UNIVERSITI PUTRA MALAYSIA

PHYLOGEOGRAPHY OF GENUS *Tenualosa* AND POPULATION STRUCTURE OF *Tenualosa toli* (Valenciennes, 1847) INFERRED FROM CYTOCHROME B MITOCHONDRIAL DNA

PUVANESWARI PUVANASUNDRAM

FP 2018 16

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By

PUVANESWARI A/P PUVANASUNDRAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

March 2017

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DEDICATION

To my beloved father and mother

Mr. Puvanasundram a/l VM Rajagopal and Mrs. Subethra a/p Perumal

Family members:

Jeevaperagasi a/p Puvanasundram

Sumathi a/p Puvanasundram

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PHYLOGEOGRAPHY OF GENUS *Tenualosa* AND POPULATION STRUCTURE OF *Tenualosa toli* (Valenciennes, 1847) INFERRED FROM CYTOCHROME B MITOCHONDRIAL DNA

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PUVANESWARI A/P PUVANASUNDRAM

March 2017

Chairman : Yuzine bin Esa, PhD
Faculty : Agriculture

This study was conducted to examine the systematic and evolutionary relationship among members of genus *Tenualosa* as well as genetic diversity of *Tenualosa toli* from selected populations in Sarawak using cytochrome *b* (cyt *b*), mitochondrial Deoxyribonucleic acid (mtDNA). A total of 111 *T. toli*, 24 *T. macrura* and four *T. ilisha* samples were obtained. All *T. toli* and *T. macrura* samples were collected from various localities in Sarawak and imported samples were collected from Satok market, Sarawak. Samples of *T. toli* were collected from Sebuyau (N= 25), Sadong Jaya (N=20), Satok market (N=9), Batang Lupar (N=20), Daro (N=12) and Mukah (N=25). Samples of *T. macrura* on the other hand, were collected from Sadong Jaya (N= 7), Kota Samarahan (N=11) and Daro (N=6). *Tenualosa ilisha* samples were obtained from Bangladesh. These samples in the form of muscle tissue and fin clips were stored in 95% ethanol before being stored in -20°C freezer for long term storage. Polymerase Chain Reaction (PCR) was conducted on samples using cyt *b* primer and products were then sent for sequencing. All the sequences obtained were first validated through Basic Local Alignment Search Tool (BLAST) analysis. Phylogenetic analysis supported the monophyletic status between the three species of shad. Pairwise genetic distance (13.9%-15.3%) between species in genus *Tenualosa* supports the taxonomic status of *T. toli* and *T. macrura* as distinct species.

Nucleotide diversity in all populations were low (0.001) and the haplotype diversity ranged from the lowest value of 0.417 (Imported Toli shad) to the highest value of 0.656 (Sebuyau samples). Gene flow (Nm) was equal to infinite among all the populations of *T. toli* in Sarawak showing very high gene flow between them due to homogeneity of terubok population from different parts of Sarawak as *T. toli* move from different rivers for spawning. Pairwise fixation index (Fst) values for genetic differentiation among population showed significant levels of genetic differentiation in all comparisons between imported samples from India of *T. toli* population and samples obtained from Sarawak. However, there were no significant differences recorded in

pairwise Fst values in most comparisons among populations of Toli shad collected from Sarawak. Analysis of molecular variance (AMOVA) results revealed that the majority of variance including percentage of variation was among population variance. Negative Tajima's D value was obtained for samples collected from Sebuyau, Batang Lupar, and Daro as well as imported samples which could be due to recent bottleneck events leading to population expansion. Fu's FS showed negative value for samples obtained from Sebuyau, Batang Lupar and imported samples where this value is expected due to recent population expansion. The utilization of mtDNA cyt *b* in this study has managed to provide an insight on the genetic makeup of *T. toli* collected from five different locations in Sarawak. This study supports the high genetic differences between the imported and locally collected samples where it could mean that the imported samples belong to a different gene pool or breeding groups. This study showed that cyt *b* is one of the suitable genes which could be employed for the purpose of species identification, phylogeography and population structure studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

FILOGEOGRAFI GENUS *Tenualosa* DAN STRUKTUR POPULASI *Tenualosa toli* (Valenciennes, 1847) MENGGUNAKAN MITOKONDRIA GEN SITOKROM B

