletchea, 2009). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) mapping have been successfully applied in various fish species and fish products identification (Arahishi 2005; Akasaki et al. 2006). In this technique, an amplified fragment will be digested with selected restriction endonucleases, resulting in few smaller fragments of different sizes. The different fragments are then separated by agarose gel electrophoresis. This method is relatively simple, robust, much easier to perform and inexpensive than PCR-sequencing. The main disadvantages are that incomplete digestion may occasionally occur and intraspecific variations could delete or create additional restriction sites. (A.K Lockley, 2000) PCR-RFLP method has been successfully applied in identification of the authentication of 18 commercial canned tuna (Teletchea, 2009).

Research studies on ornamental fishes are very scanty since this type of fish is considered as a pet. Ornamental fish is referring to a tropical species including finfish, such as

goldfish, aquatic invertebrates and amphibians. Commonly ornamental fishes are live in aquarium and not suitable for sport fishing purposes or for food. Ornamental fish usually consists of attractive colourful fishes of various characteristics such as body colour, morphology and mode of taking food. Ornamental fish consists of nine popular groups namely *Cyprinids, Cobitids, Cypinodontidss, Anabantids, Peocilids, Characins, Achilids, Osteoglossids* and *Callhthyids* (Department of Fisheries Malaysia, 2008).

Malaysia ornamental fish industry is still new and demands for ornamental fish is growing faster. One of the important ornamental fish group is *Peocilids*. As classified by Parenti (1981), *Peocilids* belongs to the family of *Poeciliidae* and under the subfamily of *Poeciliinae. Poeciliidae* is a widespread and diverse group of small-sized fishes that includes 22–29 genera and more than 200 species (Lucinda, 2003). Ornamental fish species under *Peocilids* groups are Molly, Guppy, Platy, Swordtail and Mino.

In this study, mitochondrial 12S rRNA gene was used to characterize five ornamental fish species under *Peocilids* group based on PCR-RFLP. As reported by Di Finzio (2007), PCR-RFLP has an ability to identify fish species based on species specific pattern generated after treated by specific restriction enzyme.

1.2 Objectives

1.2.1 General objective:

To characterize the ornamental fish species (*POECILIIDAE)* based on PCR-RFLP mitochondrial DNA 12S rRNA gene

1.2.2 Specific objectives:

a. To extract genomic DNA from ornamental fish species

b. To perform PCR amplification of mtDNA 12S rRNA gene of ornamental fish species using specific primers

c. To assign the ornamental fish species based on variation pattern generated by PCR-RFLP

CHAPTER 2: LITERATURE REVIEW

The application of molecular techniques has provided new and better insights into the taxonomy, population structure, and conservation management of species (Nguyen et al., 2006). Several molecular methods has been utilized for species identification such as DNA sequencing, PCR-RFLP (Polymerase Chain Reaction by Restriction fragment length polymorphism), Restriction amplified polymorphic DNA (RAPD), Real time PCR and Amplified fragment length polymorphism (AFLP) (Pereira et al., 2008). Ryan and Esa (2006) has stated that molecular markers can provide reliable and consistent results for rapid identification among species, levels of genetic variability, levels of gene flow and population subdivisions as well as understanding factors contributing to fitness. Species identification based on molecular methods requires only a very small quantity of DNA from any tissue such as scale, fin clip, muscle and meat for analysis using polymerase chain reaction (PCR) technology (Avise, 1994). This advantage is crucial particularly for endangered, protected, or remnant populations or species where non-destructive sampling is required for genetic analysis (Ward, 2000). As stated by Esa et al. (2007) a molecular DNA marker has the advantage over morphological and biochemical traits due to precision in detecting relationships among and within population of various organisms.

Traditionally, fish identification is based on morphological features such as shape, colours, size and appearance (Zhang and Hanner, 2012). In many cases, these morphology features are removed by industrial processing of fillet fish or canned fish (Di Finzio, 2007). In addition, fish also has diverse developmental stages which create difficulty in

identification based morphological characteristics (Victor et al, 2009). One of the methods used in fish identification is DNA barcoding system. DNA barcoding system based on amplification of partial sequence of cytochrome oxidase I and standardized and universal method (Hebert et al., 2003). Many studies has reported that intraspecific variation of COI barcodes is generally pretty small and clearly discriminable from interspecific variation (Ward et al., 2009).

