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DNA BARCODING OF MALAYSIAN MARINE FISH, PHYLOGENETICS AND PHYLOGEOGRAPHY OF THE SNAPPERS (PERCIFORMES: LUTJANIDAE)

by

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ADIBAH BINTI ABU BAKAR

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS

BI	Bayesian Inference
BOLD	Barcode of Life Data system
bp	Base pairs
COI	Cytochrome oxidase subunit 1
cyt b	Cytochrome b
DoF	Department of Fisheries
EGR	Early Growth Response
EP	Eastern Pacific
EPB	Eastern Pacific Barrier
FAO	Food and Agriculture Organization
FRI	Fisheries Research Institute
GenBank	Genetic sequence database
IO	Indian Ocean
IOP	Isthmus of Panama
IP	Indo-Pacific
I*ss*	Index of substitution saturation
I*ss.c*	Critical index of substitution saturation
K2P	Kimura-2-Parameter
Max	Maximum
$MgCl_2$	Magnesium chloride
Min	Minimum
min	Minute
ML	Maximum likelihood

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Mya	Million years ago
NJ	Neighbour-Joining
No.	Number
PCR	Polymerase chain reaction
RH	Rhodopsin
SE	Standard error
sec	Second
sensu	In the sense of
tmrca	The most recent common ancestor
TTE	Terminal Tethyan Event
WA	Western Atlantic

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LIST OF SYMBOLS

n	Sample size
µL	Microliter
^{0}C	Degree Celsius
E	East
M	Molar
mg/ml	Milligram per milliliter
mM	Millimole
N	North
P	Probability value
R^2	Regression value
rpm	Rotation per minute
s	Transition
U	One unit of taq polymerase
U/µL	Unit of taq polymerase per microliter
v	Transversion

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PENGKODAN DNA IKAN MARIN DI MALAYSIA, FILOGENETIK DAN FILOGEOGRAFI BAGI IKAN 'SNAPPER'

(PERCIFORMES: LUTJANIDAE)

ABSTRAK

Kajian ini dilaksanakan dengan menggunakan teknik Barkod DNA yang menawarkan potensi yang baik sebagai alat pengecaman untuk klasifikasi tangkapan ikan di Malaysia. Secara keseluruhan, kajian ini berjaya untuk menkodkan sebanyak 107 spesies, 69 genus, 36 famili dan 10 order ikan komersial Malaysia. Juga didapati bahawa, ikan jenahak, ikan bulu, ikan bayan dan ikan biji nangka dikesan mengalami penspesiesan 'cryptic' atau terdiri daripada spesies yang tidak diketahui. Hasil daripada kajian ini menunjukkan bahawa klasifikasi secara morfologi sahaja didapati tidak selalunya menjurus tepat kepada spesies individu atau kumpulan spesies. Pengenalan konvensional sehingga peringkat spesies yang menggunakan ciri morfologi didapati sukar bagi genus ini terutama bagi kes-kes berkaitan spesimen juvana dan dewasa yang mempamerkan variasi warna. Menggunakan gen COI, nilai perbezaan genetik yang tinggi (K2P = 6.1%) diperolehi antara kumpulan *L. lutjanus*, LL1 dan LL2, serta analisis seterusnya menggunakan jujukan COI dan *cyt b* menunjukkan tapak penggantian nukleotid diagnostik bagi setiap kumpulan. Oleh itu, pada masa ini, kajian ini mendedahkan bahawa wujud satu leluhur yang mengandungi takson yang tidak dikenali bagi 'kompleks ikan jenahak berbaris kuning'. Sejumlah 3612 bp jujukan yang selanjar dengan kombinasi dua gen mitokondria (654 bp gen COI dan 1116 bp gen cyt b) dan dua gen nuklear (897 bp gen RH dan 945 bp gen EGR1) telah digunakan untuk membina semula kerangka filogenetik Lutjanidae yang komprehensif. Hasil dari kajian semasa juga

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menunjukkan bahawa 'lutjanids' menunjukkan distribusi monofiletik tidak resiprokal dalam rantau Pasifik Timur (EP), Indo-Pasifik (IP), Lautan Hindi (IO) dan Atlantik Barat (WA). Dengan penggunakan penentukuran berasaskan fosil sebagai kekangan terhadap model jam molekul bagi analisis biogeografi dalam kajian semasa, didapati bahawa 'lutjanids' WA, EP dan IO adalah berasal dari leluhur Indo-Pasifik.

