MOLECULAR CHARACTERIZATION OF ORNAMENTAL FISH SPECIES (POECILIIDAE) USING MITOCHONDRIAL DNA12S rRNA AND 16S rRNA GENES

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SITI NORSYAWAZNI BINTI GHAZALI

SCHOOL OF HEALTH SCIENCES

UNIVERSITI SAINS MALAYSIA

HEALTH CAMPUS

16150, KUBANG KERIAN

KELANTAN, MALAYSIA

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SITI NORSYAWAZNI BINTI GHAZALI 112131

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LIST OF SYMBOLS

Symbols	Referring
bp	Base pair
mg/mL	Milligram per millilitre
µL	Microliter
g	Gram
%	Percentage
°C	Degree Celsius
Tm	Temperature

LIST OF ABBREVIATIONS

Abbreviation	Full Name
DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
HMW	High-Molecular-Weight
PCR	Polymerase Chain Reaction
ddH_2O	Double-distilled water
dNTPs	Deoxynucleotide triphosphate
$MgCl_2$	Magnesium Chloride
EDTA	Ethylenediaminetetraacetic acid
$(NH_4)_2SO_4$	Ammonium sulphate

Abstract

Mitochondrial DNA (mtDNA) ribosomal genes have been used as molecular markers for fish species identification in many studies. In this study, two mtDNA ribosomal genes namely 12S rRNA and 16S rRNA have been utilized to characterize ornamental fish species from *Peocilidae* family. Five ornamental fish species namely *Poecilia sphenops*, *Poecilia reticulata*, *Limia vitatta*, *Xiphophorus hellerii* and *Xiphophorus maculatus* were selected. PCR amplification was performed and DNA sequencing was carried out in order to study the genetic relationship of fish species. Phylogenetic tree results showed that two different group of fish species were found with one groups consists of *Limia vitatta*, *Xiphophorus hellerii* and *Xiphophorus maculatus*. The second group consists of *Poecilia sphenops* and *Poecilia reticulata*. Interestingly, both mtDNA ribosomal genes showed similar result for neighbor joining tree.

Abstrak

Ribosomal gen mitokondria DNA (mtDNA) telah digunakan sebagai penanda molekul dalam banyak kajian untuk mengenalpasti spesies ikan. Dalam kajian ini, dua ribosomal mtDNA gen bernama 12S rRNA dan 16 rRNA telah digunakan telah digunakan untuk mencirikan spesies ikan hiasan dari kumpulan Peocilids. Lima spesis ikan hiasan iaitu *Peocilia sphenops*, *Peocilia reticulata*, *Limia vitatta*, *Xiphophorus hellerii* dan *Xiphophorus maculatus* telah dipilih. Amplifikasi PCR telah dilakukan dan penjujukan DNA telah dijalankan untuk mengkaji hubungan genetik spesis ikan. Hasil kajian menunjukkan bahawa dua kumpulan berbeza ikan spesies dijumpai dengan satu kumpulan terdiri daripada *Limia vitatta*, *Xiphophorus hellerii* dan *Xiphophorus maculatus*. Kumpulan kedua terdiri daripada *Peocilia sphenops* dan *Peocilia reticulata*. Menariknya, kedua-dua gen ribosomal mtDNA menunjukkan keputusan yang sama untuk pohon *neighbour joining.*

CHAPTER 1 : INTRODUCTION

1.1 Introduction

Aristotle utilized a system of classification by grouping the animals according to type of reproduction and possession or lack of red blood. In the middle of 18th century, the Swedish naturalist Carolus Linnaeus had proposed the nomenclature system for classification. In this system, each species represent by two names; first name indicating genus and the second name indicating species (The Columbia Electronic Encyclopedia, 2012).

Generally, identification and classification of animal species is a key prerequisite for numerous biological studies. As stated by Fre´zal and Leblois (2008), the identification of species depends on the knowledge held by taxonomists and they cannot cover all taxon identification. Species identification strictly on the basis of morphological characters alone is quite unreliable, because of considerable geographical and ecological variability (Tsigenopoulos and Berrebi 2000; Siraj *et al.* 2007).