Other markers that utilized for species identification include mitochondrial cytochrome B, hypervariable region and ribosomal genes 12S rRNA and 16S rRNA. According to Vences et al. (2006), mitochondrial DNA 12S rRNA and 16S rRNA are highly conserved in sequence which makes them very suitable for study at interspecies and interspecies discrimination. These ribosomal RNA genes is also important in study of genetic variability and inbreeding, parentage assignment, strains and species identification and the construction of high- resolution genetic linkage maps (Lui and Cordes, 2004). Study by Siddapa et al. (2013) showed that even in closely related species both of these genes able to discriminate since these genes inherit information without recombination. These mitochondrial 12S rRNA and 16S rRNA genes has been used in identification of many species including birds, reptiles, mammals, fishes and amphibians (Yang et al., 2014).

PCR-RFLP approach based on mitochondrial DNA polymorphism has been established for species identification of common farm animals, poultry animals and livestock species (Abdel-Rahman and Ahmed, 2007). Compared to DNA sequencing, this method has been proposed as one of the most efficient methods in terms of cost, detection power and applicability to large scale screening (Pereira et al., 2008). PCR-RFLP has been applied

to detect fraudulent substitutions of commercially valuable fishes with others less expensive in manufactured fish products (Di Finzio, 2007). As mentioned by Kocher et al. (1989), this method consists of digestion of the primary PCR product with restriction endonucleases which target polymorphic regions within the amplicon to produce a species-specific pattern of fragments that can be easily identified based on their size. One of the disadvantages of PCR-RFLP it required prior knowledge of the sample analysed (Teletchea et al., 2009). Lin and Hwang (2007) have successfully utilized this technique to identify eight species of *scombroids* by using five restriction enzymes.

CHAPTER 3: METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagents

All reagents, chemicals, commercial kits and consumables used in this study are listed in Table 3.1.

3.1.2 Buffer preparations

3.1.2.1 Proteinase K (20mg/ml).

Two mg of Proteinase K was mixed with 1 mL deionized water.

3.1.2.2 10X Tris Borate EDTA Buffer

TBE Buffer stock solution was prepared by dissolving 107.8 g Tris base, 7.4g Na_2EDTA and 46 g Boric Acid in 800 mL of ddH_20. The pH was adjusted to 8.3 by adding Boric Acid. The solution was made up to 1000 mL by adding ddH_20. The 10X TBE Buffer was autoclaved and stored at ambient temperature.

3.1.2.3 0.5X TBE Buffer

TBE buffer 0.5X working solution was prepared by diluting 50 mL of 10X TBE buffer in 950 ml ddH_20.

3.1.2.4 70% Ethanol

The solution was prepared by mixing 30 mL of ddH_20 with 70 mL of absolute ethanol and was stored at ambient temperature.

Tables 3.1 Lists of kits and consumables

Reagents	**Company Name/Supplier**
Magnesium Chloride ($MgCl_2$)	Biosyintech, Fermentas, Lithuania
10X PCR $(NH)2SO_4$	Biosyintech, Fermentas, Lithuania
5U/µl DNA Taq Polymerase	Sigma, Singapore
Primers	Biosyintech, Fermentas, Lithuania
10mM dNTPs	Biosyintech, Fermentas, Lithuania
Chemicals	**Company Name/Supplier**
Agarose gel powder	Promega, USA
Denatured absolute ethanol (95%)	Teraslab Sdn. Bhd.
Boric Acid	Germany
100 base pair Allelic ladder	Invitrogen Corperation, USA
Tris EDTA (Ethylenediaminetetaacetic acid)	Sigma, Singapore
Ethidium Bromide	Sigma, Singapore
Consumable	**Company Name/Supplier**
Micropipette 10µl, 200µl, and 1000µl	Gilson,USA
Pipette	Teraslab Sdn. Bhd.
Latex Gloves	Teraslab Sdn. Bhd.
Microcentrifuge tube 1.5 µl	Odonixx Labware, USA
Thin wall PCR tube 0.2 ml	Odonixx Labware, USA
Kits	**Company Name/Supplier**
QIAamp DNA Mini Kit	QIAGEN, USA
Gene All PCR SV Kit	General Biosystem, Seoul, Korea
QIAquick® PCR Purification Kit	QIAGEN, USA

