



**ANALYSIS OF MITOCHONDRIAL CYTOCHROME OXIDASE I (COI) GENE
AND MICROSATELLITES FOR GENETIC CHARACTERIZATION OF GREY
EEL-TAIL CATFISH (*Plotosus canius* HAMILTON) IN MALAYSIA**

By

NIMA KHALILISAMANI

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

November 2015

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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

**ANALYSIS OF MITOCHONDRIAL CYTOCHROME C OXIDASE I (COI)
GENE AND MICROSATELLITES FOR GENETIC CHARACTERIZATION
OF GREY EEL-TAIL CATFISH (*Plotosus canius* HAMILTON) IN
MALAYSIA**

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November 2015

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Plotosus canius (Hamilton, 1822), commonly known as grey eel-tail catfish or locally known as ikan sembilang or semilang, in Malay, is an important marine species in Malaysia, normally consumed as food fish. However, genetic information on the population structure, stock identification and phylogenetic relationship among *P.canius* populations are still lacking, which could have negative implications on their sustainable conservation and fisheries management throughout Malaysian coastal waters. Therefore, this study was conducted to characterize the genetic structure of *P.canius* in Malaysia by utilizing the mitochondrial Cytochrome c Oxidase I (COI) gene fragment and a set of five microsatellites loci derived from the Australian freshwater eel-tail catfish (*Tandanus tandanus*). In order to fulfill the requirement of the present study, a total of 130 samples of *P. canius* were collected across five selected populations throughout the coastal area of Malaysia. To conduct the COI analysis, a partial fragment (655 bp) of the mitochondrial COI gene, also known as barcoding gene, was sequenced, while five microsatellites loci were selected from the cross species amplification studies originally developed from *T.tandanus*. Subsequently, associate statistical applications were performed to estimate and infer their phylogenetic relationship, genetic variability and population structure.

The experimental and statistic results of the mitochondrial COI analysis revealed that the haplotype diversity varied from 0.395 to 0.771 and nucleotide diversity ranged from 0.033 to 0.65, which pointed out the improbability of mutation effects on gene flow. Moreover, the interesting finding of this study was the sharing of common haplotype KR086940 between populations of Selangor and Johor of Peninsular Malaysia with the Sarawak population of Borneo. This scenario likely reflects a historical genetic connectivity between Peninsular Malaysia and Borneo populations due to the geological history of Southeast Asia during Pleistocene era. Nevertheless, the high pairwise F_{ST} rates (0.05417 - 0.62504) and consequently high genetic variations among *P.canius* populations in Peninsular Malaysia and their relatives in Borneo suggested the absence of contemporary gene flow among the current populations. Phylogenetic and F_{ST} analyses clearly resolved the genetic relationships

between *P.caninus* and *P.lineatus*, supporting their status as distinct species, while all *P.caninus* sequences were diagnosed as belonging to a single species.

Microsatellites results show a considerable heterozygote insufficiency in all populations, with the average observed heterozygosity (H_o) value of 0.2168, which was lower than the standard heterozygosity in marine population ($H_o= 0.79$). This alongside the high F_{is} values estimation, high pairwise differentiation among populations and low within population variations might be related to several factors such as small sample size, inbreeding and/or non-random mating system. Besides, the discrepancies between the mitochondrial COI and nuclear microsatellites results found in this study pointed towards the sensibility of mitochondrial DNA in detection of variation.

In conclusion, the current study has managed to provide a better understanding on the genetic characterization, molecular phylogeny, evolutionary kinship, and population structure in genus *Plotosus*, in general, and *P.caninus*, in particular. Future studies should encompass more geographical and sampling sites, larger population sizes per site, and utilization of more mitochondrial and nuclear hypervariable markers. Finally, the results of this study should be necessary highlighted for appropriate fisheries management and conservation of *P.caninus* in Malaysia.

