GENETIC VARIATION OF *Lagocephalus lunaris* SPECIES PUFFER FISH USING MITOCHONDRIAL CYTOCHROME *b* GENE

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Biotechnology)

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Declaration

I hereby declare that this thesis entitled “Genetic Variation of *Lagocephalus lunaris* Species Puffer Fish Using Mitochondrial Cytochrome *b* Gene” is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature:

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Date:
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List of Abbreviations

*L. lunaris* - *Lagocephalus lunaris*

TTX – tetrodotoxin

mtDNA – mitochondrial DNA

cyt *b* gene - cytochrome *b* gene

PCR - Polymerase Chain Reaction

MgCl₂ - Magnesium chloride

dNTP - Deoxyribonucleotide triphosphates mixture

MEGA - Molecular Evolutionary Genetic Analysis

N-J - Neighbour-Joining

MP - Maximum Parsimony

DnaSP - DNA Sequence Polymorphism
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Genetic Variation of *Lagocephalus lunaris* Species Puffer Fish Using Mitochondrial Cytochrome *b* Sequences

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ABSTRACT

This study examines the genetic characteristic of *Lagocephalus lunaris*, an important indigenous puffer fish species in Sabah. The sequence analyses were carried out using 450 base pairs of the mitochondrial cytochrome *b* gene. A total of 60 samples were collected from two locations in the coastal areas of Sabah; Kudat (Pulau Mandi Darah) and Tawau. Phylogenetic analyses were done using two methods, Neighbor-joining (NJ) and Maximum Parsimony (PM). The result of phylogenetic analysis indicated the presence of two clusters of puffer fish; one consisted of samples only from Kudat, whereas another cluster consisted of samples from Tawau and a single sample from Kudat. A total of 9 haplotypes were found, with only one haplotype (H4) being shared between the two populations. The results also showed the presence of limited genetic variation among the two populations. Overall, this study indicated the potential of using cyt *b* mtDNA gene in detection of small genetic variation, or synonymous substitution between populations.

Key words: *Lagocephalus Lunaris*, puffer fish, mtDNA cytochrome *b*, genetic variation.

ABSTRAK


*Kata kunci: *Lagocephalus lunaris*, ikan buntal, gen mtDNA sitokrom *b*, variasi genetik.*
1.0 Introduction

Recent evidence showed that a great number of vertebrate species have been designated at risk and DNA-based studies on threatened or endangered species have been one of the major interests in gathering information on the diversity, conservation biology and population genetics analyses (Avise 1996; Snow and Parker 1998), due to they usually served as food and income resource. Also, puffer fish have become an interesting model among scientists interested in mapping and sequencing vertebrate genomes due to their highly compact genome about 400Mb (Elgar et al., 1996) and their tetrodotoxin. In this study, the genetic variation of Lagocephalus lunaris puffer fish populations from the coastal areas of Sabah will be studied to determine the genetic characteristics of their populations.

According to McGinley (2008), genetic variation is defined as variation in alleles/haplotypes of genes, which occurs in both within and among populations. Variation within population is variations of genomes between members of species, while variation among population is variation between groups of species thriving in different parts of the world as a result of genetic mutation or gene flow. Otherwise, geographic variation may be also due to differences in selective pressures, genetic drift or gene flow. Hence, genetic variation is important because it provides the “raw material” for natural selection (McGinley, 2008) which assists in maintaining genetic variation in balanced polymorphisms and accesses the studies of genetic diversity.

Moreover, many molecular techniques have been developed to measure the genetic variation of population genetic in various animal taxa (Hoelzel and Dover, 1991), such as DNA sequencing, Restriction Fragment Length Polymorphism (RFLP), isozymes electrophoresis, microsatellites and Random Amplified Polymorphism DNA (RAPD).
DNA sequencing is one of the most powerful tools for measure of genetic variation among fish population and species (Hoelzel and Dover, 1991).