3.1.2.5 Ethidium Bromide (10mg/mL)

Ethidium Bromide solution was prepared by dissolving 1g of ethidium bromide in 100 mL ddH_20 and stored in amber colored bottle

3.1.2.6 Orange G loading dye

A total of 0.125 g of orange G and 20 g of sucrose were dissolved in 30 mL distilled water. The solution was made up 50 mL by adding distilled water. It then aliquot into 1.5 mL microcentrifuge tube and kept at 4C

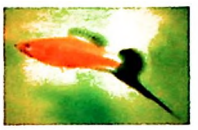
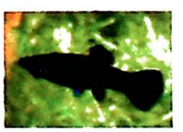
3.1.2.7 100 base pair DNA ladder

The 100 bp ladder consists of ten chromatographs purified individual DNA fragments between 100 and 1000 bp.

3.1.3 Samples collection

A total of five ornamental fish species were used in this study was belong *Peocilids* group. The scientific name for selected ornamental fish species was shown in Table 3.2.

Table 3.2: List of ornamental fish species used in this study

No.	Scientific name	Common Name
1.	*Xiphophorus maculatus* 	Swordtail fish
2.	*Xiphophorus hellerii* 	Platy fish
3.	*Poecilia reticulata* 	Guppy
4.	*Limia Vitatta* 	Cuban Limia Fish
5.	*Poecilia sphenops* 	Black Molly

3.2 Methods

3.2.1 DNA Extraction

DNA extraction from fish tissue was done using QIAGEN kit, following the recommended procedure provided by the manufacturer. A total of 25 mg of tissue was cut into small pieces and placed in 1.5 ml microcentrifuge tube. A total of 180 µl Buffer ATL was added to the tube. Then, 20 µl Proteinase K was added to the tube. The solution and sample were mixed thoroughly by vortexing. The sample was then incubated at 56 °C until tissue completely lysed. During incubation, the sample was vortexed occasionally to disperse the sample. After that, the sample was vortexed for 15 seconds. Buffer AL with volume of 200 µl was added to the sample and vortex for mixing. Then, 200 µl of 95% ethanol was added and mixed by vortex. The mixture (includes any precipitate) was transferred into DNeasy Mini spin column and placed in a 2 ml collection tube. Then centrifuged at 6000 x g for a minute was performed. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube. Buffer AW1 with volume of 500 µl was added and the spin column was centrifuged at 6000 x g for a minute. The flow-through and the collection tube were again discarded. The DNeasy Mini spin column was placed in a new tube and 500 µl Buffer AW2 was added and the spin column was centrifuged at 13000 rpm for three minutes, then, the flow-through was discarded. The same collection tube was reutilized, extra centrifuged at 13000 rpm for a minute was carried out to ensure no excess ethanol was leaved. DNeasy Mini column was placed in a clean 1.5 micro centrifuge and buffer AE of 100 µl volume was then pipetted directly onto DNeasy membrane. The tube was left at room temperature for a minute and was centrifuged at 6000 x g for a minute. The collection tube containing a genomic DNA (after spin column was discarded) was stored at -20 °C for further used.

3.2.2 Agarose Gel Electrophoresis of DNA Extraction

The presence of high molecular weight DNA (HMW) was checked using agarose gel electrophoresis. A 1% agarose gel was prepared by adding 1 g agarose gel powder into 100 ml of 0.5X TBE buffer and heated in microwave oven for 3 minutes. The agarose gel solution was allowed to cool down to 60°C before adding 1.5 µl of ethidium bromide (10 mg/ml).

The agarose gel solution was poured into horizontal electrophoresis unit and was allowed to solidify at ambient temperature for 30 minutes. A total of 2 µl of extracted genomic DNA was mixed with 1.5 µl of Orange G loading dye and was loaded into the agarose gel. The electrophoresis was performed at 80 V for 45 minutes. The presence of HMW was observed under UV light using Image Analyzer (UVP Bioimagine System).

3.2.3 DNA Quantification

The extracted genomic DNA of fish species were quantified using spectrophotometer. Five µl of DNA samples was mixed with 45 µl of TE buffer (pH 8.0). Then, the optical density (O.D) value was observed under 260 nm wavelengths. The reading for extracted genomic DNA of fish species was recorded for further used in PCR amplification.

