ASSESSMENT OF THE MOLECULAR DIVERSITY OF PELIAN (*TOR TAMBRA*) IN SABAH USING MITOCHONDRIAL AND MICROSATELLITE DNA MARKERS

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**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2023**

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2023

UNIVERSITI MALAYSIA SABAH
BORANG PENGESAHAN STATUS TESIS

JUDUL : **ASSESSMENT OF THE MOLECULAR DIVERSITY OF PELIAN (*TOR TAMBRA*) IN SABAH USING MITOCHONDRIAL AND MICROSATELLITE DNA MARKERS**

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries, and references, which have been duly acknowledged.

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DEGREE : **DOCTOR OF PHILOSOPHY IN APPLIED SCIENCE**

FIELD : **BIOTECHNOLOGY**

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ACKNOWLEDGEMENT

I would like to take this opportunity to express my deepest gratitude to God, then to my supervisors Assoc. Prof. Dr. Kenneth Rodrigues, Dr. Christopher Voo Lok Yung and Datuk Dr. Ahemad Sade for their invaluable guidance, patience, and encouragement until the end of my academic journey.

I would like to thank the Department of Fisheries Sabah especially to Datin Dr. Shuhadah Mustapha and University Malaysia Sabah especially to Prof. Dr. Lee Ping Chin, for contributing their precious time in providing guidance, moral support and valuable information regarding the genetic variation of Pelian in Sabah until the completion of this research. I would like to thank my family especially to my late parents, siblings and Carlos Rakem and my children, Clevelence Carlos and Hayden Carlos for their love, understanding, support and encouragement until the completion of my academic journey.

My gratitude also goes to all my colleagues especially to En. Mohammad Khadafi Ibrahim, Mrs. Steffiana Jipanin, Mrs. Rejainie Lahaji, Mr. Norjan Daim, Mr. Anan Abas and the late Dr. Joseph Reynold James Nandu who were willingly and sincerely putting up with me during the study. Finally, my gratitude goes to Lucy, the stray dog that always amuses me when I am down.

Helena Biun
19 April 2023

ABSTRACT

Tor tambra is a cyprinid riverine fish known as Pelian in local Sabahan, Malaysia Borneo, which known to have aquaculture and conservation worth. Applying conservation strategies and a sustainable management program necessitates a comprehensive understanding of genetic diversity and population structure. Eighteen wild Pelian populations were analyzed based on microsatellite markers and mtDNA D-loop. Pelian possesses microsatellite gene diversity and allelic richness of 0.40-0.68 and 2.63-6.10, respectively and mtDNA D-loop haplotype and nucleotide diversities of 0.000 to 0.911 and 0.001 to 0.021, respectively. Pairwise Fst values for microsatellite and Φst values for mtDNA D-loop were significant ($P<0.05$) between many population pairs. Microsatellites analyses of molecular variance, AMOVA analyses showed variances were found within populations (60.85% and 50.74% for grouping by watershed and Crocker Trusmadi Range, CTR, respectively) whereas mtDNA D-loop region revealed most variances were among groups (95.63% and 73.77% for grouping by watershed and CTR, respectively) with significantly high Φst and Fst values. Mantel tests supported weak patterns of differentiation as a result of isolation by distance. Analyses on genetic distance-based relationships (NJ, MP, UPGMA, Bayesian, haplotype network) and model-based clustering (BAPS, STRUCTURE) were consistent with partitioning the overall populations into two well differentiated cluster and further sub-structured according to watershed. Isolation by distance, river fragmentation and historical isolation by CTR significantly explain the genetic population structure. The genetic stock identification and delineation of the biological management units (MU) and conservation units (ESU) can used in identifying and prioritizing populations in need of management and conservation intervention. These data can also be used by the resource manager in decision-making and strategy-formulation process regarding Pelian stock enhancement programs. Information on the genetic variation can be used in the determination of base-line stocks for selective breeding program. However, prior to the adoption of management and conservation measures, there are still knowledge gaps from other disciplines that needs to be explored in order to support and complement the genetic results and to further enhance the management, conservation and breeding strategies formulations.