The choices of genetic marker for inferring population connectivity and isolation can be grouped into two categories, frequency markers and sequence markers. Occasionally, mitochondrial DNA sequences have usually served as sequence markers to date, which derive their power from the ability to infer relationship between alleles (Michael, 2009). Mitochondrial DNA sequences are frequently utilized for inferring phylogenetic relationships among organism, due to their properties of having a large copy number, faster evolutionary rate, maternal inheritance, smaller molecular weight, and a lack of introns (Brown et al., 1979; Moritz et al., 1987).

Besides, many studies showed that mitochondrial DNA polymorphisms were useful genetic markers, for studies of genome evolution, stock structure and gene pool conservation of fish species (Ferris and Berg, 1987; Esa and Ryan, 2006). Among genes in the mitochondrial DNA, the cytochrome b gene has been widely used as a genetic marker for population-level identification (Johns and Avise, 1998). Hence, the mitochondrial cytochrome b gene should be able to provide useful insight into the genetic variation and characteristic of Lagocephalus lunaris puffer fish populations.

On the other hands, successful dispersal between populations leaves a genetic wake that can reveal historical and contemporary patterns of connectivity in marine (Swearer et al., 2002). However, marine populations were once seen as demographically open wide, with genetic isolation among populations over the long term hard to come by. Over the past decade, marker-capture studies, chemical tagging studies, and detailed modeling of realistic currents and larval survival have all reinforced a view that successful dispersers may travel far less than their apparent potential, even for species with quite long pelagic
development (Hellberg et al., 2002). The reasons due to several barriers such as larval dispersal, geographic distance and oceanic currents, which may affect connectivity and restrict the genetic drift or gene flow among the marine populations.

Previous differentiation genetic studies suggest that the role of larval dispersal is often tempered by adult ecology, that changes in genetic differentiation with geographic distance are limited by disequilibrium between drift and migration, and that phylogeographic breaks reflect shared barriers to movement in the present more than common historical divisions (Michael, 2009). Recurring complications in the studies also include the presence of cryptic species, selection on markers, and a failure to account for differences in heterozygosity among markers and species.

Hence, the objective of this study is to characterize the genetic structure of two Lagocephalus lunaris puffer fish populations from east coastal areas of Sabah by analyzing the cytochrome b nucleotide sequences of mitochondrial DNA.
2.0 Literature Reviews

2.1 Puffer Fish

Puffer fishes derived from their ability to inflate themselves with water or air when they feel threatened. They are sensitive, scale-less, spineless and specialized fish (Malli et al., 2004). According to Malli et al. (2004), there are three different types of puffer fish those that live in fresh water, such as in ponds, the upper reaches of rivers, lakes, and streams; in the brackish waters of river estuaries, deltas, the lower reaches of a river and mangrove-type habitats or salt water (marine), that is living in the ocean. Unfortunately, puffer fish are the second most poisonous vertebrate in the world, they are belong to the family tetraodontidae due to containing a poison called tetrodotoxin that stored in the liver and reproductive organ (Simon et al., 2009).

3.1.1 Lagocephalus lunaris puffer fish

*Lagocephalus lunaris* is a type of puffer fish that lived in demersal, oceanodromous, or brackish marine. They mostly distributed along the tropical climate areas such as Indo-West Pacific: Red Sea and Persian Gulf to South Africa, east along the continental shores to western Pacific, from Japan to Australia and Southeast Atlantic like south coast of South Africa.

Beside, this type of species was toxic that may lead to lethal when people consuming them (Berry and Hassan, 1973) due to all of its body tissues exhibited toxicity levels exceeding the safety limit for human consumption (10 mouse units/g). Nevertheless, tetrodotoxin (TTX) was identified as the main toxin in this species, which is a lethal marine
toxin with no known antidote (Hwang and Noguchi, 2007) and mainly caused a serious health issue in many coastal areas of the Asia-Pacific region.

