Preliminary Molecular Study on Yellow Pufferfish *(Xenopterus naritus)* and Green-Spotted Pufferfish *(Tetraodon nigroviridis)*

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DECLARATION

No portion of the work referred in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
mtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
RE	Restriction Enzyme
Cyt b	Cytochrome b
CTAB	Cetyltrimethylammonium Bromide
EtOH	Ethanol
$MgCI_2$	Magnesium Chloride
dNTP mix	Deoxyribonucleic Triphosphate Mix
ddH_2O	Deionized Distilled Water
NaCI	Sodium Chloride

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Pufferfish can be divided into 3 families namely Triodontidae, Tetraodontidae and Diodontidae. Family Tetraodontidae consists of 176 species and 27 genera (Dawes, 2005) which include the freshwater, brackish and marine species. They can defend themselves from other predators by body inflation. Pufferfish body especially the viscera, is made up of tetrodotoxin (Nelson, 2006). It is also known as blowfish, swellfish and globefish. Pufferfish is mostly found in warm, shallow tropical and sub-tropical water of west India, Japan, China, Philippines, Mexico, Taiwan and as well as Southern Asia. Freshwater species of pufferfish spawn in pairs whereby the male will guard the eggs or fry (Dawes, 2005). Invertebrates, fishes or plants (such as algae) are the common types of food consumed by the pufferfish.

Yellow pufferfish, *Xenopterus naritus* is a freshwater species which is discriminate from others type of puffer by having prominent yellowish, bronze or golden colouration especially towards the lower part of the body (Albert and Annie, 2004). In Malaysia, it is found to be abundance in the state of Sarawak in areas of *Batang Sadong*, *Batang Lupar*, *Batang Krian* and *Batang Saribas* besides other Asian countries such as China, Vietnam, Thailand and Burma (Berra, 2001). However, Atack (2006) had reported that the yellow pufferfish also known as

“Buntal Pisang” is a coastal as well as estuarine species of pufferfish whereby it can be found at *Sg. Kuap* (Kuching) and most of the brackish rivers in Sarawak. Yellow pufferfish is known to be omnivorous species. Eggs are being laid in nest during spawning season.

Green-Spotted pufferfish, *Tetraodon nigroviridis* is another type of pufferfish with a number of spots distributed at the upper part of its body. It is a brackish water species. In Sarawak, it can be found at *Sungai Bako* and known as “Buntal Bako” which is representing the river where it is found (Atack, 2006). Besides that, it can also be found in India, Indonesia, Sri Lanka and Thailand. It is a carnivorous species of pufferfish. Green-spotted pufferfish will show aggression towards its own kind. The spawning event occurs in the brackish water whereby the eggs are laid on particular substrate and will be guarded by the male.

Morphological identification has its limitation in identify particular species of organisms especially those from the processed, degraded or incomplete samples. Cheng *et al*. (1975) had reported that different developmental stages (for example, different ages group will have different colourations) had contributed to different species names given to the same species of pufferfish. Moreover, the physical appearances (morphological characteristics) of particular species of pufferfish, on the other hand is incapable of revealing any genetic variation among its species over time.

A fossil record is another application used in determining the evolutionary time of particular species of fish. However, Yamanoue *et al.* (2006) reported that the records are incomplete because the molecular divergence time estimated often surpass the time estimated through the used of fossil records. For example, divergence time between *Torafugu* and green-spotted pufferfish with molecular clock of *Cytochrome b (Cytb)* gene is 18-30MYA in comparison of 84.4MYA with fossil records used. Thus, in order to overcome this limitation, genetic study had been applied to gain more robust data on all kind of organisms.

Deoxyribonucleic acid (DNA) had been known as the storage form of genetic information in an organism and is used in the application of molecular techniques mainly on the analysis of evolutionary divergence in vertebrates. This genetic information had particular configuration which in turn form the variation among the individuals in a population. Therefore, no individuals from the same species will have exactly the same set of DNA sequences. In order to make the genetic analysis work easier, selection on the genetic material used in analysis is important and must be compatible with the research purposes. *Hsing et al.* (2001) used the partial *Cyt b* sequence of mitochondrial DNA (mtDNA) instead of the whole *Cyt b* sequence in identification of endangered animals. This is because endangered animals could be available commercially in many forms including powder form. DNA from powdered bone samples is usually very limited and can easily be degraded.