Oleh

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Kajian ini dijalankan untuk mengetahui hubungan evolusi dan sistematik antara ikan daripada genus *Tenualosa* dan juga kepelbagaian genetik antara *Tenualosa toli* daripada populasi yang dipilih di Sarawak menggunakan sitokrom *b*, mitokondria *Deoxyribonucleic acid* (DNA). Sebanyak 111 sampel *T. toli*, 24 sampel *T. macura* dan empat sampel *T. ilisha* telah diperoleh. Kesemua sampel *T. toli* dan *T. macrura* diperoleh daripada beberapa lokasi di Sarawak manakala sampel yang diimport diperoleh dari Pasar Satok, Sarawak. Sampel *T. toli* yang digunakan dalam kajian ini diperoleh dari Sebuyau (N= 25), Sadong Jaya (N=20), Pasar Satok (N=9), Batang Lupar (N=20), Daro (N=12) dan Mukah (N=25). Sampel *T. macrura* pula diperoleh dari Sadong Jaya (N= 7), Kota Samarahan (N=11) and Daro (N=6). Sampel *T. ilisha* pula diperoleh dari Bangladesh. Kesemua sampel dalam bentuk tisu otot dan sirip ini disimpan di 95% ethanol sebelum disimpan di dalam peti sejuk bersuhu -20°C untuk jangka masa panjang. *Polymerase Chain Reaction* (PCR) telah dijalankan menggunakan primer sitokrom *b* dan sampel dihantar untuk proses penjujukan DNA. Kesemua jujukan yang diperoleh disahkan menerusi Basic *Local Alignment Search Tool* (BLAST). Analisa filogenetik mengesahkan status monofiletik di antara tiga spesies di bawah Genus *Tenualosa*. Jarak genetik di antara spesies-spesies dalam genus *Tenualosa* (13.9%-15.3%) mengesahkan status taksonomi *T. toli* dan *T. macrura* sebagai spesies yang berbeza.

Kepelbagaian nukleotida di antara semua populasi adalah rendah (0.001) dan kepelbagaian haplotaip pula adalah di antara 0.417 (ikan *T. toli* yang diimport) dan 0.656 (sampel *T. toli* dari Sebuyau). Nilai *gene flow* (Nm) pula di antara kesemua populasi *T. toli* di Sarawak adalah infiniti menunjukkan aliran gen yang tinggi di antara kesemua populasi ini kerana ikan ini bergerak dari sungai untuk bertelur dan ini menyebabkan kehomogenan populasi terubok. Nilai *Fixation index* (Fst) di antara sampel *T. toli* yang diimport dan sampel *T. toli* dari Sarawak menunjukkan perbezaan

genetik di tahap yang signifikan. Tiada perbezaan yang signifikan dijumpai dalam kalangan *T. toli* di Sarawak. Nilai analisis varian molekular (AMOVA) yang tinggi menunjukkan bahawa majoriti daripada varian dan peratusan tinggi variasi genetik adalah berpunca daripada perbezaan di antara populasi. Nilai Tajima's D yang negatif yang diperoleh dari sampel di Sebuyau, Batang Lupar, Daro dan sampel yang diimport mungkin berlaku akibat proses 'bottleneck' yang seterusnya menyebabkan pengembangan populasi. Nilai negatif Fu's FS yang diperoleh dari sampel di Sebuyau, Batang Lupar dan sampel diimport juga menunjukkan berlakunya pengembangan populasi. Penggunaan sitokrom *b*, DNA mitokondria dalam kajian ini mampu memberikan informasi mengenai genetik *T. toli* yang diperoleh dari lima populasi berbeza di Sarawak. Kajian ini yang membuktikan kepelbagaian genetik yang tinggi di antara sampel *T. toli* dari Sarawak dan sampel yang diimport menunjukkan sampel yang diimport adalah daripada kumpulan gen yang berbeza. Kajian ini juga membuktikan keberkesanan penggunaan sitokrom *b* dalam mengetahui identiti species, filogeografi and kajian struktur populasi.