Study by Herbert *et al.* (2003), indicate four main significant limitations for species identification based on morphological characters. First, species recognition sing phenotypic plasticity and genetic variability in the characters can lead to incorrect identifications. Second, this method overlooks morphologically puzzling taxa, which are common in many groups. Third, many individuals cannot be identified since the morphological keys are often effective only for a particular life stage or gender and fourth, misdiagnoses of the species still can occur.

Traditionally, the studies of variation of a species are based on morphological characters. Molecular DNA based marker, allow species identification through the analysis of a small segment of the genome and aids the classification of closely related species that cannot be done by morphological identification method (Herbert *et al.*, 2003). Multiple DNA based approaches have been developed for species identification, including DNA hybridization, restriction enzyme digestion, random PCR amplification, species-specific PCR primer use, and DNA sequencing (Yang *et al.*, 2014). The application of DNA based markers has several advantages such as more resistant and thermostable than protein though involve in various process (canning and heating). Second, DNA can be retrieved from any substrate because it is present in almost all cells of an organism. Unlike to protein, DNA provides more information due to degeneracy of the genetic code and the presence of many non-coding regions (Teletchea, 2009).

The application of molecular techniques such as DNA sequencing has provided a new insight into identification of animal species. Molecular based DNA markers provide a reliable technique, accurate and consistent results for rapid identification among species (Ryan and Esa 2006). The use of molecular method has the advantage over morphological and biochemical traits due to precision in detecting relationships among and within population of various organisms. In addition, it requires only a very small quantity of DNA from any tissue (gill, scale, fin clip or muscle) for analysis using polymerase chain reaction (PCR) technology (Avise, 1994). According to Ward (2000), this advantage is crucial particularly for endangered, protected, or remnant populations or species where complete sample is required for genetic.

Deoxyribonucleic acid (DNA) is a complex molecule within a living cell. It was first isolated in 1869 by a German biochemist named Frederich Miescher and in 1953, a double helix shape of DNA has been proposed by James Watson and Francis Crick (Rettner, 2013). DNA is the hereditary material in humans and almost all other organisms. Most DNA is located in the cell nucleus (where it is called nuclear DNA); only a small amount of DNA can be found in the mitochondria cell (where it is called mitochondrial DNA or mtDNA). Moreover, according to Mohd-Shamsudin *et al.* (2011), molecular DNA based marker especially mitochondrial DNA markers offer a better resolution of systematic relationship among species.

1.1.1 Mitochondrial DNA

Mitochondria is tiny in structure, surrounded by two membranes and it located at outside the nucleus, but still within the cell. The main function of mitochondria is to produce the energy required for the cell. Mitochondria have own DNA molecules, which entirely separated from nuclear DNA known as mitochondrial DNA (mtDNA). The feature of high copies number per cell made mtDNA is easier to retrieve from low-quantity and/or degraded biological samples than nuclear DNA. Moreover, in most species, mtDNA is uniparentally inherited without recombination.

Animal mtDNA is a small, extrachromosomal genome, typically ~16 kb in size. Animal mtDNA contain 36 or 37 genes, which are two for ribosomal RNAs (12S rRNA and 16S rRNA), 22 for transfer RNAs (tRNAs) and 12 or 13 subunits of multimeric proteins of the inner mitochondrial membrane (Boore, 1999) (Figure 1). MtDNA possess own organelle specific DNA which play a role in replication, transcription and translation systems. Mitochondrial DNA is histone-free, has limited repair ability, and therefore has a relatively

high mutation fixation rate (5–10 times than nuclear DNA) (Jansen *et al.*, 2000). Although mtDNA has evolved faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships.