There are a few studies done on the molecular characteristic of pufferfish mostly from the *Takifugu rubripes* species (Fernandes *et al.*, 2007; Kai *et al.*, 2005; Neafsey & Hartl, 2005; Song *et al.*, 2001; Caldas *et al.,* 1998; Trower *et al.,* 1995), *Takifugu pseudommus* (Song *et al.*, 2001), *Tetraodon nigroviridis* species (Yamanoue *et al.*, 2006; Gen *et al.*, 2006), *Tetraodon fluviatilis* species (Mandrioli *et al.*, 2000) and more. These studies basically involve the studies of genetic variation, population genetics and phylogenetic.

1.2 Statement of Problems

Even though there are a lot of studies done on puffer, there is still lacking of molecular study on the *Xenopterus naritus* and *Tetraodon nigroviridis* especially those from the Sarawak Rivers. *Sungai Bako* is distant from *Batang Saribas* in term of geological allocation. Genetic studies on these species might reveal vital information regarding its existence or evolution as well by natural selection and geographical isolation. Moreover, yellow pufferfish of the *Batang Saribas* had been heavily harvested in recent years for human consumption. In future, these molecular genetic studies might be useful in overcoming the problem of over-harvesting of yellow pufferfish. Molecular data is also useful for rapid species identification.

1.3 Objectives

Objective of this study were:

(1) To optimize the molecular biology protocols namely Total Genomic DNA Extraction, Polymerase Chain Reaction (PCR) and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) for the yellow pufferfish and green-spotted pufferfish from Sarawak.

(2) To establish molecular markers for the yellow pufferfish and green-spotted pufferfish from the Sarawak rivers.

Upon completing this study, the established molecular marker might be useful in future rapid species identification.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy

Phylum: Chordata
Class: Actinopterygii
Order: Tetraodontiformes
Family: Tetraodontidae
Genus: *Xenopterus*
Species: *naritus*
Common name: Yellow Puffer

Figure 2.1.1: Taxonomy of Yellow Pufferfish**.**

Phylum: Chordata
Class: Actinopterygii
Order: Tetraodontiformes
Family: Tetraodontidae
Genus: *Tetraodon*
Species: *nigroviridis*
Common name: Spotted Green Puffer

Figure 2.1.2: Taxonomy of Green- Spotted Pufferfish.

(Source: http://www.fishbase.org)

2.2 General Facts

2.2.1 Molecular Study

Pufferfish is being selected as a model used for the study of vertebrate's genome because its genome is compact and lack of repetitive sequences (Mandrioli *et al.,* 2000). Thus, comparing the genomes of different species of pufferfish with others organisms will guide future approaches to the understanding of gene function, regulation and evolution (Yamanoue *et al.*, 2006).

2.2.2. Pufferfish Study in Sarawak

According to Albert and Annie (2004), there is a need to assess the *Xenopterus naritus,* yellow puffer from the *Batang Saribas,* Sarawak in terms of their ecology and biology. This information is important for future breeding of this species. The use of sophisticated technology in harvesting the fish practised at *Batang Saribas* might cause depletion of that species. Comprehensive sets of data are needed in order to solve this problem effectively.

2.3 Selection of Genetic Materials in Analysis

There are a number of sources of genetic materials use in the molecular analysis; nucleus, mitochondrial (animals) and chloroplast (plants). According to Farias *et al*. (2001), the mitochondrial DNA (mtDNA) is frequently used in evolutionary study. There are a few reasons for this explanation: (1) mtDNA evolves at a rapid rate compare to other genes, (2) it is maternally inherited and (3) only a single mtDNA genotype exists in an individual. For instance, Takehana *et al.* (2003) had revealed that 82.8% of substitutions occur on the third codon position, 13.5% on the first codon position and 3.7% on the second codon position of the *cytochrome b* sequence in Medaka (*Oryzias latipes*) fish. Gen (2006) reported that the mitochondrial genome size of *T. nigroviridis* is 16,462bp long whereby the *cytochrome b* gene is 1143bp long.