ACKNOWLEDGEMENT

First and foremost, I would like to thank Universiti Putra Malaysia (UPM) for giving me the opportunity to further my studies in Masters Degree, and also for providing the required space and equipments to run my research throughout my studies. I would also like to thank my supervisor Assoc. Prof Dr Yuzine Esa for guiding me throughout my research journey by giving constructive comments. A special thanks to my co-supervisors Dr Ina Salwany Binti Md Yasin and Dr S. M. Nurul Amin for assisting me throughout my studies. I am also grateful for the assistance provided by Prof Madya Dr. Khairul Adha A. Rahim and staff as well as postgraduate students from Faculty of Resource Science and Technology (FRST) Universiti Malaysia Sarawak. My gratitude to all the staff in Aquaculture Department, Universiti Putra Malaysia for their assistance. I would also like to acknowledge my laboratory mates namely Nurnadia Marshita Binti Abdul Aziz, Nor Syakina binti Nor Rahim for your assistance and idea whenever I face problem regarding my research. I have learnt a lot from them. Thank you to Diyana Nadhirah Khairul Parman for assisting me in some laboratory works which were new to me. I am also grateful to Nur Jasmin Mohd Yaminudin, Nur Ain Sofea Mohd Taher and Zalikha Mohad Hoszaini for being such wonderful friends and assisting me whenever I needed help.

A very special thanks to my parents Mr Puvanasundram s/o Rajagopal and Madam Subethra d/o Perumal who have never stopped believing in me and supported me through thicks and thin throughout my masters journey, my sisters, Madam Jeevaperagasi and Madam Sumathi for being a role model and back bone of my life. I am who I am now because of my family. Thank you for all the tolerance you gave me. I love you all and thank you so much for everything.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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°C	Degree celcius
Δ	Transversion
%	Percentage
®	Registered trademark
™	Trademark symbol
©	Copyright
-ve	Negative
µl	Microlitre
µM	Micromolar
A	Adenosine
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Hierarchical analysis of molecular variance
BBA	Binding buffer
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
C	Cytosine
CE	Capillary electrophoresis
CEPA	Community Education and Public Awareness
CLB	Cell Lysis Buffer
CNI	Close neighbour interchange
CWA	Column Wash Solution
Cyt *b*	Cyrochrome *b*
DAMBE	Data Analysis in Molecular Biology and Evolution

ddH20	Double distilled water
DNA	Deoxyribonucleic acid
DnaSp	Software package for comprehensive analysis of DNA polymorphism data
dNTP	Deoxyribonucleotides
Dxy	Nucleotide substitution per site
et al	And others
FAO	Food and Agriculture Organization of the United Nations
Fst	Fixation index
G	Guanine
HPLC	High Performance Liquid Chromatography
HTL	*Tenualosa ilisha* haplotypes
HTM	*Tenualosa macrura* haplotypes
HTT	*Tenualosa toli* haplotypes
IEF	Isoelectric fusing
IUCN	International Union for Conservation of Nature
kg	Kilogramme
Kxy	Pairwise nucleotide difference
LC	Least Concern
LIFDCs	Low-income food deficit countries
LKIM	Lembaga Kemajuan Ikan Malaysia
M	Molar
MCMC	Markov Chain Monte Carlo
MEGA	Molecular Evolutionary Genetics Analyses
min	minutes

mg	Miligramme
$MgCl_2$	Magnesium chloride
ml	Millilitre
ML	Maximum likelihood
mM	milimolar
MP	Maximum Parsimony
MSN	Minimum spanning network
mtDNA	Mitochondrial DNA
N	Number of samples
NCBI	National Centre for Biotechnology Informaation
ng	Nanogramme
NJ	Neighbour Joining
Nm	Gene flow
nuDNA	Nuclear DNA
P	Probability
PCR	Polymerase Chain Reaction
PBS	Phosphate-buffered saline
PK	Proteinase K
PopArt	Population Analysis with Reticulate Trees
PUFAs	Polyunsaturated fatty acids
RAPD	Randomly Amplified Polymorphic DNA
R	Transition to transversion ratio
Rpm	Revolutions per minute
s	Seconds
SSR	Simple Sequence Repeat

SL	Standard length
T	Thymine
TBE	Tris base, boric acid and EDTA
TRIP	Terubok Rehabilitation Integrated Programma
TL	Total length
UV	Ultraviolet
V	Volt
x	Transition
X	Times

