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DNA BARCODING AND INFERRED GENETIC DIVERSITY OF EPINEPHELINAE GROUPERS FROM PENINSULAR MALAYSIA USING MITOCHONDRIAL CYTOCHROME C OXIDASE I GENE AND MICROSATELLITES

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

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This study was conducted with the purpose of validating the species identity and genetic diversity of groupers from Peninsular Malaysia. All samples were collected from across the Peninsular Malaysian waters and stored in ethanol (95%) for short term storage before being placed into -20°C freezer. All steps were done in a sterile environment to avoid any contamination of samples. A total of 131 individuals comprising of 12 species from three genera were collected from coastal areas of Johor (N=4), Kedah (N=12), Kelantan (N=4), Malacca (N=4), Negeri Sembilan (N=2), Pahang (N=14), Perak (N=15), Selangor (N=22) and Terengganu (N=54). All 12 species were identified as *Cephalopholis boenak, C. formosa, C. miniata, Epinephelus areolatus, E. bleekeri, E. coioides, E. corallicola, E. erythrurus, E. fuscoguttatus, E. poecilonotus, Plectropomus leopardus* and *P. maculatus*.

The first part of the study was on DNA barcoding analysis using Cytochrome c Oxidase I gene on all 131 collected individuals. Deoxyribonucleic acid extraction and Polymerase Chain Reaction were done to all collected samples before being sent for sequencing. The successful sequences were validated through National Center for Biotechnology Information database (using BLAST: Basic Local Alignment Search Tool) and most of the sequences were correctly matched with their respective grouper species (identity index ≥95%), except for eight individuals which showed discrepancy in taxonomic identification. A barcode gap was present and the lowest genetic pairwise distance was between *P. leopardus* and *P. maculatus* (4.5%), while the highest distance was between *C. miniata* and *P. leopardus* (23.8%). The phylogenetic trees showed that there were two main clades present which separated the genera *Cephalopholis* and *Epinephelus* with genus *Plectropomus*. Sister clades were found between *P. leopardus* and *P. maculatus* with strong bootstrapping confidence level. This concludes that species with the same genus tend to be closely related.

In the second part of the study, five sets of microsatellite markers which were used for genetic diversity analysis showed polymorphism in the three selected species

(*Epinephelus areolatus, E. bleekeri* and *E. coioides*: N=64) from two populations (East Peninsular Malaysia and West Peninsular Malaysia). PCR annealing temperatures are different for all species. The markers were substituted into labeled primers, and all successful PCR products of the polymorphic markers were sent for fragment analysis (genotyping). The highest number of alleles per locus can be found in *E. areolatus* populations ranging from seven to 32 alleles, followed by *E. bleekeri* populations ranging from four to 23 alleles, and lastly *E. coioides* populations which ranged from five to 15 alleles. An estimation of Fixation index (F_{ST}) value for all microsatellite loci showed a small data. The highest value of percentage variation was in *E. areolatus* (79.74%), followed by *E. coioides* (67.88%) and *E. bleekeri* (67.47%). In addition, the population structuring for all three species showed a similar pattern. Hence, the two populations for all species did not show significant differences which indicated that all individuals were in the same gene pool. In conclusion, DNA barcoding approach is suitable for species validation and phylogenetic study among groupers and polymorphic microsatellites is proven to be suitable for determining the genetic diversity of a species.

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

BARKODING DNA DAN KEPELBAGAIAN GENETIK IKAN KERAPU EPINEPHELINAE DARI SEMENANJUNG MALAYSIA MENGGUNAKAN MITOKONDRIA GEN *SITOKROM C OKSIDES I* DAN MIKROSATELIT.