2.4 Molecular Biology Techniques

2.4.1 Total Genomic DNA extraction

Total genomic DNA can be extracted from variety of body part with a range of methods; the liver cells of *Tetraodon fluviatilis* with the standard phenol-chloroform procedures (Mandrioli *et al.*, 2000), muscular tissues of pufferfish with phenol-chloroform method (Ishizaki *et al*., 2005), muscular tissues of *Tetraodon nigroviridis* with Qiagen DNeasy tissue kit (Qiagen) (Yamanoue *et al*., 2006), tissue samples of *Tetraodon rubripes* and *Tetraodon pseudomus* using NucloeoSpin columns (BD Biotech) (Neafsey & Hartl, 2005), muscle tissue of the genus *Takifugu* pufferfish with phenol-chloroform and chloroform-isoamyl method (Song *et al*., 2001) and muscle tissue or whole body of juveniles of *Tetraodon rubripes* by using standard phenol-chloroform technique (Kai *et al*., 2005).

2.4.2 Polymerase Chain Reaction (PCR)

Grunenwald (2003) had reported that there were a few variables that could optimize the PCR amplification reaction namely annealing temperature, primer concentration, template concentration, $MgCI_2$ concentration, extension time and cycle number. Besides that, selection of the forward and reverse primer also plays a role in optimizing the PCR amplification. Dieffenbach & Dveksler (1995) stated that, the primers should exactly complementary to the template DNA. For example, forward primer of

16SarL (5’-CGCCTGTTTATCAAAAACAT-3’) and reverse primer of 16SbrH (5’-CCGGTCTGAACTCAGATCACGT-3’) had been used by Song *et al.* (2001) and Ishizaki *et al.* (2006) in species identification of pufferfish by amplifying the *16S* gene of the mitochondrial DNA (mtDNA). In addition, GludG-L14724 (5’-TGACTTGAARAACCAYCGTTG-3’) a 21bp forward primer and CB2H-H15175 (5’-CCCTCAGAATGATATTTGTCCTCA-3’) a 24bp reverse primer also commonly used to amplify partial of the *Cytochrome b* mtDNA gene in aquatic organisms. (Martin & Palumbi, 1993).

2.4.3 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) is another technique used in genetic analysis. It functions through the reorganization of cutting sites in the targeted DNA sequence. Different DNA sequences will require specific types of restriction enzyme (RE). There is a lot of RE available commercially and has successfully provided PCR-RFLP profiles. For example, *CfoI* and *BstF5I* (Mirhendi *et al.*, 2005), *BsiYI* and *NdeII* (Pardo & Villareal, 2004), *Eco32I* and *Eco105I* (Aranishi *et al.*, 2005), *SmaI*, *NcoI* and *ApaI* (Hubalkova *et al.*, 2007) as well as *RsaI, AluI,* and *DdeI* (Goswami *et al.*, 2005). Longer targeted DNA fragment require longer time for complete digestion with the RE.

2.4.4 Gel Electrophoresis

Gel electrophoresis is important in deciding the succession of particular technique that applied because it will show the band of fragments with different sizes (measure in base pair) in the gel; the most commonly used gel is the agarose gel. Larger molecule of DNA will move slower compare to small molecule of DNA through the gel matrix. High concentration of agarose gel used will results in low resolution while low concentration of agarose gel used will have a high resolution. *Song et al.* (2001) reported that, 1 % agarose gel was suitable in study to separate the amplified *16S* gene fragment (598bp). In comparison, for separation of smaller DNA fragments (for example, less than 254bp after digested with restriction enzyme), a 2% agarose gel was applied (Ishizaki *et al.,* 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

Batang Saribas and *Sungai Bako* were selected as sampling sites because (1) they are easy to access by road, (2) previous study and local people reported about the abundance of pufferfish in these areas and (3) capture methods used by the local people proved to be successful in collecting the pufferfish.