ABSTRAK

PENILAIAN KEPELBAGAIAN MOLEKUL PELIAN (TOR TAMBRA) DI SABAH MENGGUNAKAN PENANDA DNA MITOKONDRIA DAN MIKROSATELIT

Tor tambra, ikan sungai siprinid juga dikenali sebagai Pelian, mempunyai potensi akuakultur dan nilai pemuliharaan. Memahami kepelbagaian genetik dan struktur populasi adalah asas pelaksanaan langkah-langkah pemuliharaan dan program pengurusan mampan. Lapan belas populasi Pelian liar dianalisis berdasarkan penanda mikrosatelit dan gelung D mtDNA. Pelian mempunyai kepelbagaian gen mikrosatelit (0.40-0.68) dan kekayaan alel (2.63-6.10) dan (0.000 hingga 0.911) haplotip gelung D mtDNA dan (0.001 hingga 0.021) kepelbagaian nukleotida. Nilai Fst berpasangan untuk nilai mikrosatelit dan Φst untuk gelung D mtDNA adalah signifikan ($P<0.05$) untuk banyak pasangan populasi. Analisis mikrosatelit varians molekul, analisis AMOVA mendedahkan bahawa kebanyakan varians ditemui dalam populasi (60.85% dan 50.74% untuk pengumpulan oleh tadahan air dan Crocker Trusmadi Range, CTR, masing-masing) manakala rantau gelung D mtDNA mendedahkan kebanyakan varians adalah antara kumpulan (95.63% dan 73.77% untuk pengumpulan oleh tadahan air dan CTR, masing-masing) dengan nilai Φst dan Fst yang sangat tinggi. Ujian Mantel menyokong corak pembezaan yang lemah akibat pengasingan dengan jarak. Analisis mengenai hubungan berasaskan jarak genetik (NJ, MP, UPGMA, Bayesian, rangkaian haplotaip) dan pengelompokan berasaskan model (BAPS, STRUCTURE) adalah konsisten dengan membahagikan populasi keseluruhan kepada dua kluster yang dibezakan dengan baik dan sub-berstruktur selanjutnya mengikut tadahan air. Pengasingan mengikut jarak, pemisahan sungai dan pengasingan sejarah oleh CTR menjelaskan struktur populasi genetik. Pengenalpastian stok genetik dan persempadanan biologi unit pengurusan dan unit pemuliharaan boleh digunakan dalam mengenal pasti dan pengutamaan populasi yang memerlukan intervensi. Data-data ini boleh digunakan membuat keputusan dan menggubah strategi mengenai program peningkatan stok Pelian. Variasi genetik digunakan dalam penentuan stok program pembiakan terpilih. Namun, terdapat jurang pengetahuan dari disiplin lain perlu diterokai untuk melengkapkan keputusan genetik dan meningkatkan strategi pengurusan, pemuliharaan dan pembiakan.

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LIST OF SYMBOLS

~ - Approximately

/ - Per

< - Less than

> - More than

≤ - Less than or equal to

` - Prime

% - Percent

α - Alpha

β - Beta

δ - Delta

ɸ - Phi

^{0}C - Degree celcius

LIST OF ABBREVIATIONS

IUCN - International Union for Conservation Nature

ESU - Evolutionary Significant Unit

MU - Management Unit

GSI - Genetic Stock Identification

sp. - Species

NJ - Neighbour-joining

UPGMA - Unweighted Pair-group Method using Arithmetic Averages

ML - Maximum Likelihood

mtDNA - Mitochondrial Deoxyribonucleic Acid

Ne - Effective Population Size

HWE - Hardy-Weinberg equilibrium

ESS - Effective sample sizes

MCMC - Markov chain Monte Carlo

CI - Confidence Intervals

myr - million years

mya - million years ago

SMH - Stream Hierarchy Model

IBD - Isolation by Distance

AMOVA - Analysis of Molecular Variance

BLAST - Basic Local Alignment Search Tool

km - kilometer

m - meter

bp - Base pairs

g - Gram

H_20 - Water

h - Hour

kb - Kilobase pairs

mg - Miligram

min - Minutes

ml - Mililitre

µg - Microgram

µl - Microlitre
ng - Nanogram
PCR - Polymerase chain reaction
sec - Seconds
v. - version

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The genus *Tor* of cyprinid fish, also known as the Mahseer, is well-known for its game fish (Ng, 2004), high commercial worth, and conservation concern (Nguyen, 2008). It also has the potential for aquaculture (Ingram et al., 2005). The native Dusuns in Sabah, Malaysian Borneo, refer *Tor* species as "Pelian." Local names for *Tor* include Empurau, Semah, and Kelah in Sarawak and Peninsular Malaysia, respectively. Laos, Cambodia, Yunnan, Jawa, Kalimantan, Sumatra, Yunnan, Malaysia, Thailand, and Vietnam are its native countries (Kottelat, 2001). This species is native to the river basins of Malaysian Borneo and is likely the most common *Tor* species found there (Esa et al., 2006). It was demonstrated that samples collected from Malaysian Borneo may represent a new species of *Tor* based on the mtDNA COX1 phylogenetic tree (Inger & Chin, 2002; Walton et al., 2016). To distinguish the *Tor* species from Sabah in this study, however, and in accordance with Inger and Chin (2002), it will be written as Pelian.