Figure 1: Samples of *Lagocephalus lunaris*

In this study, the puffer fish species used as below:

Family: Tetraodontidae

Genus: Lagocephalus

Species: *Lagocephalus lunaris*

Local Name: Butete, Lunartail puffer, blowfish, Buntal Pisang Kasar

2.2 Polymerase Chain Reaction (PCR)

PCR has been employed extensively in the medical and biological sciences since it was formally introduced at the Cold Spring Harbor 51st Symposium on Quantitative Biology (Mullis *et al.*, 1986). The description of the PCR in the mid 1980’s was therefore hailed as a major scientific breakthrough as it become possible to amplify sufficient quantity of
specific region of DNA (Yue and Orban, 2001). The specific region of DNA can be a single gene, part of a gene or a non-coding sequence.

Nevertheless, the PCR was an *in vitro* method for the enzymatic synthesis of specific DNA sequences (Fox *et al.*, 1991). It used two specific oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA during cellular DNA replication with DNA polymerase (Henry, 1992). One of primers being complementary to the 3’ end on one DNA strand and the other one complementary on the 3’ end on the opposite strand. Oligonucleotide primer is single stranded DNA sequences of 20-30 nucleotides that serve as points of attachment for the DNA polymerase (Kolmodin and Birch, 2002)

Besides, PCR process requires a repetitive series cycle of heating and cooling (Henry, 1992) which involved three fundamental steps that defines as one PCR cycle. In each cycle, heating separate the two-stranded DNA as denaturation, two oligonucleotide primes are annealed to complementary segment on the DNA, and DNA polymerase direct synthesis of the complementary DNA sequence between two primers as extends to produce copies that can serve as templates in subsequent cycles. Theoretically, every cycle doubles the amount of target copies. Given the capacity to detect the presence of particular specific gene sequences from extremely minute quantities of DNA (Fox *et al.*, 1991), hence PCR had an enormous impact in both basic and diagnostic aspects of molecular biology.

### 2.3 DNA Sequencing

The first DNA sequences were obtained by academic researchers, using laborious methods based on 2-dimensional chromatography in the early 1970s. Following the development of
dye-based sequencing methods with automated analysis, DNA sequencing has become easier and orders of magnitude faster (Maxam and Gilbert, 1977). The term DNA sequencing refers to optimal method of population comparison in both of high resolution and facilitations interpretation, which determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a molecule of DNA (Hoelzel and Dover, 1991). The double-strand DNA was initially separated by heat, then the primers annealing and extension. However, the difference between them is DNA sequencing involves a termination step, which is a percentage of the copies ended at each of the position in the sequence and accomplish by adding a small portion of dideoxynucleotide in the reaction.

In fact, DNA sequencing provides the greatest resolution for detecting genetic variation and phylogenetic relationship between species and population. Consequently, it is a powerful tool for measurement of genetic characterization among population and species (Henry, 1992). The advent of DNA sequencing has significantly accelerated biological research and discovery. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of the human genome, in the Human Genome Project.

2.4 Methods in Phylogenetic Analysis

The term sequence analysis in biology implies subjecting a DNA or peptide sequence to sequence alignment, sequence databases, repeated sequence searches, or other bioinformatics methods on a computer. Occasionally, there are three basic methods were used to construct the phylogeny analysis, which is neighbor-joining method (a distance based method), maximum parsimony method and maximum likelihood method.
The neighbor-joining method identifies the closest pairs of taxonomic units by the distances between them (Saitou and Nei, 1987; Tamura et al., 2007). A pair of these neighbors is defined to be two units connected through a single node in an unrooted bifurcating tree, where two branches join at each interior node. This method continues by successive clustering of the lineages, setting branch lengths as the lineages join. The tree does not assume a constant substitution rate as an evolutionary clock.

Parsimony methods are based on character values observed for each species, rather than the distances between the sequences (Edwards and Cafalli-Sforza, 1963; Fitch, 1971). For each topology, the sequences at each node are inferred to be those that require the least number of changes to give each of the two immediately descendant sequences. The parsimony approach is used also in describing intraspecific phylogeny of mtDNA molecules (Fitch, 1971). Within this method, it is possible to illustrate the most parsimonious tree, polytomies and homoplasmic sites in a single network that relates the sequence based haplotypes (Bandelt et al., 1995).