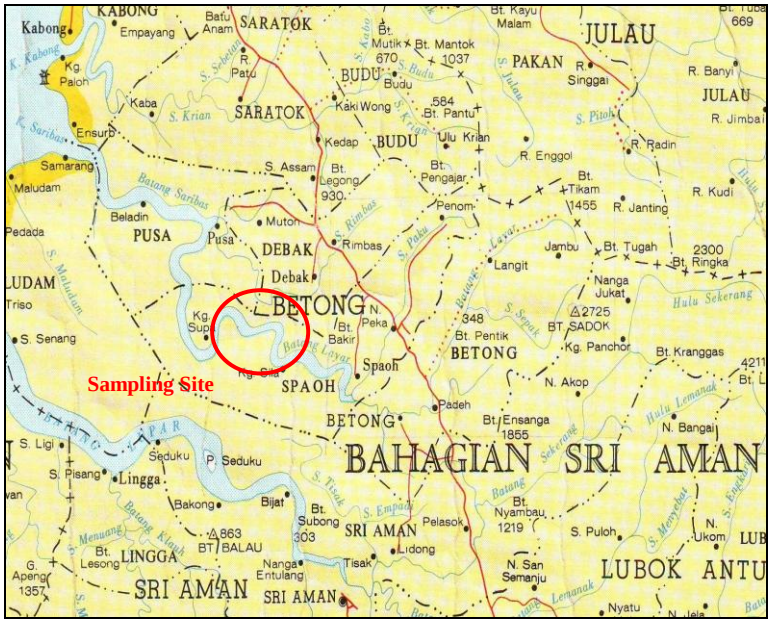

Figure 3.1.1: Map showing the *Batang Saribas*, sampling site for the *Xenopterus naritus* (yellow pufferfish).

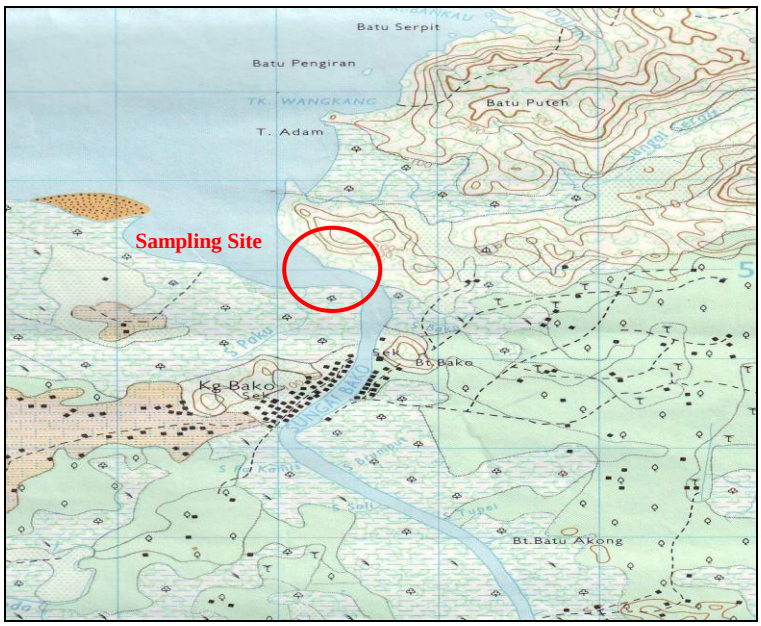

Figure 3.1.2: Map showing the *Sungai Bako*, sampling site for the *Tetraodon nigroviridis* (green-spotted pufferfish).