Recently, mitochondrial genes such as cytochrome oxidase B (cyt B), cytochrome oxidase I (COI), hypervariable region and ribosomal genes 12S rRNA and 16S rRNA have been proposed as alternatives DNA markers for species identification. Both nuclear and mtDNA is available for species identification. The feature of high mutation rate occupied by mitochondria allows the discrimination of even closely related species (Kocher *et al.*, 1989). MtDNA 12S rRNA and 16S rRNA genes also have been used as a molecular markers in identification of mammals, birds, shrimp, and other species using species-specific primers (Yang *et al.*, 2014). According to Di Finizio *et al.* (2007), ribosomal RNA genes also extensively applied to solve problems of food authentication and widely utilized in phylogenetic reconstructions for a variety of fish species at different taxonomical levels.

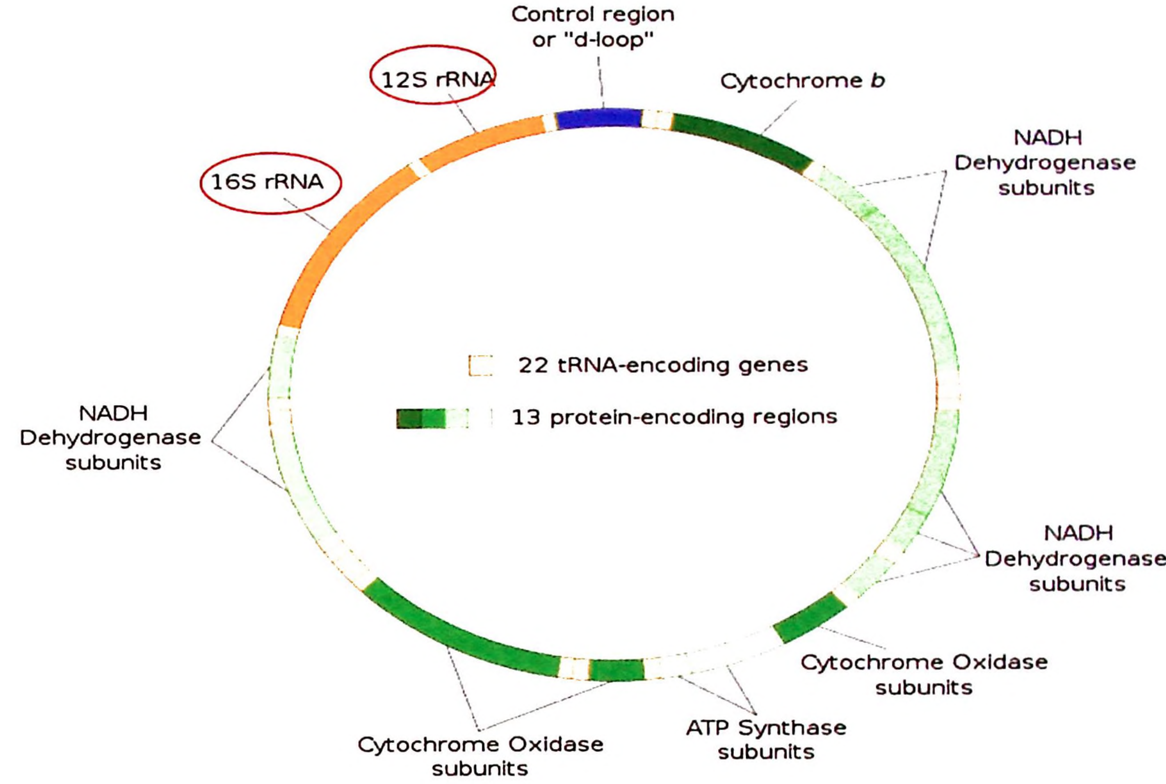

Figure 1.1: Representative diagram of an animal mitochondrial DNA genome showing the ribosomal gene 12S rRNA and 16S rRNA (red circles) (http://upload.wikimedia.org/wikipedia/commons/thumb/3/3e/Mitochondrial_DNA_en.svg/300px-Mitochondrial_DNA_en.svg.png)