Oleh

NURNADIA MARSHITA BINTI ABDUL AZIZ

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Kajian ini telah dijalani untuk mengesahkan identiti spesies dan kepelbagaian genetik ikan kerapu yang diperoleh dari Semenanjung Malaysia. Kesemua sampel dikumpul dari pelbagai lokasi perairan Semenanjung Malaysia dan disimpan di dalam etanol (95%) untuk penyimpanan jangka masa yang pendek sebelum disimpan di dalam peti sejuk -20°C. Kesemua langkah-langkah telah dijalankan di dalam persekitaran yang telah dinyah kuman bagi mengelakkan sampel dari tercemar. Pengekstrakan Asid Deoksiribonukleik dan proses *Polymerase Chain Reaction* (PCR) telah dilakukan terhadap semua sampel yang telah dikumpul sebelum dihantar untuk penjujukan. Sebanyak 131 individu yang terdiri daripada 12 spesies dari tiga genera telah dikumpul dari kawasan perairan Johor (N=4), Kedah (N=12), Kelantan (N=4), Melaka (N=4), Negeri Sembilan (N=2), Pahang (N=14), Perak (N=15), Selangor (N=22) dan Terengganu (N=54). Kesemua 12 spesies telah dikenalpasti sebagai *Cephalopholis formosa, C. boenak, C. miniata, Epinephelus areolatus, E. bleekeri, E. coioides, E. corallicola, E. erythrurus, E. fuscoguttatus, E. poecilonotus, Plectropomus leopardus* dan *P. maculatus*.

Bahagian pertama kajian ini adalah mengenai barkoding DNA dengan menggunakan gen *sitokrom c oksides I* terhadap 131 individu yang telah di kumpulkan. Jujukan yang berjaya telah disahkan melalui pangkalan data *National Center for Biotechnology Information* (menggunakan BLAST: *Basic Local Alignment Search Tool*) dan kebanyakan jujukan telah dipadankan dengan spesies kerapu masing-masing (indeks identiti ≥95%), kecuali lapan individu yang telah menunjukkan percanggahan dalam identifikasi taksonomi. Jurang barkod dan jarak dari segi pasangan genetik yang paling rendah adalah antara *P. leopardus* dan *P. maculatus* (4.5%), manakala yang paling tinggi adalah antara *C. miniata* dan *P. leopardus* (23.8%). Pokok filogenetik menunjukkan bahawa terdapat dua cabang utama yang memisahkan genera *Cephalopholis* dan *Epinehelus* dengan genus *Plectropomus*. Cabang ditemui antara *P. leopardus* dan *P. maculatus* dengan nilai *bootstrapping* yang tinggi. Ini menunjukkan bahawa spesies dari genus yang sama mempunyai hubungan yang rapat di antara satu sama lain.

Di bahagian kedua kajian pula, terdapat lima pasang penanda mikrosatelit yang telah digunakan untuk menganalisa kepelbagaian genetik yang telah menunjukkan bahawa ketiga-tiga spesies (*Epinephelus areolatus*, *E. bleekeri* dan *E. coioides*: N=64) dari dua populasi yang berbeza (Timur Semenanjung Malaysia dan Barat Semenanjung Malaysia) adalah polimorfik. Suhu PCR *annealing* berbeza mengikut spesies. Penanda mikrosatelit telah diubah menjadi penanda berlabel dan semua produk PCR yang berjaya menghasilkan polimorfik dihantar untuk analisis fragmen (*genotyping*). Jumlah alel per lokus yang tertinggi boleh dijumpai di populasi *E. areolatus* di antara tujuh hingga ke 32 alel, populasi *E. bleekeri* di antara empat ke 23 alel, dan populasi *E. coioides* di antara lima ke 15 alel. Anggaran nilai indeks penetapan (F_{ST}) untuk kesemua lokus mikrosatelit menunjukkan data yang kecil. *E. areolatus* (79%) menunjukkan nilai yang tertinggi dalam peratus variasi di ikuti oleh *E. coioides* (67%), dan *E. bleekeri* (67%). Tambahan lagi, penstrukturan populasi untuk ketiga-tiga spesies menunjukkan corak yang hampir sama. Sehubungan dengan itu, kedua-dua populasi untuk kesemua spesies tidak menunjukkan perbezaan yang ketara, di mana kesemua individu berada dalam kolam gen yang sama. Kesimpulannya, pendekatan barkoding DNA sesuai untuk pengenalpastian spesies dan kajian filogenetik antara kerapu, dan mikrosatellit polimorfik menunjukkan ianya sesuai untuk penentuan kepelbagaian genetik sesuatu spesies.