(Adapted from Aquatic Vertebrate Laboratory Unimas)

3.2 Samples Collection

Ten individuals of each yellow pufferfish *(Xenopterus naritus)* (which its size ranges between 25 to 30cm for the female and 10 to 15cm for the male) and green-spotted pufferfish (*Tetraodon nigroviridis)* (which its size ranges from 10 to 12cm for both sexes) were collected from *Bt. Saribas* and *Sg. Bako* respectively with help from local fishermen after failed in attempts to catch the pufferfish using gill nets. Pufferfish were kept in aerated tank while they were transported back to Aquatic Vertebrate Laboratory of Unimas. However, some the fish were unable to sustain the induced pressure, alternative solution for maintaining the DNA quality was carried out namely storing the samples in the cooler box prior to transferring back to Unimas. Photographs were captured *in-situ* for record as well as for fish identification. In the laboratory, small portion of the samples tissue (approximately 10 cubic millimetres) was transferred into a blank eppendorf tube and eppendorf tube with 90% ethanol, followed by freezing in -20^{o}C for further use.

3.3 Methodology

3.3.1 Total Genomic DNA Extraction

CTAB protocol modified from Grewe (1993) was used in the total genomic DNA extraction of pufferfish tissue. Approximately 1-2 cubic millimetres of fish tissue was minced nicely and transferred into 1.5 ml labeled eppendorf tube (H_1) which contains 700µl of CTAB buffer. A volume

of 5µL of Proteinase K (20mg/ml) was transferred into tube H_1 and followed by incubation in water bath at 60^0C for less than one hour (depend on the tissue sizes; larger size requires more longer time to completely digested by the Proteinase K) until the fish tissue was completely dissolved and digested. Then, a volume of 700µL of chloroform-Isoamyl alcohol was added into tube H_1 and shake gently for 2 minutes. Centrifugation was carried out at 13000 rpm (round per minute) for 10 minutes and 3 layers of phase were formed. A volume of 500µL of upper aqueous phase of the centrifuged tube H_1 was transferred into a new labeled tube (H_2) and mixed by inversion. Then, a volume of 500µL of absolute (100%) ethanol (EtOH) was added into tube H_2 and stored in -20^OC freezer for 30 minutes. Next, tube H_2 was centrifuged at 13000 rpm (round per minute) for 10 minutes upon taken out from the refrigerator. Tube H_2 was removed from the machine centrifuge and absolute EtOH was poured out. A volume of 500µL of cold 70% ethanol and 25µL of 3M Sodium Chloride (NaCI) were added into tube H_2 and mix by inversion. Tube H_2 was centrifuged at 13000 rpm (round per minute) for 10 minutes. EtOH was poured out and cool to room temperature for 10 minutes as to dry the pellet of DNA. DNA pellet was re-dissolved in 30µL (depend on the sizes of the pellet) of deionized distilled water (ddH_2O) and store at -20^OC freezer for future use in gel electrophoresis.

3.3.2 Polymerase Chain Reaction (PCR)

Master Mix consists of all the appropriate concentrations and amount of reagents use in PCR reaction was put into an eppendrof tube (Table 3.3.2.1). Reaction number is highly depending on the desirable number of samples. For example, 5 samples of pufferfish were used. Six (5 samples plus 1 negative control) times 22.8μL were equal to 136.8μL of reagents in the master mix tube. This would mean that each aliquot tube had 22.8μL of master mix solution. However, 0.2μL *Taq* polymerase and 2μL of DNA extraction product were added separately into each aliquot tube to form a total volume of 25μL reagents. On the other hand, 2μL of ddH_2O was added into the negative control tube instead of template DNA. Negative control tube was important in ensuring if any contamination occurred during the mixing steps.

Table 3.3.2.1: Master Mix profiles for *Cyt b* mtDNA and *16SI* mtDNA genes.

Master Mix:	*Cyt b* mtDNA Gene	*16S* mtDNA Gene
Deionized Distilled Water (ddH_2O)	15.3μL	15.3μL
10 x Reaction Buffer	2.5μL	2.5μL
Deoxyribonucleic Triphosphate (10mM dNTP) Mix	1.0μL	1.0μL
Magnesium Chloride (50mM $MgCI_2$)	1.50μL	1.50μL
Forward Primer (10nM)	(GludgL)1.25μL	(16Sar)1.25μL
Reverse Primer (10nM)	(CB2H)1.25μL	(16Sbr)1.25μL
Total	22.80μL	22.80μL