1.1.2 Ornamental fish

Ornamental fish is described as an aquatic animal kept in the aquarium or a garden pool and not suitable for fishing. Ornamental fish comprises fishes, invertebrates such as coral, crustaceans (e.g., crabs, hermit crabs, shrimps), mollusks (e.g., snails, clams, scallops), and also live rock (Livengood and Chapman, 2007). More than 550 varieties of ornamental fish belonging to 250 species are cultured in Malaysia. Many ornamental fish belong to freshwater habitat. In Malaysia, 95% of cultured ornamental fish are exported .Ornamental fish consists of nine families namely *Cyprinids, Cobitids, Cypinodontidss, Anabantidis, Poecilids, Characins, Cichlids, Osteoglossid and Callchthyids. Peocilids* family become the second highest ornamental fish exported after aquatic plant (Departmnet of Fisheries Malaysia, 2008)

Poecilids also known as *Poeciliidae* belong to a single family of freshwater fish known as the *Poeciliidae* (Monks, 2007). The family *Poeciliidae* or the subfamily *Poeciliinae* as classified by Parenti in 1981, is a widespread and diverse group of small-sized fishes that includes 22–29 genera and more than 200 species (Lucinda, 2003). *Poeciliidae* is one of four groups of *Cyprinodontiform* order fishes that evolved internal fertilization (Parenti, 1981). *Poeciliidae* family consists of guppies, mollies, platies, and swordtails. All *Poeciliidae* family shares a similar body shape with a distinctly upturned mouth, and in most cases the males are substantially smaller than the females (Monks, 2007). Males also tend to be more brightly colored, and also have a modified anal fin known as a *gonopodium* used to fertilize the female. Guppies and mollies belong mostly to the genus *Poecilia* while the swordtails and platies belong to the genus *Xiphophorus*.

1.2 Significances of Study

The ornamental fish is popular as aquarium fish in Malaysia due to their uniqueness of physical appearance, size and color. It is important to study on genetic affinity of the ornamental fish especially *Peociliidae* family since there are insufficient studies on assessment of genetic background of this fish family. It has been reported that in *Peociliidae* family, hybridized species occurred between guppy-molly (Monks, 2007). Therefore, by using molecular DNA method, hybridized species can be distinguished from wild type species. This is the first study on genetic background of ornamental fish from *Peociliidae* group conducted using mtDNA ribosomal genes 12S rRNA and 16S rRNA.

1.3 Objectives

1.3.1 General objective

a. To characterize the ornamental fish species (*Poeciliidae*) based on mitochondrial DNA 12S rRNA and 16S rRNA genes.

1.3.2 Specific objectives

a. PCR amplification of partial sequencing mtDNA 12S rRNA and 16S rRNA genes of ornamental fish species.
b. To analyze the sequencing data for polymorphism.
c. To study the genetic affinity among them.

1.4 Flow Chart of the Study

Sample Collection

DNA Extraction from tissue

Agarose Gel Electrophoresis of extracted DNA

DNA Quantification using Spectrometer

PCR amplification of partial sequence of mtDNA 12S rRNA and 16S rRNA

Agarose Gel Electrophoresis of PCR Products

Purification of PCR product

Sequencing and data analysis

CHAPTER 2 : LITERATURE REVIEW

Recently, a number of new methods have been developed and utilized for fish species identification. According to Bossier (1999), morphological features are more suitable for identification of fresh fish but in situation of processed fish this method is not suitable since they do not retain enough morphological characteristics for identification purpose. Traditional method based on separation and characterization of specific protein using electrophoretic techniques such as isoelectric focusing (IEF) (Rehbein, 2004) and capillary electrophoresis (CE) (Kvasnicˇka, 2005) are proved to be reliable and easy to be used in food identification, but not suitable for heat-treated products as thermal treatment as in canning, smoking or drying leads to irreversible loss of solubility (Quinteiro *et al.*, 1998; Rehbein *et al.*, 1999). Application of techniques based on the analysis of nucleic acids such as mitochondrial DNA or nuclear DNA offer an advantages over protein-based techniques since they are not dependent on tissue source, age of the individual or/and sample damage (Irwin *et al.*, 1991; Yang *et al.*, 2014).