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DNA BARCODING OF MALAYSIAN MARINE FISH, PHYLOGENETICS AND PHYLOGEOGRAPHY OF THE SNAPPERS (PERCIFORMES:LUTJANIDAE).

ABSTRACT

This study, implemented DNA Barcoding which offers great potential as a reliable identification tool to classify catches in Malaysia. Overall, this study had successfully barcoded a total of 107 species, 69 genera, 36 families and 10 order of commercial Malaysian fishes. It is found that, the snappers, threadfin fishes, parrot fishes and goatfishes sampled were detected to either experience cryptic speciation or consist of unknown/undescribed species. Conventional identification to species level using morphological characters were found to be difficult for this genera especially in cases where juvenile and adult specimens exhibit variation in colouration. Using COI gene, deep genetic divergence (K2P = 6.1%) values was obtained between LL1 and LL2 groups of *L. lutjanus* and the subsequent analysis of both COI and *cyt b* sequences revealed diagnostic nucleotide substitution sites exclusively to each group. Thus, at present, this study exposed that at least one lineage represents a currently unrecognized taxon of the 'yellow-lined snapper complex'. A total of 3612 bp aligned sequences corresponding to the combinations of two mitochondrial genes (654 bp of the COI gene and 1116 bp of cyt b gene) and two nuclear genes (897 bp of the RH gene and 945 bp of the EGR1 gene) were also employed to reconstruct a comprehensive phylogenetic framework of the Lutjanidae. Results from current study did indicate that lutjanids presented a non-reciprocal monophyletic distribution within Eastern Pacific (EP), Indian Ocean (IO), Indo-Pacific (IP) and Western Atlantic (WA) regions. Using a fossil-based calibration to constraint the relaxed

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molecular clock model for biogeography analysis in current research, it is found that WA, EP and IO lutjanids derived from Indo-Pacific lineages.

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CHAPTER 1

INTRODUCTION

The marine fisheries sector plays an important role in the Malaysian economy, contributing to the national Gross Domestic Product (GDP), employment and foreign exports and representing a rich source of protein for Malaysians. Located on the edge of the "coral triangle" and home to an estimated >2000 species of marine fishes (Allen, 2008), the marine waters surrounding the Malay coastlines that support these activities are some of the most biodiverse regions in the world.

Approximately 200-300 species of marine fishes are landed in the major Malaysian landing sites, with an average of 50-100 species being displayed for sale daily in fish markets (DoF, 2014). Additional species may appear seasonally, with certain species predominating market landings during the monsoons, while other permanent resident species of estuaries, bays and reef areas are landed throughout the year (DoF, 2014). The diversity of wild species harvested and variety of fisheries operations in the country makes assembling accurate detailed catch data challenging.

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This thesis covers three areas of investigation in which each is focused on resolving specific issues. The objectives of current study are as follows:

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1) Large-scale DNA Barcoding assessment of commercial marine fishes in malaysian water: An application for sustainable fishery management.
2) Phylogenetic analysis of *Lutjanus* species (Pisces: Lutjanidae) in Malaysian fisheries catch.
3) Phylogenetic and historical biogeography analyses of the family Lutjanidae, using multi-gene approach and fossil-calibrated tree.

Finally, overall discovery was summarized and concluded in Chapter 7 along with description of future recommendations.

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CHAPTER 2

LITERATURE REVIEW

2.1 Fish diversity in the marine realm

A considerable amount of literature has been published on the importance of marine sphere. Most research to date has tended to focus on the economic and ecological values of marine biota especially as a source of food and indicator of environmental health. Furthermore, publications on major threats to marine biodiversity which include overharvesting, habitat degradation, pollution, global warming, biological invasions and anthropogenic stressors (Costello *et al.*,, 2010) are also increasingly gaining coverage. Still, much of the literature until today, fails to identify the current number of marine species in the ocean. Although scientists have estimated the ocean to contain approximately 2.2 million species (Mora *et al.*, 2011), it is actually challenging to measure the incredible diversity that lies beneath the waves.