Most river systems in Sabah have headwaters and tributaries where Pelian can be found (Ahmad et al., 2006; Inger & Chin, 2002; Jimmy et al., 2005). Pelian habitat includes both fast-moving rivers in the plains and rocky, pebbly, or stony streams and rivers in the mountains. These types of waterways are frequently preferred by Pelian. Plant matter, including leaves, flowers, and fruits, makes up the majority of Pelian natural diet. Pelian spawning takes place in streams up to 500 metres above sea level and involves several spawners. Schools of Pelian begin migrating upstream from lower reaches of rivers when the breeding season begins, which occurs during the monsoon rains. While bigger adult Pelian are primarily found in the lower reaches of rivers, where they occupy the deepest parts of pools, sub-adult Pelian can be found in the higher sections of the water level at both reaches of rivers (Ng, 2004).

Pelian culture-based fisheries in Sabah were encouraged by the success in breeding Empurau and Semah using hatchery-based broodstock in Sarawak, Malaysian Borneo (Ingram et al., 2005), as well as the significant advantages of implementing the Tagal system, a local community-based management, and conservation efforts. To date, the Tagal system serve as the main conservation program that managed to give positive impacts to the abundance of Pelian stocks. The Ministry of Science, Technology, and Innovation has begun a technique transfer program for the fertilization and reproduction of Pelian (Max, 2010) with the objective of producing fish for the market and raising young fish for stock-enhancement projects. *Tor* sp. have a significant market in Sarawak and Peninsular Malaysia as a high-value food fish. Pelian is a commercially important inland fish species in Sabah with market prices between 15 and 50 Malaysian Ringgit (roughly 4 to 12 US Dollar) per kilogram. *Tor* is a newly emerging fish in the leisure fishing industry associated to ecotourism (Nguyen et al., 2009). Pelian emerged as a popular ecotourism destination, especially in Kg. Moroli Luanti Sabah, which is located 131 kilometres from Kota Kinabalu. It was claimed that Pelian was a cryptic species of conservation value. Pelian differs genetically from the Sarawak Rivers' *Tor* sp. on Borneo Island (Esa et al., 2006).

1.2 Research Problem

Although *Tor tambra* is now categorised on the IUCN Red List as "data deficient", overfishing, logging, deforestation, agriculture, and river modification caused by humans are all contributing to the species' overall population decline (Kottelat, 2012). Due to overfishing, habitat destruction, pollution, and dams, *Tor tambra* is considered a highly vulnerable species in Malaysia (Chong et al., 2010; Wong, 2003). In the 1950's, Pelian were sighted to reach 40 cm long (Inger & Chin, 2002). Despite the population recovery, the typical Pelian today measures between 20 to 30 cm in length. As a result, it is important to characterise the amounts of wild populations genetic variation and comprehend their population genetic structure.

In Sabah, there is no population genetic study on the genetic variation of Pelian wild populations was ever conducted. Studies of genetic variation were conducted mainly in Sarawak, Malaysian Borneo populations. *Tor douronensis* population genetic analysis in Sarawak, Malaysian Borneo employing mitochondrial DNA and microsatellite markers has been demonstrated in (Esa et al., 2006; Esa et al., 2011; Md Adzhar & Hassan, 2015; Nadiatul et al., 2011; Nguyen, 2008; Nguyen et al., 2006). The studies showed mitochondrial and microsatellites genetic diversity ranging from 0.00-0.900 (haplotypes diversity, h), 0.000-0.016 (nucleotide diversity, п) and 0.06-0.60 (Hardy–Weinberg expected heterozygosity, He) 1.64-5.17 (allelic richness, Ar), respectively. The patterns of low intra-population variation and high inter-population variation amongst *Tor douronensis* populations might be due to small population, historical bottleneck, or the existence of physical impediments that restrict population movement (Esa et al., 2006; Nguyen et al., 2006). In general, all studies showed the presence of private haplotype and alleles in some populations, high population differentiation (Fst = 0.147-1.000) and high degree of population sub-structuring, which was in general accordance with geography and habitat fragmentation. These findings show there has been isolation among populations from different river basins with restricted or no migration, possibly due to large geographical areas and the presence of geographical barriers or disconnected river systems.