As mentioned by Teletchea (2009), there are three advantages of using DNA based technique over protein analysis. First, DNA is more resistant and thermostable than proteins though DNA might be altered by various processes such as canning and heating. Second, DNA could potentially be retrieved from any substrate because it is present in almost all cells of an organism. Third, DNA provides more information that protein due to the presence of non-coding region and the degeneracy of genetic code. Due to that DNA-based identification techniques have been proved to be powerful tool for fish species identification. Most importantly, a DNA-based identification system is more rapid and informative than the

traditional morphological-based methods and can be a crucial tool especially in highly critical field of conservation biology (DeSalle and Amato, 2004).

As stated by Kochzius (2009) and Teletchea (2009), mitochondrial DNA (mtDNA) genes are promising markers for fish species identification when compared to nuclear genes due to its several special features of mtDNA. Mitochondrial DNA occupy high copy number in each cell as well as small in size 15-20 Kb which made mtDNA successful to recover from limited or degraded samples (Hubert *et al.*, 2008). The features of maternal inheritance pattern without recombination (Sangthong and Jondeung, 2003) and rapid mutation rate made mtDNA suitable as a tool for studying phylogeny and genealogy of taxa through matrilineage (Moore, 1995). All these mtDNA characteristics make it useful for analysis of processed samples (Alberts et al., 1990). Several mtDNA markers such as cytochrome B, cytochrome oxidase I, hypervariable region and ribosomal genes (12S rRNA and 16S rRNA) have been used in species identification.

Mitochondrial DNA cytochrome oxidase I (COI) has been used as a standard genetic marker for fish identification as well as for birds, reptiles and mammals (Yang *et al.*, 2014). A short segments 648 bp from 5' end of the mitochondrial gene cytochrome oxidase I (COI) gene has been used as a uniform region for species identification (Fre´zal and Leblois, 2008). The use of standard region system in animal species identification known as DNA barcoding (Herbert *et al.*, 2003). This standard regions DNA barcoding represents a more standardized method that can be applied across a broader swath of living taxa (Herbert *et al.*, 2003). Previous study had shown that DNA barcode widely applied in the identification of marine and freshwater fishes (Steinke *et al.*, 2005; Ward *et al.*, 2005), forensic sciences (Dawnay *et al.*, 2007), molecular systematic (Hardman, 2005) as well as seafood products identification (Botti and Giuffra, 2010; Lowenstein *et al.*, 2010; Steinke *et al.*, 2009). Disadvantages of

DNA barcoding is that it may not be effective in phylogenetic tree reconstruction (Wong *et al.*, 2011) and species with conserved sequence (Kitano *et al.*, 2007).

Study has shown that mitochondrial cytochrome b and ribosomal RNA have been extensively applied to solve problems of food authentication (Lockley and Bardsley, 2000) as well as to phylogenetic reconstructions for a variety of fish species at different taxonomical levels (Di Finizio *et al.*, 2007). Recently, mitochondrial cytochrome b and ribosomal genes has been applied in detection of fraudulent substitutions of commercially valuable fishes with others less expensive in manufactured fish products (Di Finizio *et al.*, 2007). Study by Ludwig *et al.* (2004) has showed that the application of small subunit of ribosomal RNA gene as a standard method for identifying microbial organisms (Ludwig *et al.*, 2004).

The mitochondrial ribosomal genes including 12S rRNA and 16S rRNA and nuclear ribosomal genes such as 28S rRNA, 5.8S rRNA and 18S rRNA are widely used as genetic markers for phylogenetic analyses (Wang *et al.*, 2000). According to Hillis and Dixon (1991), ribosomal RNA sequences have been used to infer phylogenies across a very broad spectrum, from studies among the lineages of life to relationships among closely related species and populations. In fact, these two mitochondrial ribosomal genes share similar structures and functions in organisms ranging from bacteria to humans, despite their sequences exhibit numerous inter- and intraspecific nucleotide variations (Yang *et al.*, 2014). Previous study has showed that by using species specific primer of mitochondrial 12S rRNA and 16S rRNA they are successfully identify mammals, birds, shrimps and other species (Yang *et al.*, 2014). As suggested by Yang *et al.* (2014), it is necessary to perform species identification with both the 12S rRNA and the 16S rRNA mitochondrial genes since these two sequences are highly conserved

DNA sequence-based identification utilizes the refined Sanger sequencing method which is still the ‘‘gold standard’’ but requires samples that contain DNA of only one specimen (Kochzius *et al.*, 2010) . Contrast to next generation sequencing methods which are enabling the analysis of mixed samples, but need highly sophisticated and expensive equipment (Kochzius *et al.*, 2010). Currently, DNA sequencing analysis is the most used method for molecular species identification (Pereira *et al.*, 2008).