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In recent years, scientists have been making serious headway in trying to understand the marine diversity. One of the most extensively studied groups of organism in the marine realm is fish (Mora *et al.*, 2003). Fish represent a keystone in present-day monitoring of environmental health of marine ecosystems (Thomsen *et al.*, 2012). More than 50,000 available species names of fishes have been documented, with over 31,000 of them currently regarded as valid species. Eschmeyer (2010) reported that new marine species are being catalogued at a rate of about 100–150 per year. For easy access to broad information on fish, there are

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several online websites which can be used. The two most referred sites are the Catalog of Fishes (Eschmeyer and Fong, 2016) and FishBase (Froese and Pauly, 2016).

2.2 Assessing the diversity through conventional methods

Great effort has been devoted to the study of fish species identification for several decades. Traditionally, external morphological features, including body shape, pattern of colours, scale size and count, number and relative position of fins, number and type of fin rays, or various relative measurements of body parts (Strauss and Bond, 1990) are utilized for classification. However, major drawbacks from only using morphological criteria for fish identification is the limited characters for differentiation purposes in certain taxa (Callejas and Ochando, 2001). Teletchea (2009) also informed that even with whole specimens, there might be only small interspecific variations. Although many improvements have been made to comprehend such weakness especially by adding more phenotypic characters, morphological ambiguities still persist during species recognition research.

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Examples of identication issues regarding dependency on morphological characters are not uncommon. In 2002, Iff At, demonstrated that number of gill rakers, can be used to differentiate morphologically similar species of Korangi Creek mullets. However, in a later study, Lindsey (1981) deduced that gill raker characteristics is highly influenced by environment through her experiment with coregonids. She discussed that though gill-raker count is less subject to phenotypically induced variation from environmental influences than are most

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morphological characters, it is actually not insusceptible. She advised that when employing even relatively stable characters such as gill raker counts, one should be aware of the influence of the biological effect that exists with them. Besides the usage of gill rakers, analysis of otoliths is an alternative to describe fish species (Pierce and Boyle, 1991; Granadeiro and Silva, 2000). Otoliths are commonly referred to as "earstones" or "fish ear bones". However, the main limitation of this tool are it is destructive, meaning that the extraction of otoliths kills the fish, and otoliths can easily break during extraction/ manipulation. Moreover, otolith analysis is also very difficult because of the concave form of the otoliths and overall variability of its shape.

Vecchione *et al.* (2000) reported that there are many factors that affect fish identification. The most significant factors include experience level of identifiers, reliable taxonomic references, distinction of morphological characters, condition of specimens, life stages of specimen and cryptic speciation. Based on these, they suggested that a more uniform system should be developed. It is notably known that erroneous identification used in analysis or publication can seriously affect future inferences. As species identification is of paramount importance especially to monitor biodiversity (Vecchione *et al.*, 2000), researchers have attempted to improvise conventional methods for identifying fish species without relying exclusively on morphological features.

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2.3 Molecular approach for marine fish identification

Knowlton (1993) pointed out that it is no surprise that scientists took the opportunity provided by the advancement of molecular methods to clarify many ambiguities in conventional taxonomy. Problems in morphological diagnosis are usually associated in identification of the early life stages such as eggs and larvae. Furthermore, large phenotypic plasticity and sexually dimorphic species as well as cryptic species that are widely distributed in marine systems also contribute to the complications.

At the onset, molecular methods used in species identification, including fish, were based on the separation and characterization of specific proteins using electrophoretic techniques, such as isoelectric focusing (IEF) (Rehbein, 1990) and capillary electrophoresis (CE) (Kvasnička, 2005), high performance liquid chromatography (HPLC) (Hubalkova *et al.*, 2007) and even immunoassay systems, such as EnzymeLinked ImmunoSorbent Assay (ELISA) (Asensio and Montero, 2008). These techniques have been widely reviewed (Mackie *et al.,* 1999; Civera, 2003; Moretti *et al.,* 2003; Hubalkova *et al.,* 2007). Even though most of the mentioned methods are of considerable value in certain instances, they are not suitable for routine sample analysis because proteins lose their biological activity after animal death (Telechea, 2009) and their presence and characteristic depend on the cell types (Asensio and Montero, 2008). This has now caused attention to turn towards DNA as a source of information. As an alternative to protein analysis, DNA-based identification methods have currently been explored and extensively developed (Teletchea *et al.,* 2005).

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