CHAPTER 1

INTRODUCTION

The global total capture of fishery production in 2014 was 93.4 million tonnes, where 81.5 million tonnes were from marine waters and 11.9 million tonnes were from inland waters according to Food and Agriculture Organization of the United Nations (FAO, 2016). On the other hand, per capita consumption of fish in 2013 for developing regions were 18.8 kilogramme (kg) whereas for low-income food deficit countries (LIFDCs) were 7.6 kg. The total marine capture production in Malaysia specifically, for the year 2014 was 1.46 million tonnes which was 1.7% lower compared to 2013. Besides that, Malaysia seems to be one of the top 25 producers and main group of farmed species in 2015, where the total aquaculture production which includes both inland and marine aquaculture was 521 thousand tonnes (FAO, 2016) thus portraying the significance of this sector in Malaysia.

Clupeiformes is globally known as one of the most important commercial fisheries. This order is classified into five different families namely Denticipidae, Pristigasteridae, Engraulidae, Clupeidae and Chirocentridae (Froese and Pauly, 2004). Genus *Tenualosa* which is one of the member of family Clupeidae consists of five main species worldwide but only two species (*Tenualosa toli and Tenualosa macrura*) could be mainly found in Malaysia especially in East Malaysia also known as Borneo. *Tenualosa toli* commonly known as 'terubok sungai' is one of the most important species in Sarawak fisheries as it has a lot of economical and cultural value. This fish has various other local names such as 'bekawal', 'bengkalis', 'temparik', 'terubuk padi', 'terubuk payau', 'terubuk mulut besar' and Chinese herring (Atan *et al*., 2010).

The order Clupeiformes which includes anchovies, herrings, sardines, menhadens, shads and other members plays a major role in world fisheries as the global capture production of Clupeiformes was 19,000,000 tonnes in 2003, which constitutes 25% of total annual catch (FAO, 2003). Likewise, in 2014, there were many Clupeid species listed under the major species and genera in marine capture production (FAO, 2016). One of the twenty highest imported fish in Malaysia for the year 2015 was terubok fish where it is imported in frozen form [Fisheries Development Authority of Malaysia (LKIM, 2015)]. 'Terubok' is mostly sold as salted fish in East Malaysia which is a very famous delicacy.

Overall, among the total 31.2 million Malaysian citizens, it has been recorded that each person consumes about 31.5 kg of fish in a year. This portrays the importance of fish as a frequently opted source of protein compared to meat which would usually be taken occasionally. Fish is believed to be one of the most important sources of protein for people around the world as 17% of intake of animal protein in 2013 was fish. Consumption of fish reduces the risk of heart disease, strokes, some cancer and other ailments (Sikorski, 1990). One of the highly desired nutrient in fish is the omega-3 polyunsaturated fatty acids (PUFAs) which has many benefits to human such as

reducing the risk of coronary heart diseases, mild hypertension, prevent cardiac arrhythmias and sudden death (Sidhu, 2003).

In the International Union of Conservation of Nature (IUCN) red list of threatened species, the status of both *T. toli* and *T. macrura* has yet to be assessed, but the status of *T. ilisha* is written as least concern (LC) and the population trend of this fish is stated as decreasing. Eventhough the status of *T. toli* has yet to be assessed it is highly recommended that preventive conservation related programme should be conducted in advance. In Sarawak, conservation and rehabilitation programme focuses more on the Big Mouth 'Terubok' or also known as *T. toli* as it is the species that is being over exploited. Terubok sungai is highly targeted by fishermen especially the ovaries of spawning females as it is highly valuable (Milton *et al*., 1997; Willman *et al*., 1989).

For the purpose of fish stock identification, one of the conventional methods utilized is by comparing morphological characters of fish. Examples of mostly used characters are number of scales in lateral series or relative body depth (Ferris and Berg, 1987). Due to various developmental stages of fish mainly larvae and juvenile where their morphological characteristics seems difficult to be used as a base for species identification, molecular marker based identification mainly Deoxyribonucleic acid (DNA) characters based identification is more appropriate to be utilized (Teletchea, 2009).