CHAPTER 3 : METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

All the chemicals, reagents, 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 1ml deionized water.

3.1.2.2 10X Tris Borate EDTA buffer (10X TBE)

TBE buffer stock was prepared by dissolving 107.8 g Tris base, 7.4 g EDTA $(NH_4)_2SO_4$ and 46 g Boric acid in 800 ml of ddH_2O. The pH was adjusted to 8.3 by adding Boric acid. The solution was made up to 1000 ml by adding ddH_2O. 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_2O.

3.1.2.4 70% Ethanol

The solution was prepared by mixing 30ml of ddH_2O with 70ml of absolute ethanol and was stored at ambient temperature.

3.1.2.5 Ethidium bromide (10mg/ml)

Ethidium bromide solution was prepared by dissolving 1 g of ethidium bromide in 100 ml ddH_2O and stored in amber colored bottle.

Table 3.1: List of all chemicals, reagents, commercial kits and consumables that used in this study

Reagents	**Company Name/Supplier**
Magnesium Chloride ($MgCl_2$) 10X PCR $(NH)_2SO4$ 5U/µl DNA Taq Polymerase Primers 10mM dNTPs	Biosyintech, Fermentas, Lithuania Biosyintech, Fermentas, Lithuania Sigma, Singapore Biosyintech, Fermentas, Lithuania Biosyintech, Fermentas, Lithuania
Chemicals	**Company Name/Supplier**
Agarose gel powder Denatured absolute ethanol (95%) Boric Acid 100 base pair Allelic ladder Tris EDTA (Ethylenediaminetetaacetic acid) Ethidium Bromide	Promega, USA Teraslab Sdn. Bhd. Germany Invitrogen Corperation, USA Sigma, Singapore Sigma, Singapore
Consumable	**Company Name/Supplier**
Micropipette 10µl, 200µl, and 1000µl Pipette Latex Gloves Microcentrifuge tube 1.5 µl Thin wall PCR tube 0.2 ml	Gilson,USA Teraslab Sdn. Bhd. Teraslab Sdn. Bhd. Odonixx Labware, USA Odonixx Labware, USA
Kits	**Company Name/Supplier**
QIAamp DNA Mini Kit Gene All PCR SV Kit QIAquick® PCR Purification Kit	QIAGEN, USA General Biosystem, Seoul, Korea QIAGEN, USA

3.1.3 Sample Collection

In this study, five fish samples from *Poecilidae* family were selected. Each fish species was duplicates for the confirmation of the genetic purpose. *Poecilidae* family consist of three distinct genuses of fish species (Table 3.2). The scientific name for each of the fish species was referred to the "Prosedur Operasi Piawai: Pemeriksaan Konsaian Ikan Hiasan Air Tawar Yang Dimport" by Malaysian Quarantine and Inspection Services (2013).

Table 3.2: List of *Poeciliidae* family fish species

Genus Name	Common Name	Scientific Name	Picture
Limia	Cuban Limia fish	*Limia vitatta*	
Poecilia	Guppy fish	*Poecilia reticulata*	
	Black Molly fish	*Poecilia sphenops*	
Xiphophorus	Platy fish	*Xiphophorus maculatus*	
	Swordtail fish	*Xiphophorus hellerii*	

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 minute, 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 leaved 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 in -20 °C for further used.

3.2.3 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).

3.2.4 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.

3.2.5 Primers

In this study, the published primers were used for PCR amplification of mitochondrial DNA 12S rRNA and 16S rRNA gene (Palumbi *et al.*, 1996). The details of the primers sequences were displayed in Table 3.3.