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

**ANALISIS GEN MITOKONDRIA CYTOCHROME C OXIDASE I (COI)
DAN DNA MIKROSATELIT UNTUK PENCIRIAN GENETIK IKAN
SEMBILANG (*Plotosus canius* HAMILTON) DI MALAYSIA**

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Plotosus canius (Hamilton, 1822), yang biasanya dikenali sebagai “grey eel-tail catfish” atau lebih dikenali sebagai ikan sembilang atau semilang dalam bahasa Melayu, merupakan sejenis spesies ikan marin yang popular di Malaysia, selalunya ditangkap sebagai makanan. Walau bagaimanapun, maklumat mengenai struktur populasi, identifikasi stok dan perhubungan filogenetik antara populasi-populasi *P.canius* masih belum ada, yang boleh memberi implikasi yang negatif untuk pemuliharaan dan pengurusan perikanan secara mampan untuk spesies tersebut di sepanjang pesisir pantai Malaysia. Untuk memenuhi keperluan kajian ini, sejumlah 130 sampel *P.canius* telah ditangkap dari lima populasi terpilih di sepanjang pesisir pantai di Malaysia. Untuk analisa gen COI, satu bahagian (655bp) dari gen mitokondria COI, juga dikenali sebagai “gen barkod”, telah menjalani penjujukan, sementara sejumlah lima lokus mikrosatelit telah dipilih hasil dari kajian amplifikasi rentas spesies yang asalnya diperolehi dari *Tandanus tandanus*. Seterusnya, aplikasi statistik dan analisa yang berkaitan telah dilakukan untuk menganggar dan memperolehi maklumat tentang perhubungan filogenetik, variabiliti genetik dan struktur populasi.

Keputusan analisis COI mendedahkan yang kepelbagaian haplotip berbeza-beza daripada 0.395 kepada 0.771 dan kepelbagaian nukleotida berbeza-beza daripada 0.033 kepada 0.650, yang menegaskan ketidakmungkinan kesan mutasi terhadap aliran gen. Tambahan pula, penemuan yang menarik dari kajian ini ialah perkongsian bersama haplotip KR086940 di antara populasi-populasi Selangor dan Johor dari Semenanjung Malaysia dengan populasi Sarawak dari Borneo. Senario ini berkemungkinan besar menunjukkan perhubungan genetik masa lampau antara Semenanjung Malaysia dan Borneo, yang berkaitan dengan sejarah geologi Asia Tenggara semasa era Pleistocene. Walaubagaimanapun, nilai F_{ST} yang tinggi dan variasi genetik yang tinggi di antara populasi-populasi *P.canius* dari Semenanjung Malaysia dengan populasi-populasi di Borneo mencadangkan tiada lagi aliran gen kontemporari di antara populasi-populasi tersebut. Analisis filogenetik dan F_{ST} (0.05417 - 0.62504) jelas menyelesaikan perhubungan genetik di antara *P.canius* dan *P.lineatus*, dengan menyokong status mereka sebagai spesies yang berlainan, sementara semua jujukan *P.canius* didiagnosis sebagai satu spesies.

Keputusan mikrosatelit menunjukkan defisit heterozigot yang besar dalam kesemua populasi, dengan nilai purata heterozigositi yang didapati (H_o) serendah 0.217, yang menunjukkan nilai yang rendah berbanding nilai heterozigositi piawai dalam populasi marin ($H_o = 0.79$). Ini bersama-sama dengan nilai F_{ST} yang tinggi, perbezaan genetik antara populasi yang tinggi manakala perbezaan dalam populasi yang rendah, berkemungkinan besar adalah berkaitan dengan beberapa faktor seperti saiz sampel yang kecil, pembiakan dalaman dan/atau sistem pengawanan tidak rawak. Di samping itu, perbezaan di antara keputusan COI mitokondria dan mikrosatelit nuclear di dalam kajian ini menunjukkan ke arah kepekaan DNA mitokondria dalam mengesan perubahan.

Kesimpulannya, kajian ini telah berupaya memberikan kefahaman yang lebih baik terhadap pencirian genetik, filogeni molecular, perhubungan evolusi, dan struktur populasi dalam genus *Plotosus*, secara am, dan *P.caninus*, secara khusus. Kajian di masa depan harus merangkumi lokasi persampelan dan geografi yang lebih luas, saiz sampel yang lebih besar per lokasi, dan penggunaan lebih banyak penanda-penanda mitokondria dan nuclear. Akhirnya, hasil dari kajian ini seharusnya diketengahkan untuk pengurusan perikanan dan pemuliharaan *P.caninus* yang sewajarnya di Malaysia.