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I certify that a Thesis Examination Committee has met on 5 January 2017 to conduct the final examination of Nurnadia Marshita binti Abdul Aziz on his thesis entitled "DNA Barcoding and Inferred Genetic Diversity of Epinephelinae Groupers from Peninsular Malaysia using Mitochondrial Cytochrome C Oxidase I Gene and Microsatellites" 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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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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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Name of Chairman of Supervisory Committee: Yuzine bin Esa, PhD

Signature: _____
Name of Member of Supervisory Committee: Aziz bin Arshad, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ºC	Degree Celsius
%	Percent
&	And
µl	Microlitre
-ve	Negative control
-th	Is a suffix which forming ordinal and fractional numbers
ABGD	Automatic Barcode Gap Discovery
Amova	Analysis of Molecular Variance
BLAST	Basic Local Alignment Search Tool
BOLD	The Barcode of Life Data System
BOLD-IDS	BOLD Identification System
bp	Base pair
C.	*Cephalopholis*
COI	Cytochrome c Oxidase I
CITES	The Convention on International Trade in Endangered Species of Wild Fauna and Flora
ddH2O	Double distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, and dUTP)
E.	*Epinephelus*
EDTA	Ethylenediaminetetraacetic acid
et al.	And others
EtBr	Ethidium bromide
FISH-BOL	The Fish Barcode of Life Initiative
F_{ST}	Fixation index
g	Gram
h	Hour
i.e.	For example,
ISSR	Inter-simple Sequence Repeat
kb	Thousand base pairs
kg	Kilogram
m	Meter
mg	Milligram
MgCl2	Magnesium chloride (stabilizer)
ml	Milliliter
M	Molar
Min	Minute
mM	Milimolar
N	Number of samples
N/A	Not available
Na	Size range / Number of alleles
ng	Nanogram
NN	Nearest neighbor
NUMT	Nuclear mitochondrial DNA segment
P	Probability
P.	*Plectropomus*
PCR	Polymerase Chain Reaction

pH	Potential hydrogen
RAPD	Random Amplified Polymorphic DNA
RNase	An enzyme that promote RNA breakdown
RM	Ringgit Malaysia
s	Second
SE	Standard error
STRs	Short Tandem Repeats
Ta	Temperature of annealing
TBE	A buffer; Tris/Borate/EDTA
UV	Ultraviolet light
V	Volt
VNTRS	Variable Number Tandem Repeats
xg	Earth gravitational force

CHAPTER 1

INTRODUCTION

Groupers are widely spread across the ocean and have become one of the most consumed fish in the world. In Malaysia, there are approximately 59 species of grouper (eight genera) from 159 species (21 genera) of groupers that can be found worldwide. Groupers are important in commercial, artisanal and fisheries of tropical and subtropical seas as they provide an economic value to the country. It has become one of the commercialized fish in food trading and as an income source for the coastal residents nearby and sometime as delicacies for the people outside the areas. It is also being commercially traded both locally and internationally. Therefore, the price has increased due to its demand throughout the years.

Serranidae fishes have been traditionally identified based on visible morphological, meristic and anatomical characters (Chakkaravarthy *et al*., 2011). Morphological and genetic identification methods cannot be separated as both methods co-exist to make a complete detail on species identification analysis. The results obtained from molecular method promises an accurate identification of fish species.

According to the Fisheries Development Authority of Malaysia (LKIM), groupers were placed in spot number 12 in the highest 20 imported species or product in raw category list with 3,506 million ton of product whereas in the list of highest 20 export species or product based on raw category, groupers were in the 11th place in the year 2014. Although the amount of fish landing increases by years, unfortunately, the price of grouper in wholesale and market sale decreases during this few years. This situation might be due to the increase in amount of fish landing of other popular fishes as there are competitions in sales between them. The wholesale decreases 1% with final value of RM23.58 per kg., while, market sale price decreases 8% until the value reached RM24.13 per kg. According to Food and Agriculture Organization of the United Nations or FAO (2014), Malaysia is at number 15 in marine capture fisheries in ranking. Groupers were mostly caught using fishing rod and some were caught using nets.

On the other hand, aquaculture had set its foot in Malaysia centuries ago. It has become one of the country's income contributors. Generally, aquaculture has many benefits that can contribute directly or indirectly to the farmers, consumers and even to the country. Aquaculture assist in maintaining household food security, poverty alleviation, income for coastal residents and country, and providing food sources in fish product without threatening the wild fish. The number of grouper fish either in the captive or in the wild depends on several aspects such as human activities and natural disaster. There are many efforts that have been done to overcome these problems, for example, national law enforcement, public awareness and preservation and conservation of the grouper.

