



UNIVERSITI PUTRA MALAYSIA

***MOLECULAR PHYLOGENY OF MALAYSIAN GROUPERS INFERRED
FROM MITOCHONDRIAL (Cytochrome b) AND NUCLEAR (Tmo-4c4
AND
Rag-1) GENES***

NOR SYAKINA BINTI NOR RAHIM

FP 2017 45



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By

NOR SYAKINA BINTI NOR RAHIM

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

May 2017

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

MOLECULAR PHYLOGENY OF MALAYSIAN GROUPERS INFERRED FROM MITOCHONDRIAL (Cytochrome b) AND NUCLEAR (Tmo-4c4 AND Rag-1) GENES

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May 2017

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Groupers are known as one of the important marine and estuarine fishes. They are widely distributed in tropical and subtropical open waters. Difficulty in netting them in wild has increased their demand in the market thus resulting in overexploitation of these fishes. The classification also difficult for most species from this family due to their homology characteristics which is most notably among closely related species which makes the conservation and management of these fishes ambiguous. This study aimed to elucidate the phylogenetic relationship of wild grouper in Peninsular Malaysia. Sample collection was done for nine sites which are from Terengganu (N=24), Kelantan (N=6), Perak (N=9), Pahang (N=10), Perlis (N=1), Perlis (N=1), Kedah (N=5), Selangor (N=26), Pahang (N=10), Malacca (N=3) and Johor (N=2) coastal areas. Samples were identified based on their morphological characteristics. Molecular phylogeny study was done inferred using Mitochondrial Cytochrome *b* (Cyt *b*), Nuclear Tmo-4c4 and Recombination activating gene-1 (Rag-1) genes and combined Mitochondrial and Nuclear genes. The results revealed a total 86 individuals belonging to 12 species representing three genera which are *Epinephelus*, *Cephalopholis* and *Plectropomus*. The phylogenetic trees were constructed for Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), Minimum Evolution (ME), and Bayesian Inference (BI) which were implemented by Molecular Evolution Genetic Analysis 7.01 (MEGA) and MrBayes 3.2.1. One consistent result across all the analyses was the monophyletic status of the subfamily of Epinephelinae. It is revealed that genus *Plectropomus* sister to the rest genera. Genus *Epinephelus* was clustered into two clades which is constant for all analyses. The separation confidence level between cluster *Epinephelus*, *Cephalopolis* and *Plectropomus* were 100% respectively for BI analysis based on combined dataset. Molecular data revealed that combined dataset helps increases the phylogenetic resolution compared to single gene phylogeny where the dataset well resolved the deeper node within genus *Epinephelus* compared to single gene analyses. Nevertheless, several genetic relationships remain unresolved particularly among morphologically similar species, for instance between *Epinephelus fario* and *E. longispinis* and species with limited number of individuals which is *E. coioides*. This study have aids in giving

baseline information regarding groupers particularly in Malaysia which related to the area of taxonomy, phylogeny and genetic diversity which are important for their appropriate sustainable management, for conservation as well as aquaculture purposes



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

FILOGENETIK MOLEKUL KERAPU MALAYSIA MENGGUNAKAN GEN MITOKONDRIA (Sitokrom b) DAN NUKLEAR (Tmo-4c4 DAN Rag-1)