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LIST OF ABBREVIATIONS

A	Ampere
AMOVA	Analysis of Molecular Variance
Ar	Allele richness
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CA	Correct Assignment
COI	Cytochrome c Oxidase I
d.f	Degree of freedom
<i>F_{is}</i>	Inbreeding Coefficient
<i>F_s</i>	F-statistics
He	Expected heterozygosity
Ho	Observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
I.A.M	Infinite Allele Model
JHR	Johor
ML	Maximum Likelihood
mM	Millimole
MP	Maximum Parsimony
MSN	Minimum Spanning Network
mtDNA	Mitochondrial DNA
Na	Number of allele
Ng	Nanogram
NJ	Neighbour-Joining
NSN	Negeri Sembilan
P	Probability
QTL	Quantitative Trait Locus
SBH	Sabah
SGR	Selangor
S.M.M	Stepwise Mutation Model
SWK	Sarawak
THA	Thailand
T.P.M	Two Phase Model
V	Voltage
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Family Plotosidae are known as elongated bony fishes, which mostly dominated the West Pacific and Indian Ocean coastal area (Ferraris, 2007). Among them, genus *Plotosus* is comprised of approximately 9 different types of Plotosid catfishes including: *P.lineatus*, *P.canius*, *P.limbatus*, *P.nkunga*, *P.papuensis*, *P.abbreviatus*, *P.japonicus*, *P.nhatrangensis* and *P.fisedoha* (Usman et al. 2013; Prithiviraj & Annadurai, 2012). Until now, two species of genus *Plotosus* (*P.lineatus* and *P.canius*) are described in Malaysia (Usman et al. 2013) that could be found in tropical to temperate aquatic ecosystems (Prithiviraj & Annadurai 2012; Kumar 2012). *Plotosus canius* (Hamilton, 1822), which is regionally known as grey eel-catfish, black eel-tail catfish, canine catfish or Indian catfish in its dispersal area (Prithiviraj, 2014; Usman et al. 2013; Riede, 2004; Khan et al. 2002) is diagnosed a member of genus *Plotosus*, family Plotosidae (Froese & Pauly, 2014).

Plotosus canius have been morphologically distinguished by the significant extended and well-developed nasal barbels into the eye diameters and the nape (Usman et al. 2013; Prithiviraj & Annadurai, 2012). Comprehensive morphological account of grey eel-tail catfish have been described them as tapered eel-like, compressed, long, and elongated catfish species, with a fairly large head. Their colour pattern could be varied from dark brown in dorsal region to lighter colours in abdomen (Usman et al. 2013; Kumar, 2012). They have been mainly dispersed in estuaries, freshwater rivers, lagoons, and shallow waters of Australia and South-eastern Asia (Carpenter, 1999), specifically in Australia, Papua New Guinea, Malaysia, Philippines, Thailand, Myanmar, Bangladesh, Sri Lanka and India (Usman et al. 2013; Kumar, 2012).

They were described as amphidromous and demersal fishes, which can live in marine, brackish and freshwater habitats with frequent predictable and cyclical migration in relatively more than 100 km distances on which could not be categorized as breeding migration (Riede, 2004). Adults could be typically found in estuaries, lagoons and merely in riverine habitats, while their juveniles mostly migrate in massive aggregations toward coastal waters (Carpenter, 1999) for feeding and growing for the duration of a couple of months until fully-grown adults usually return to freshwater in order to feed and grow more to preparing for reproduction (Riede, 2004).

Official fisheries statistics have recorded the total catchment of *P.canius* and *P.lineatus* with the rates of lower than one ton in West and East coasts of Peninsular Malaysia, while the peak season of fisheries were recorded in May for west coast and October in East coastal region. However, lack of recent official fisheries records on their catchment, made a great obstacle on determination of both species fisheries status in Malaysia, fisherman observations suggest the seasonal catchment patterns with

constant reduction in fisheries (Usman et al. 2013). Therefore, it seems that grey eel-tail catfish is on severe necessity of conservation studies specifically from regional perspective in Malaysia due to fact that there is a massive gap in precise research on family Plotosidae and *P.caninus* in specific (Usman et al. 2013; Prithiviraj & Annadurai, 2012). Hence, the conducted research have been carried out to answer the questions on:

- What is the level of genetic variation within populations of *P.caninus* in Malaysia?
- To what extent does the level of genetic diversity differ between *P.caninus* and *P.lineatus*?
- Does population structure analysis of *P.caninus* show the different genetic diversity patterns in Malaysia inferred from mitochondrial DNA and microsatellite markers?