2.5 Mitochondrial DNA

Animal mitochondrial DNA is a small circular molecule about 15 to 20 kb and composed of about 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs, the latter coding for proteins mainly involved in the electron transport and oxidative phosphorylation of the mitochondria. Nevertheless, mitochondrial DNA has many advantages as the best molecular marker used in studying genetic variation (Esa et al., 2008). For example, it evolves faster than nuclear DNA (Brown et al., 1982), probably due to inefficient replication repair. Different regions of the mitochondrial genome evolve at different rates
allowing suitable regions to be chosen for the question under study (Brown et al., 1979; Moritz et al., 1987).

Secondly, it is maternally inherited in most species and does not recombine. Occasionally, individuals are homoplasmic for one mitochondrial haplotype, which means that each molecule as a whole usually has a single genealogical history through maternal lineages (Hoelzel and Dover, 1991). Besides, some aforementioned studies of genome evolution, stock structure and gene pool conservation of fish species (Ferris and Berg, 1987) just proved.

2.6 Cytochrome b

Since mitochondrial DNA approaches had dominated molecular systematic in the late 1970s and 1980s (Avise, 1994), the most popular sequence in phylogenetics are the cytochrome b (cyt b) and cytochrome oxidase 1 (COI) genes, which are utilized for a species and family level analysis (Kartavtsev et al., 2007). Among genes in mitochondrial DNA sequences, the cytochrome b sequences have been utilized widely as a “molecular clock” to estimate the chronology of speciation in several taxa (Smith and Patton, 1993) and also as a genetic marker for species-level identification (Johns and Avise, 1998).

Cytochrome b is one of the cytochromes involved in the electron transport in the respiratory chain of mitochondria. It contains eight transmembrane helices connected by intramembrane or extramembrane domains. The cytochrome b gene is the most widely used gene for phylogenetic work for several reasons. Although it evolves slowly in terms of non-synonymous substitutions, the rate of evolution in silent positions is relatively fast (Irwin et al., 1991).
The wide use of cytochrome \( b \) has created a status as a universal metric, in the sense that studies can be easily compared. Cytochrome \( b \) is thought to be variable enough for population level questions, and conserved enough for clarifying deeper phylogenetic relationships. However, the cytochrome \( b \) gene is under strong evolutionary constraints because some parts of the gene are more conserved than others due to functional restrictions (Meyer and Wilson, 1990). Most of the variable positions seem to be located within the coding regions for transmembrane domains or for the amino- and carboxy-terminal ends (Irwin et al., 1991). So far, cytochrome \( b \) has been the most prevalent source of sequence data in fish studies.
3.0 Materials and Methods

3.1 Samples Collection and Preservation

*Lagocephalus lunaris* were collected from two east coastal areas in Sabah (Pulau Mandi Darah, Kudat and Tawau) between July 2009 and August 2009. Each of populations consisted of 30 individual of puffer fish. The collected specimens were immediately frozen or preserve in ethanol, followed by air transportation to UNIMAS and subsequently stored at -20 °C until required for genetic analyses.

Figure 2: Sampling location in two populations in East Coast of Sabah. Population 1 derived from Pulau Mandi Darah, Kudat and population 2 derived from Tawau

Population 1
(Pulau Mandi Darah, Kudat)
N = 30 samples

Population 2 (Tawau)
N = 30 samples
3.2 DNA Extraction

Total DNA was extracted from muscle tissue using a modified CTAB method (Grewe et al., 1993). 150 µl of the tissue sample (20-50mg) was added into each of 1.5ml microcentrifuge tube that contained 700 µl CTAB followed by addition of 8 µl of 20mg/µl proteinase-K (20 mg) into each tube. The solution was briefly mixed and incubated at 55 ºC for 3 hours until the tissue was completely dissolved.