Table 3.3: List of the primers used in this study

Primers	Primer sequences (5'-3')	Primer Tm	Region
L1067F	AAACTGGGATTAGATACCCCACTAT	67 °C	12S rRNA
H1478R	GAGGGTGACGGGCGGGCGGTGTGT	67 °C	
L2510F	GCCTGTTTATCAAAAACAT	58 °C	16S rRNA
H3080R	CCGGTCTGAACTCAGATCACGT	58 °C	

3.2.6 PCR amplification of mtDNA 12S rRNA and 16S rRNA gene

Mitochondrial DNA 12S rRNA and 16S rRNA genes were PCR amplified using specific primers mentioned in Table 3.3. PCR amplification was carried out in 0.2 ml thin wall PCR tubes containing the following mixtures: 2 µl of 10X PCR buffer $NH_4(SO_4)_2$, 2 µl of 25 mM $MgCl_2$, 0.32 µl of 10mM dNTPs mix, 3 µl of genomic DNA (10ng/ µl), 1 µl of 10 pmol of each primer, 0.3 µl of Taq polymerase (5U/ µl) and 10.38 µl of ddH_2O was added to make up final volume 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, final extension at 72 °C for 5 minutes and hold at 4 °C. The amplified PCR product was kept at 20 °C until further use.

3.2.7 Agarose Gel Electrophoresis of Amplified PCR Products

A 1% agarose gel was prepared by adding 1 g of agarose 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 1.0 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 2 µl of PCR products was mixed with 2 µl Orange G dye and was loaded into agarose gel. 2 µl of 100 base pair DNA ladder was loaded into the first well. The electrophoresis was performed at 90V for 45 minutes. The presence of PCR products were visualized under UV light using Image Analyzer.

3.2.8 PCR Product Purification

PCR product was purified using Gene ALL PCR SV kit. A total of 5 volumes of PB buffer were added to 1 volume of PCR product. The PCR product mixture was transferred into SV column attached to 2 ml collection tube and centrifuged at 13000 rpm for 30 seconds.

The filtrate in the collection tube was discarded and the SV column was placed back to the same collection tube.

The process was continued by adding 700 µl buffer NW into SV column and was centrifuged at 13000 rpm for 30 seconds. The filtrate in the collection tube was placed back to the SV column and again centrifuged at 13000 rpm for additional 1 minute. The SV column was then transferred into a new 1.5 ml centrifuge tube and 30 µl of EB buffer was added and was left at ambient temperature for 1 minute before centrifuging at 13000 rpm for 1 minute. The purified PCR product was analyzed by agarose gel electrophoresis prior sending for sequencing.

3.2.9 Analysis of the Sequences

The polymorphisms reported in this study were analyzed using MEGA 4 and BioEdit ver. 4.0 software against the Reference Sequence obtained from GeneBank (www.ncbi.nlm.nih.gov/pubmed). The presence of polymorphism such as transition, transversion, insertion and deletion were recorded. The neighbor-joining tree was constructed using Kimura 2-parameter distance model via Mega 4 software.

CHAPTER 4 : RESULTS

4.1 Agarose gel electrophoresis of extracted genomic DNA.

Figure 4.1: Electropherpgram of extracted HMW DNA

Lane 1: *Xiphophorus hellerii*

Lane 2: *Xiphophorus maculatus*

Lane 3: *Poecilia reticulata*

Lane 4: *Limia vitatta*

Lane 5: *Poecilia sphenops*

Lane 6: 1 kb DNA ladder

4.2 Agarose gel electrophoresis of amplified PCR products

PCR amplification of mtDNA 12S rRNA and 16S rRNA gene was performed using primers 12S rRNA F1 and R1 and 16S rRNA F1 and R1 respectively. PCR amplification of mtDNA 12S rRNA gene was generated amplicons at about 410 bp (Figure 4.2) and about 570 bp of PCR products was observed for PCR amplification of 16S rRNA gene (Figure 4.3).