The gene content of mitochondria-genomes is highly conserved throughout the evolution of metazoan (Wolstenholme, 1992) which is one of the main criteria that qualifies mitochondrial DNA (mtDNA) marker's application in stock and species identification. Besides that, molecular markers such as mtDNA markers are also suitable to resolve phylogeography relationships and population structure as well as genetic variation analysis in fish species. Phylogeographic studies involve geographic and evolutionary processes which portrays the genetic divergence of the population. This is because, molecular sequences has a very simple structure where there are mainly four bases that are subjected to mutation, selection and drift (Kocher and Stepien, 1997) which makes it highly conserved. Mitochondrial DNA has been utilized to investigate significant genetic divergences among population across geographical regions as well as to conduct demographic study to identify any form of population expansion (Ma *et al*., 2010).

In some research, nuclear and mitochondrial markers are used hand in hand to investigate the phylogeographic structure of a particular species (Hammer *et al*., 2010). There are disprecancies on mtDNA biogeographical data compared to nuclear DNA (nuDNA) mainly due to adaptive introgression of mtDNA, demographic disparities and sex-biased asymmetries, hence the idea of combining both of this markers is believed to provide a much stronger and reliable molecular data for both phylogenetic and phylogeographic analysis (Toews and Brelsford, 2012). Thus, both mtDNA and nuDNA marker are suitable for utilization in molecular related studies.

Significance of the study

Currently there are not many studies which cover on the genetic attribute of *T. toli* eventhough there seem to be more genetic related studies on its congener, *T. ilisha*. Various studies such as population genetic study, allozyme and morphological variation, genetic variation and many more studies were conducted on Hilsa shad (Behera *et al*., 2015; Mazumder and Alam, 2009; Salini *et al*., 2004; Rahman and Naevdal, 2000). The decline in the population of *T. toli* calls for more genetic study for the purpose of conservation. One of the earliest study which focuses solely on *T. toli* is the study by Blaber *et al*., (1996). This study covers on the life history of this fish. This study could be acknowledged as the basis of various studies on *T. toli*. Toli shad is subjected to overfishing as well as climate changes which affects the population level. Another importance of this current study is that *T. toli* was subjected to over-exploitation including other factors mainly environmental degradations and water pollutions. Hence the total catch landing of this fish has been reported to have depleted to a very low level due to overfishing activities (Blaber *et al*., 2005). It is also believed that the fish populations in Batang Saribas and Batang Lassa which are known as the core terubok areas in Sarawak has depleted to a low level (Khairul Adha *et al*., 2014).

Knowing the significance of *T. toli* as a foodfish as well as the characteristics of this fish as a protandric hermaphrodite which makes it unique, it is very important to identify the actual genetic makeup to know the current status of terubok fish population in Sarawak. The application of genetics in conservation is mainly to prioritize which particular population or species requires the allocated financial resources the most.

It is believed that *T. toli* in Sarawak shared the same estuarine habitat with more than about sixty other species. This lead to a rich biodiversity environment so any form of conservation programme implemented in the habitat of this fish would do a huge favour to all other species and increase the overall fish biomass production (Awang Alim *et al*., 2012). Moreover Toli shad is placed at the lowest trophic (food) level in the pyramid so the stability of the system depends on the diversity and availability of this fish.

Objectives

Therefore, there were three main objectives in this study are,

1. To reconstruct phylogenetic tree inferred from cytochrome *b*, mitochondrial DNA in order to examine the systematic and evolutionary relationship among shad of genus *Tenualosa*.

2. To examine the population genetic structure and level of genetic diversity among and within populations of *Tenualosa toli* from different geographical groups using cytochrome *b*, mitochondrial DNA.

3. To examine the population genetic structure and level of genetic diversity among and within populations of *Tenualosa toli* from different geographical groups using microsatellite DNA.

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LIST OF PUBLICATIONS

Paper journal

Puvaneswari, P. S., Esa, Y., Khairul Adha, A. R and Nurul Amin, S. M. **(2018)**. Phylogeography and Population Structure of *Tenualosa toli* inferred from Cytochrome b Mitochondrial DNA Fragment. *Journal of Environmental Biology*. (Accepted)

Proceedings

Puvaneswari, P. S., Esa, Y., Khairul Adha, A. R and Nurul Amin, S. M. **(2018)**. Phylogeography and Population Structure of *Tenualosa toli* inferred from Cytochrome b Mitochondrial DNA Fragment. In proceedings of 11th Malaysia Genetic Congress: Focus on Genes: Maximise on impact , 12 – 13 Auguest 2015, Perdana University, Serdang, Selangor, Malaysia.

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