3.2.4 Primers

In this study, the published primers were used for PCR amplification of mitochondrial DNA 12S rRNA (Palumbi et al., 1996). The details of the primers sequences were displayed in Table 3.3

Table 3.3: Primer used in amplification of mtDNA 12S rRNA of fish species

Primer	Annealing Temperature	(G+C) content (%)
Forward Primer 5'- AAA CTG GGA TTA GAT ACC CCA CTA T -3'	Tm= (7+4)2+(4+7)4 = 30+ 40 = 70 °C	40%
Reverse primer: 5'- GAG GGT GAC GGG CGG GCG GTG TGT -3'	Tm= (4+6)2+(8+2)4 =84°C	75%

3.2.5 PCR amplification

PCR is a technique where the target region in the DNA being amplified, in other words to make a larger numbers of copies of the specific DNA fragment needed. This technique also called as "DNA photocopier". PCR is prepared to produce sufficient amount of DNA required for analysis. It is rapid, low cost technique and simple method used to produce high amount of DNA from limited sources. There are three distinct steps in PCR by governed temperature starting with denaturation, annealing and extension (Figure 3.1). PCR occurs in cycling reaction and can be repeated for 30 or 40 cycles (McPherson and Meller, 2000).

PCR amplification was carried out in 0.2 mL thin wall PCR tubes containing the following mixtures: 2 µL of 10X PCR buffer NH_4 (S04)2, 2 µl of 25 mM $MgCI_2$, 0.32 µL of 10 mM dNTPs mix, 1 µL of genomic DNA (10 ng/ µL), 1 µl of 10 pmol of each primer, 0.3 µL of Taq polymerase (5U/ µL) and added with 12.38 µL ddH_20 to make up to 20 µL of total reaction mixture. The following PCR thermal cycle conditions were performed: 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 30 seconds, 70 °C /84 °C for 30 seconds, 72 °C for 30 seconds, final extension at 72 °C for 5 minutes and hold at 4 °C. The amplified PCR product was kept at 4 °C until further use.