Oleh

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Kerapu terkenal sebagai salah satu ikan penting laut dan juga muara. Ianya boleh didapati secara meluas di kawasan laut terbuka di tropika dan subtropika. Kepayahan untuk menangkap ikan liar ini menjadikan permintaan terhadap ikan ini meningkat dalam pasaran seterusnya menyebabkan ikan ini dieksploitasi secara berlebihan. Klasifikasinya juga sukar disebabkan oleh sifat-sifat homologi mereka terutamanya diantara spesies yang berhubungan kait rapat yang menyebabkan pengurusan dan pemeliharaan ikan-ikan ini menjadi rumit. Kajian ini bertujuan untuk menjelaskan hubungan filogenetik ikan kerapu liar di Semenanjung Malaysia. Sampel telah diambil dari sembilan kawasan-kawasan laut pantai di Terengganu (N=24), Kelantan (N=6), Perak (N=9), Pahang (N=10), Perlis (N=1), Perlis (N=1), Kedah (N=5), Selangor (N=26), Pahang (N=10), Malacca (N=3) and Johor (N=2). Sampel-sampel ini dikenalpasti berdasarkan ciri-ciri morfologi mereka. Filogeni molekul telah disimpulkan dengan menggunakan gen Mitokondria Cytochrome *b* (Cyt *b*), Nuklear Tmo-4c4 dan Penggabungan semula mengaktifkan gen-1 (Rag-1) dan juga gabungan gen mitochondrial dan gen-gen nuklear. Hasil daripada ini sebanyak 86 individu diperolehi tergolong daripada 12 spesies yang mewakili tiga genera iaitu *Epinephelus*, *Cephalopholis* dan *Plectropomus*. Pokok-pokok filogenetik telah dibina untuk *Neighbour-Joining* (NJ), *Maximum Parsimony* (MP), *Maximum Likelihood* (ML), *Minimum Evolution* (ME), dan *Bayesian Inference* (BI) dengan menggunakan menggunakan Molecular Evolution Genetic Analysis 7.01 (MEGA) dan MrBayes 3.2.1. Satu keputusan yang konsisten terhasil daripada semua analisis adalah tentang status monofiletik bagi Epinephelinae. Ia juga mendedahkan genus *Plectropomus* adalah adik beradik kepada genera-genera lain. Genus *Epinephelus* telah terbahagi kepada dua kelompok yang sama dengan kajian lain yang dijalankan sebelum ini. Data molekular juga membuktikan bahawa dengan gabungan dataset ianya dapat membantu meningkatkan resolusi filogenetik nod dalaman berbanding dengan analisis menggunakan satu gen. Walau bagaimanapun, beberapa hubungan genetik masih kekal tidak dapat dijelaskan terutamanya dalam kalangan spesies morfologi yang serupa misalnya, antara *Epinephelus fario* dan *E. longispinis* dan spesies dengan bilangan individunya terhad iaitu *E. coioides*. Kajian keatas ikan kerapu ini berjaya membantu dalam memberi informasi secara dasar dalam bidang berkaitan dengan

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I certify that a Thesis Examination Committee has met on 9 May 2017 to conduct the final examination of Nor Syakina bte Nor Rahim on her thesis entitled "Molecular Phylogeny of Malaysian Groupers Inferred from Mitochondrial (Cytochrome b) and Nuclear (Tmo-4c4 and Rag-1) Genes" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

μL	Microliter
μM	Micromolar
ng	nanogram
ml	milliliter
g	gram
N	Number of samples
5X	Five times
10X	Ten times
Bp	Base pair
$^{\circ}\text{C}$	Degree Celsius
%	Percent
Kb	kilobase
DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
Cyt <i>b</i>	Cytochrome <i>b</i>
Rag-1	Recombination activating gene-1
PCR	Polymerase chain reaction
NJ	Neighbour-Joining
MP	Maximum Parsimony
ME	Minimum Evolution
ML	Maximum Likelihood
BI	Bayesian Inference
IUCN	International Union Conservation of Nature
Blast	Basic Local Alignment Search Tool
Popn	Population
FAO	Food and Agriculture Organization
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
UV	Ultraviolet light
ILD	Incongruence length difference
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
AFLP	Amplified fragment length polymorphism
SNP	Single nucleotide polymorphism
EST	Expressed sequence tag
PSRF	Potential scale reduction factor
AIC	Akaike information criterion



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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The fisheries and aquaculture sector has improved and has been developed throughout the years, hence becoming one of the most important sector for economy support of the world. Food and Agriculture Organization of United Nation (FAO) stated that global total capture of marine fishery production was not much different from 2014 to 2015 which are about 80.0 million tonnes to 81.0 million tonnes (FAO, 2016). It is has showed an increases from previous year which was from marine waters. For the world fish production utilization, increment to 146.3 million tonnes was also recorded. Therefore, makes it becomes one of the fastest growing sector supplying food to the world that cater to the rising demand for the fisheries product.

According to Department of Fisheries of Malaysia (Jabatan Perikanan Malaysia, 2016), even though Malaysia have already surpassed the level of self-supply sufficiency of fish resources as a food ever since 2010, however, the fish production indeed needs to be outsourced for fish consumption in this country. Fish consumption indexed also showed an increment from 53.1 kg in 2011 and expected to be 61.1 kg in 2010 (Yusoff, 2015). In 2013, approximately 1, 482, 899 tons (RM8.336b) of marine fish were produced however, the production decreases 1.67% to 1, 458, 128 tons (RM8.785b) mostly because of excessive exploitation of marine fish (Yusoff, 2015). Our marine resource biomass had been decreasing since application of trawling by fisherman were introduced in 1960s.

Approximately about 70 percent of the world's fish species are either fully exploited or has been depleted (FAO, 2015). Higher demand for wild marine fish contribute to the overfishing of certain marine fish. Epinephelinae is a subfamily of Serranidae. Most of the species belongings to this subfamily are being overexploited and it is causes a significant depletion for some of the species. On the other hand, some of them were globally commercialized due to high demand for their meats (Zhu and Yue, 2008; Morris, *et al.*, 2000; Heemstra and Randall, 1993). Due to higher demand compared to other fishes, this subfamily of groupers has been classified as an exclusive and highly price fish in the market. Demand for their wild meat increases gradually throughout the years which is due to the difficulties in catching them because they can only be found in the deep sea.