Worldwide, *Epinephelus coioides, E. malabaricus, E. akaara, E. striatus, E. septemfasciatus, Cromileptes altivelis* and *Mycteroperca microlepis* were cultured in to

fulfill the market needs. Sabah specifically Tuaran and Sandakan are the major grow-out sites in Malaysia. Other than Sabah, there are also aquaculture activities in protected coastal areas such as in Johor, Selangor, Penang and Kedah. Groupers that have mainly cultured in Malaysia include *E. coioides, E. tauvina, E. fuscoguttatus, E. lanceolatus, Plectropomus leopardus* and *Cromileptes altivelis* (Pomeroy *et al*., 2002). Groupers are highly valuable carnivorous fish species which are typically raised in small cages at an inshore environment. The increase in demand for grouper fish will expand the future of aquaculture sector. In Indonesia, Humpback grouper was reduced in number as overexploitation and unsafe collecting method were practiced (Susanto *et al*., 2011). Therefore, aquaculture is one of the alternative methods that can minimize the pressure on natural population.

The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (2015) listed several species of grouper in endangered and critically endangered species categories. There are five grouper species that have been listed as endangered species mainly *Epinephelus striatus* (Nassau grouper), *E. akaara* (Hong Kong grouper), *E. marginatus* (Dusky grouper), *Mycteroperca fusca* (Comb grouper) and *M. jordani* (Gulf grouper). Meanwhile, three species of grouper are listed as critically endangered which are *E. itaraja* (Atlantic Goliath grouper), *E. drummondhayi* (Calico grouper) and *Hyporthodus nigritus* (Warsaw grouper). Most of these grouper species had been listed due to the drastic decrease in their population size.

There are needs to make an effort in conserving the fish which seems to be decreasing lately in number. Government and Non-Governmental Organizations (NGOs) have been making several efforts in dealing with this matter. There are several laws enforced in Malaysia to ensure the marine biological diversity in Malaysia such as Fisheries Act 1985 [Act 317], International Trade in Endangered Species Act 2008 [Act 686] and Fisheries (Marine Culture System) Regulations 1990. There are also several NGOs which participate in conserving the diversity in Malaysia. Such NGOs like Tropical Research and Conservation Centre is giving people an opportunity to volunteer in reef repair, coral planting, turtle and shark protection program, and The Reef Ball Foundation which is publicly supported by non-profit organization that helps in restoring and protecting the coral reefs. At the same time, they educate the public about the conservation program. All of these efforts directly and indirectly help to conserve the diversity of grouper fish in Malaysian ocean.

DNA barcoding is a term used worldwide for a study which involves identification of an organism either it is bacteria, plant or animal. Mitochondrial Cytochrome c Oxidase I gene or better known as COI gene is a universal gene used in this DNA barcode method in order to identify species and it is actually a subunit of the Cytochrome c Oxidase complex. COI gene has its mutation rate which is often fast enough to distinguish closely related species compared to other mitochondrial primers.

DNA barcode allows the identification of small, damaged or industrially processed material and it is useful in detecting food fraud and products taken from protected species. The Consortium for the Barcode of Life or also known as CBOL, is one of the many campaigns which enables people to understand and protect the biodiversity of the

Earth by promoting it through international partnerships worldwide (DNA Barcoding; A New Tool for Identifying Biological Specimens and Managing Species Diversity). The identification of the specimens by non-taxonomists such as students can be done through this website, which generates a global and open access library of barcode sequences.

Another ongoing campaign that has been in the spotlight is the FISH-BOL, and it is similar with BOLD system, as it gathers barcode records on all fishes. FISH-BOL stores the fish barcode as an international collaboration and the protocol for DNA sequences which are stored in its library are standardized. This campaign highlights the concern on market substitution and the limitation of the commercial fisheries. It is said to be a powerful tool in understanding the natural history and ecological interaction. In addition, they stated that DNA barcoding method allows a deeper understanding of food webs.

There are several studies that have used DNA barcoding as their main method in order to identify the species of the focus subject such as Mat Jaafar *et al*. (2012) who conducted study on DNA barcoding to reveal cryptic diversity within commercially exploited IndoMalay Carangidae from IndoMalay Archipelago (IMA). The ability to distinguish a species and to reveal cryptic species using DNA barcode had been documented. The authors stated that DNA barcodes method was a straightforward where it relies on the existing COI sequences from previous documented and archived voucher specimens. All collected specimens were examined using COI barcodes and compared to other recorded sequences from around the world and also with identification based on morphological characters. As a result, there were three described species that showed discrepancy with assigned morphological taxonomic. The Neighbor-Joining phylogenetic tree showed presence of a monophyletic clusters. Researchers also stated that these result will assist in planning, and monitoring of the conservation, and fisheries program in Indo-Malay region.