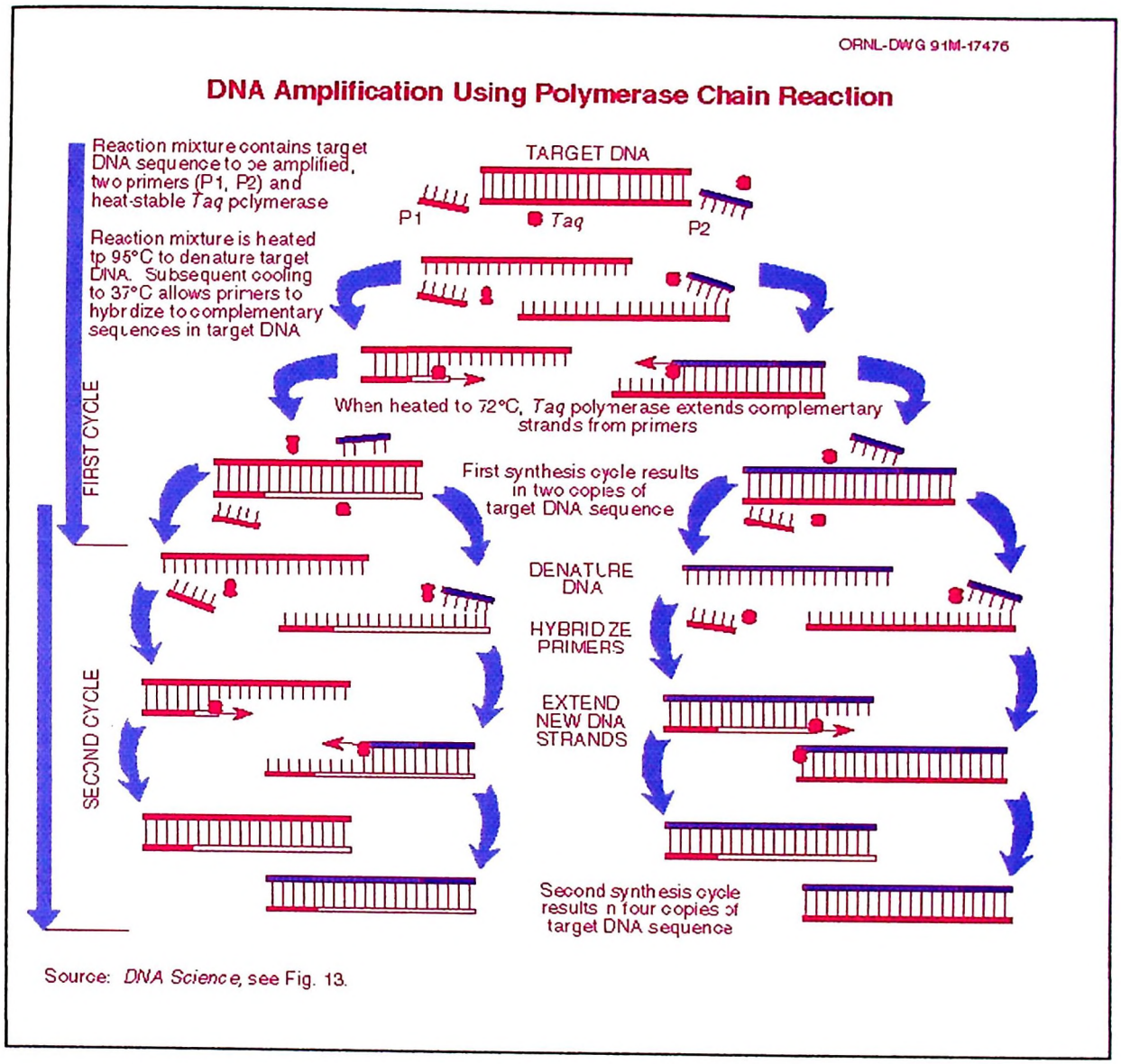


Figure 3.1: Schematic of polymerase chain reaction amplification (PCR) cycles

(Sources: http://www.animalgenome.org/edu/doe/pcr.gif

3.2.6 Agarose Gel Electrophoresis of Amplified PCR Product

A 1% agarose gel was prepared by adding 1 gram of agarose powder into 100mL of 0.5X TBE buffer and heated in microwave oven for 3 minutes. The agarose gel solution was allowed to cool down to 60 °C before adding 1.5 µL of ethidium bromide (10mg/ mL). The solution was poured onto a gel plate and was allowed to solidify at ambient temperature for 30 minutes. A total of 3µL of PCR products was mixed with 2 µL Orange G dye and was loaded into each well. One µL of 100 base pair DNA ladder was loaded into the first well. The electrophoresis was performed at 80 V for 45 minutes. The presence of PCR products were observed under UV light using Image Analyzer.

3.2.7 PCR RFLP Analysis

Restriction enzyme is site specific since its cleave DNA molecules at specific nucleotide sequences which are called restriction sites (Snustad and Simmons, 2003). The restriction sites will produce fragments which can be facilitating for characterization (Hartwell et al, 2011). There are two types of cut made by the restriction enzymes known as sticky ends and blunt ends (Snustad and Simmons, 2003) (Figure 3.2).

In the study, PCR-RFLP analysis was performed on mtDNA 12S rRNA gene in order to determine the species- specific site. The PCR product was treated with restriction endonuclease in 15 µl of total reaction (Table 3.4). The reaction mixture was incubated at 37 °C for 5 hours and the enzyme was deactivated at 65 °C for 15 minutes. The digested PCR product was loaded onto 2% agarose gel and electrophoresed at 80 V for 90 minutes.