At least four out of 12 species that were used in this study were under near threatened as reported by International Union Conservation of Nature Red List (IUCN Red List) (IUCN, 2017). The species listed as near threatened by IUCN Red List includes *Epinephelus bleekeri*, *E. coioides*, *E. fuscoguttatus* and *Plectropomus leopardus* (Appendix 1). This may be because of coastal areas in Malaysia were listed under marine

waters that had relatively high number of threatened species of groupers (Sadovy *et al.*, 2011). While, others such as *E. corallicola*, *E. erythrurus*, *E. sexfasciatus*, *E. heniochus* does not have sufficient data that available (IUCN, 2017). Based on IUCN Red List, it is showed that many other species under this subfamily are not well discovered, as many species status are still unknown such as *Cephalopholis boenak*, *C. formosa*, *E. longispinis*, *P. maculatus* (IUCN, 2017).

Aquaculture is implemented for many marine fishes that are valued in market. Those commercialized species were cultured to reduce the dependency of catching the fishes from the wild. This is also one of the solution to avoid from overfishing which occurs to many marine fishes that which is could leads towards depletion of the species. Some artificial breeding programs have already been applied in some country such as in Singapore, Thailand, Kuwait and Japan (Tookwinas, 1989). However, the aquaculture programs are difficult to be implement because the source of brood stock for aquaculture programs still depends on the wild population. Moreover, there is still lack of information about the species also makes the program more challengin. Despite these challenges, some grouper species from subfamily Epinephelinae were successfully cultured. This includes *E. fuscoguttatus*, *E. tauvina*, and *E. coioides*. However, the species that are being cultured were chosen based on the popularity of the fishes or most preferred by consumers which is most likely to be different based on the country. Even with the establishment of the culture programs, the trends of population still showed the depletion in population of certain species (IUCN, 2017). This problem is still a concern and many information on this subfamily are required as to give more understanding about this marine fishes to avoid species extinction.

Using molecular approach in taxonomic field enables the data to be fully utilized which it is can give a detailed report on the history of life (Hillis, 1987). Molecular genetic approaches is a powerful tool that can detect genetic uniqueness of individuals, populations or species (Awise, 2004). Markers are used as they can be analytical to study the genetic diversity of the organisms. Organisms subjected to mutation because of the cells within it which interacts with the surrounding that subsequently results in genetic variation which is known as polymorphism. The importance of this polymorphism is that it can gives the species ability to adapt to the changing of environment in order to keep surviving. Other factors which occurs during this process such as selection, genetic drift and genetic variation increases the differentiation at the population level, species and higher order of taxonomic groups. This polymorphism analyses were widely used as it benefits in giving highest resolution of genetic variation (Nguyen *et al.*, 2008; Nguyen *et al.*, 2006; Liu and Chen, 2003; Wang *et al.*, 2000) which can assist in species identification and understanding the population structure of a particular species.

1.2 Justification

The concern on subfamily Epinephelinae arose a few decades back, where the classification of the organisms were still based on morphological traits. As the technology on molecular studies being applied, the taxonomist tried to compare on both methods to find which methods give better resolution. Recently, researchers argue about the classification of organisms under family Serranidae. There are many incongruent in

the classification of taxa under this family when comparing between morphology and genetic data. Due to some characteristics that were not well resolved by using conventional methods of classification.

Latest study also discovered that some groupers are difficult to be identify by using only morphological traits as some of them have intraspecific variation such as color pattern (Govindaraju and Jayasankar, 2004) and some of them also have homogenous morphology (Maggio *et al.*, 2005) and some of them also have different developmental stages especially during larvae to adult (Heemstra and Randall, 1993), thus making morphological classification become difficult. These eventually can contributed to false classification of this subfamily. Thus, this study tried to answer a few questions and help to give insight information on the genetics aspects of groupers as molecular marker based identification is more reliable to be utilized. The following are the research questions;

1. Are there any differences between mitochondrial and nuclear genes?
2. Which gene between mitochondrial and nuclear gene will better resolve this subfamily?

1.3 Aims

In this study, we aimed to utilize mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) to study the phylogenetic relationship of the grouper species in Malaysia. Cyt *b*, Tmo-4c4 and Rag-1 is used as a molecular marker in order to study the relationship between wild species among subfamily Epinephelinae. This analysis can help to understand the taxonomy as well as the genetic diversity among species as it can give reliable and constant results (Esa *et al.*, 2008; Ryan and Esa, 2006). The objectives of the study are listed as below:

1. To reconstruct the phylogenetic relationship of groupers in Malaysia using mitochondrial and nuclear genes.
2. To analyze and compare the genetic composition between mitochondrial and nuclear genes.

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