There is also a study conducted by Hebert *et al*. (2003a) which suggested that DNA-based identification system using COI gene can assist in the resolution of the diversity. All COI sequences obtained were from variety of primers and also derived from different sections of the gene. The results had recognized the congeneric species of animals that usually possessed significant sequence divergence in their COI gene, as there were more species pairs which greater than 2% sequence divergence. The intraspecific divergence was established which less than those that separates the congeneric species pairs. Thus, COI gene was summarized as an important tool in species recognition which allows delineation of the regional lineages.

Microsatellites or Simple Sequence Repeats (SSRs) are universally dispersed within genome and it is known to be a co-dominant molecular genetic marker which mainly occurs in non-coding sequence. Microsatellite markers help in several genetic analyses such as species identification and population genetic. Microsatellites allow the determination of identity between individuals based on formal estimates of allele frequencies (Kirst *et al*., 2005). The higher organisms' genome contains three types of simple repetitive DNA sequences which are satellite DNAs, minisatellites and microsatellites (Madesis *et al*., 2013). Microsatellites are an informative, multi-allelic and reproducible, and usually occurs in eukaryote genomes which were suggested in

order to overcome the limitations regarding restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Garcia *et al*., 2004: Vos *et al*., 1995: Senior and Heun, 1993).

Similar with DNA barcoding, genetic diversity analysis using microsatellites had been used in many studies around the world. Mokhtar *et al*. (2011) uses microsatellites in characterization of 10 novel microsatellite loci from Brown marbled grouper (*E. fuscoguttatus*) in Malaysia. Initially there were 43 primer pairs designed from the sequences before only 10 primers were chosen as the microsatellite markers due to polymorphism. Among 10 novel microsatellite markers, there was one locus that deviated significantly from Hardy-Weinberg equilibrium and no significant linkage disequilibrium was found after Bonferroni's correction (adjusted; P=0.0051). There was no evidence of stuttering and allelic dropout in all loci. The 10 loci were suggested to be derived from the same chromosome in the genome as there was non-significance showed in genotypic disequilibrium. Therefore, these results made the chosen microsatellites suitable for other related studies which might assist in aquaculture sector of the groupers.

Another study conducted by Zhao *et al*. (2009) studied on Yellow grouper (*E. awoara*) with 12 polymorphic microsatellites. This study was conducted in order to test population with alleles per locus. Among the 12 polymorphic microsatellite markers, there were five loci which significantly deviated from Hardy–Weinberg equilibrium. In addition, there was no significant linkage disequilibrium found. Other than that, there were two additional species added in cross-species amplification analysis. The polymorphic microsatellite markers that were chosen would be useful for further investigation on a detailed genetic diversity of *E. awoara* and other related species.

Justification

There are problems surrounding grouper's identification and population genetic. Limitation in normal identification of a species occurs as this is only effective at a particular life stage or gender. The use of morphological keys demands high level of expertise and a particular amount of time. Confusion might happen as there is overlapping of the morphological characteristics between each related species (i.e. same genus or family). On top of that, mislabeling may occur in the market.

Despite all the benefits that come along with aquaculture sector, there are still some disadvantages. The sector is not expanding due to the expensive price of the fingerlings, feed supply and health management of the wild grouper. There are lack of basic knowledge on aquaculture managements and biology of the grouper. The number of wild grouper is decreasing over the past years which might be due to the lack of molecular data on genetic diversity of grouper. The lack of molecular data prevents any breeding or cross breeding to happen in order, thus, lowering the dependencies towards the higher priced wild grouper supplier.

Objectives

Therefore, there are two main objectives that were being focused in this study, which are:

1. To validate the genetic identity of grouper species (Subfamily: Epinephelinae) from Peninsular Malaysia using DNA barcoding of Cytochrome c Oxidase I (COI) gene;
2. To examine the population genetic of three grouper species (*Epinephelus areolatus, E. bleekeri* and *E. coioides*) of Peninsular Malaysian grouper using microsatellites.

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