1.2 Problem Statement

Regarding to recent published manuscripts on characterization of grey eel-tail catfish, conducted studies might review under two main categories: (1) pure biological researches on topics like fecundity, reproduction and fisheries overview and (2) genetic studies on which characterized genus *Plotosus* in general alongside with other key members of order Siluriformes.

In genetic scale, regarding to several studies on genetic characterization (Powell, 2012; Ninwichian et al. 2012; Wang et al. 2010), barcoding (Lakra et al. 2011; Zhang, 2011; Wong et al. 2011), and microsatellite isolation (Muñoz-Rojas et al. 2012; Yue et al. 2003; Watanabe, 2001) of catfish species e.g., a few researches on genetic basis of genus *Plotosus* (Zhang & Hanner, 2011; Sullivan et al. 2006), almost nothing carried out on DNA barcoding nor population genetic assignments of the species. Genetics researches can be perceived as the viable incentive to impact the evolution in ways that enhance the persistence of populations (Purvis & Hector, 2000). Much published research in the field applies genetic sampling techniques to infer population parameters from the patterns of variation in populations. The limited resolution of these inferences seems to yield limited confidence which results in conservative policies.

Despite regional significance in East and Southeast Asia (Usman et al. 2013), deficiency of genetic research (evolutionary studies and population structure) on *P.caninus* might have destructive effects on fisheries and aquaculture. Therefore, there could be numerous potential interests in study of species like stock identification, captive breeding, conservation assignments, using genetic tools to delineate their taxa, biogeographic and biodiversity hotspots, relationship of genetic and demography, interaction of mating system on genetic diversity, and inbreeding depression and hybridization negative effects on biodiversity for instance. Hence, as it can be clearly seen, there are several deficiencies on some basic genetic and conservation potentials of *P.caninus* in Malaysia. Consequently, here, we intended to answer some basic genetic questions and problems regarding to:

- The level of genetic variation within populations of *P.caninus* in Malaysia.
- The causes and effects of genetic differentiation among populations of *P.caninus* in Malaysia.
- The population structure of *P.caninus* in Malaysia.
- The extent of genetic differentiations between *P.caninus* and *P.lineatus*.

1.3 Aims and Objectives

The main objective of this work is to genetically characterize *P.caninus* populations in Malaysia through the utilization of the mitochondrial cytochrome c oxidase I (COI) gene and a set of microsatellites derived from the Australian freshwater eel-tail catfish (*T.tandanus*). In order to achieve this aim, the specific objectives are listed below:

1. To examine the population structure of *P.caninus* in Malaysia using mitochondrial COI gene.
2. To perform cross-amplification study of microsatellites derived from *T.tandanus* in *P.caninus*.
3. To evaluate the population structure of *P.caninus* using selected microsatellites.

1.3 Outline of Thesis

Chapter 2 describes an overview on biological and genetic basic of experiment. The biological section consists of taxonomy and classification, morphology and identification, habitat and distribution and significance of study in *P.caninus*, while the genetic part introduces different types of applied markers in present assignment as well as some detailed information on their general application, significance and practical application in catfish species. In general, it gives some overview on previous biological and genetic assessments and publications on *P.caninus*.

Chapter 3 deals with DNA barcoding analysis in specific, including a general introduction, detailed methodology which contains two main parts: experimental section on DNA isolation, Polymerase Chain Reaction (PCR) and gel electrophoresis and statistical implementation. At first, the different steps of mitochondrial analysis will be described. Next, the assigned outcomes will be shown and discussed in order including the main findings of thesis. At the end a general conclusion was established into the main points, problems and contributions.

In Chapter 4, firstly a brief introduction on population genetic analysis have given. Next, experimental procedure on genotyping methodology was described including microsatellite screening, fragment analysis, evaluation of genotyping error, estimation of genetic diversity, calculation of population differentiation and structure and assignment. Afterwards, main results were illustrated and accordingly discussed including the comparison of main similarities and differences of mitochondrial and microsatellite experiments. Finally, a brief conclusion of presented results followed by their contribution and vision were given.

Chapter 5 contains brief conclusion and recommendation for future work. The references, biodata and publications are attached at the end of the thesis.

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