After that, 600 µl of chloroform:isoamyl alcohol (24:1) was added into each tube and mixed briefly for 2 minutes before centrifuged at 13,000rpm for 10 minutes. The viscous aqueous phase (top phase) containing the DNA was transferred into a new clean 1.5 ml microcentrifuge tube by using a wide bore pipette tip. The sample was centrifuged for 10 minutes at 13,000rpm. The supernatant was removed from the tube by using a wide bore pipette tip after centrifugation and approximately 600 µl of cold absolute ethanol with 25 µl 3M NaCl was added. Then, centrifuged at 13,000rpm for 10 minutes and the solution were discarded carefully after centrifugation. Finally, the DNA pellet was resuspended in 35 µl distilled water (dH₂O) after the pellet was air dried.

3.3 Running Gel Electrophoresis

1% of agarose gel was used for visualising after DNA extraction. The preparation of agarose gel is as follows; 0.5 g agarose powder was weighted and added into a flask. 50 ml TBE buffer solution was added into the cone and heated using microwave for 2 minutes. The comb was inserted in the casting tray while the cone was taken out and let it cool for a while. Then, 1.0 µl ethidium bromide was added into the agarose solution and poured off into the casting tray.
After cooling for 30 minutes, the comb was taken out and wells were appeared in the gel, next inserted the gel into the tank with agarose buffer and let the gel sank. 2 µl of sample was taken out and mixed with 1 µl of loading gel by adjusted of micropipette to 3 µl on the spyrofoam, then resuspended them for several times and put into each well. 2 µl DNA ladder was also put into the well and then the electrophoresis was turned on at 90V. It was stop when the band reached the 3rd bottom line in 30 minutes and screened after that.

### 3.4 Polymerase Chain Reaction (PCR)

All the puffer fish samples were subjected to DNA amplification by using Polymerase Chain Reaction (PCR) and a 400bp of the cytochrome b mitochondrial gene fragment. Amplification was performed in a gradient cycler (Biorad) for 30 cycles and used 25 µl reaction volume, containing sterile distilled water (dH₂O), 10X PCR buffer (Promega), 50mM Magnesium chloride (MgCl₂), 5u/µl Taq DNA polymerase, 2mM Deoxyribonucleotide triphosphates mixture (dTTP, dATP, dCTP and dGTP), 10 pmol/µl Forward primers, 10 pmol/µl Reverse primer and 1µl of template DNA molecule of each samples.

The sequences of two cytochrome b primers that were used are shown as below (Palumbi et al., 1991):

GluDG-L (Forward primer): 5’- TGACTTGAAARAACCAYCGTTG -3’

CB2-H (Reverse primer): 5’- CCCTCAGAATGATATTTGTCCTCA -3’

Cycle parameter of optimization was used as followed; initial denaturation of 94 °C for 2 minutes, denaturation of 94 °C for 1 minute, annealing of 40.0 °C - 60.0 °C for 1
minute, extension of 72° C for 1 minutes and final extension of 72° C for 5 minutes. Then, 1.0% of agarose gel electrophoresis system was used to determine the best annealing temperature after optimization. Lastly, the amplification was performed using cycle parameter of optimization with the annealing temperature of 43.8 ° C, followed by the product yielded was visualized by using 1.0% of agarose gel electrophoresis system and Gene Ruler™100bp DNA ladder was used as a standard size marker.

3.5 Purification

All the PCR products were purified before sent for DNA sequencing and the purification was done by using commercial purification kit (Promega), according to the manufacturer’ instruction. An equal volume (25~28 µl) of membrane binding solution was added to the PCR reaction. A minicolumn was inserted into the collection tube and prepared PCR product was transferred into the minicolumn assembly. Then, centrifuged at 14,000rpm for 3 minute, followed by supernatant discarded and reinserted the minicolumn into the collection tube.

After that, 700 µl of membrane washing buffer (within ethanol added) was added into tube and centrifuged at 14,000rpm for 2 minute. Supernatant of tube was discarded after centrifugation and the minicolumn was reinserted into tube. Then, the DNA pellet was subjected to a second wash by using 500 µl of membrane washing buffer, followed by centrifuged at 14,000rpm for 5 minutes. Later, the supernatant was discarded and the tube was recentrifuged within the minicolumn at 14,000rpm for 1 minute to dry the tube. The minicolumn was then carefully transferred to a clean 1.5 ml microcentrifuge tube.