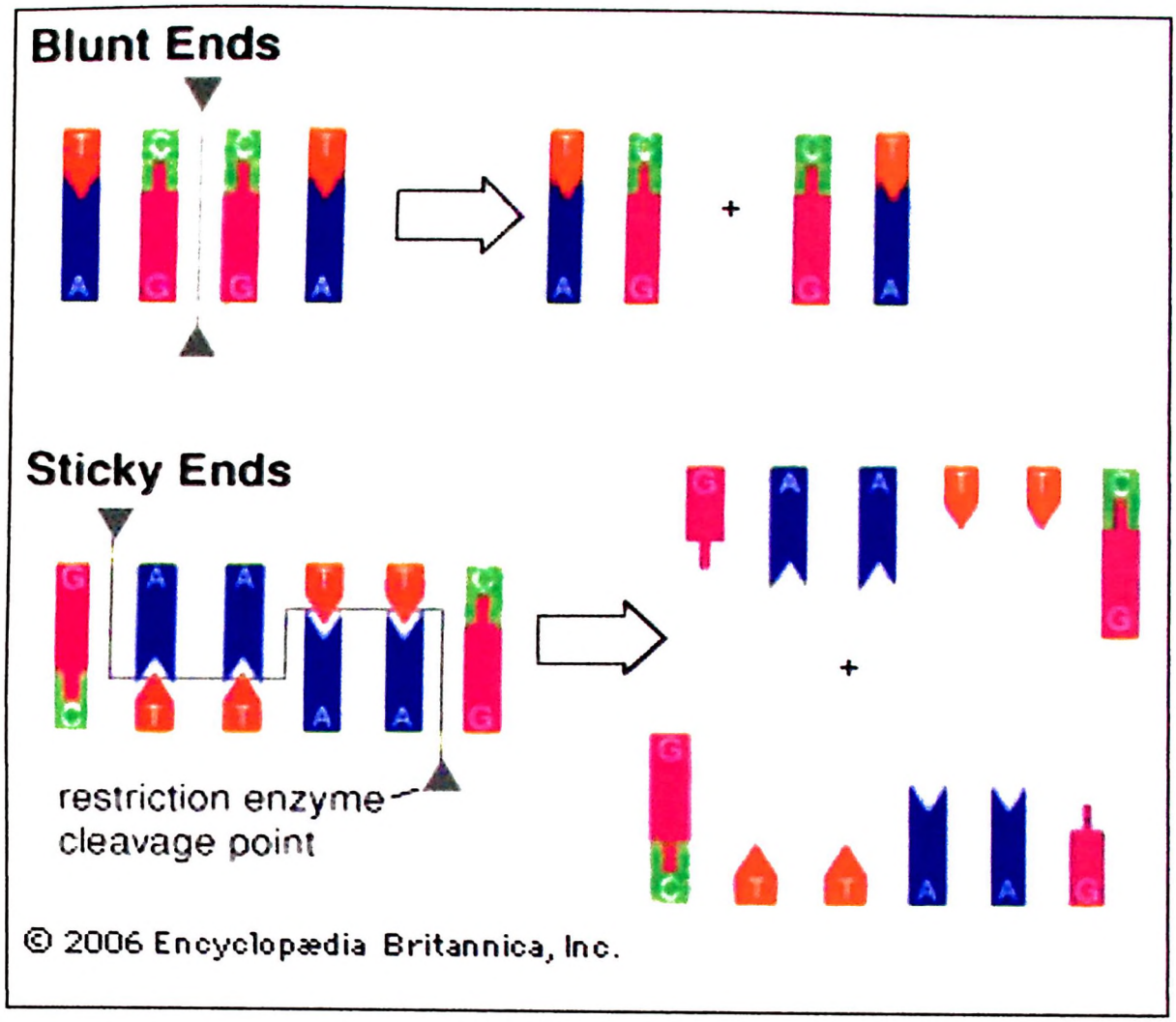


Figure 3.2: The fragment created either blunt ends or sticky end after treatment with endonucleases restriction enzyme

(Source: http://media.web.britannica.com/eb-media/46/94946-036-1CB67CC4.jpg

Table 3.4: The restriction endonuclease master mix preparation

RFLP mixture	Volume (µL)
Restriction enzymes	0.25 µL
Amplified PCR product	3.0 µL
10X RE Buffer	1.5 µL
dH_20	10.25 µL
Total	15 µL

CHAPTER 4: RESULT

Figure 4.1: Electropherogram of extracted genomic DNA.

Lane 1: *Xiphophorus maculatus*

Lane 2: *Xiphophorus hellerii*

Lane 3: *Poecilia reticulata*

Lane 4: *Limia Vitatta*

Lane 5: *Poecilia shenops*

Lane 6: 1 kb DNA ladder

4.2 Agarose gel electrophoresis of amplified PCR products

PCR amplification of mtDNA 12S rRNA gene was performed using primes 12S rRNA. A 350 bp of PCR product was amplified and loaded into agarose gel electrophoresis (Figure 4.2).

Figure 4.2: Agarose gel electrophoresis of amplified PCR product of mtDNA 12S rRNA gene

Lane 1: *Xiphophorus maculatus*

Lane 2: *Xiphophorus hellerii*

Lane 3: *Poecilia reticulata*

Lane 4: *Limia Vitatta*

Lane 5: *Poecilia shenops*

Lane 6: Negative control

Lane 7: 1 kb DNA ladder