Since there is no population genetic study on the genetic variation of Pelian wild populations conducted in Sabah, the goal of this research is to understand the genetic variation of Pelian in its natural environments. This will give an idea of how the population is doing now and help to come up with ways to manage the Pelian population and develop aquaculture in a sustainable way. This data is important because it can facilitate effective conservation program implementations such as population conservation prioritization, management spatial scale, and the determination of factors, which act as geographical barriers of population migration, leading to population differentiation. In short-term fisheries management framework, genetic stocks are analogous to management units (MUs) (Moritz, 1994) with the objective of maintaining productive fisheries resources and sustain local abundance by avoiding overexploitation whereas in long-term fisheries conservation framework, genetic stocks refers to evolutionary significant unit (ESUs) (Crandall et al., 2000; Moritz, 1994) which represent important components of adaptive diversity and long-term evolutionary potentials with the objective of preserving sustainable local populations. MU refers to exploited populations that require separate management, whereas ESU refers to special interest populations that require separate conservation. Determination of MU and ESU will be the foundation for informed management and conservation development and practice.

Owing to their locus-specificity, codominance, high information value, significant pattern, homogeneous genome coverage, relative abundance, faster mutation rate, and sample processing flexibility, microsatellite markers have become popular for population genetics studies of marine and freshwater fish species (Abdul-Muneer, 2014). Fragmentation, isolation and unbalanced sex ratios observed in wild *Tor* populations (Ng, 2004) make the populations susceptible to genetic drift, bottleneck and inbreeding processes. mtDNA markers is particularly sensitive to detect these processes. D-loop region in mtDNA, the highly polymorphic region making it useful for analysis of population genetics.

1.3 Research questions

1. Are the populations of Pelian in 10 major rivers at 18 locations throughout Sabah major river systems in Sabah genetically differentiated?
2. Do geographic barriers lead to genetic structuring of Pelian in Sabah?

1.4 Hypotheses

1. The population of Pelian in Sabah exhibit a high degree of genetic population structuring.
2. There is a correlation between levels of genetic variation and geographic distribution of Pelian in Sabah.

1.5 Objectives

This study was conducted with four main objectives as follows:

1. To infer population diversity and structure of wild Pelian populations obtained from sites throughout Sabah's major river systems using mtDNA D-loop and microsatellites loci.
2. To identify Evolutionary Significant Unit (ESU) and Management Unit (MU) for management and conservation in Pelian.
3. To identify potential wild donor populations for capture breeding in restocking programs of Pelian.
4. To identify potential wild founder populations for selective breeding program of Pelian.

1.6 Study scope

The sampling scale of study involved major drainages in Sabah collected across its habitat distribution with members from one spawning season. Sampling sites were from areas where the rivers were managed by the community (Tagal) areas. Tagal is a community-based system of river conservation in Sabah, Malaysia. It involves restricting fishing in certain areas or seasons to allow the fish to grow and breed. Tagal also helps to protect the river ecosystem and the cultural heritage of the local people.

CHAPTER 2

LITERATURE REVIEW

2.1 The Mahseer Group

As one of the biggest scale carp and a prized species of freshwater fish in Asia, mahseers are categorised into three genera: *Tor*, *Neolissochilus*, and *Naziritor*, which are members of the Cyprinidae family and Cyprininne subfamily (Nguyen et al., 2008). In accordance with the median lobe's anatomy, which is unique to this genus and absent from the other two, only *Tor* species from these three genera are considered valid mahseers (Nguyen, 2008; Pinder et al., 2019).

2.1.1 Distribution

Tor sp. is found in three biogeographical regions: South Asia (Pakistan, India, Nepal, Bangladesh), East Asia (China), and Southeast Asia (Myanmar, Laos, Vietnam, Thailand, Cambodia, Indonesia, Malaysia). *Tor* sp. live along all of Asia's major rivers, including the Ganges, Brahamaputra, Indus, Irrawaddy, Mekong, Changjian, and rivers in the now-underwater Sunda Basin islands of Borneo, Java, and Sumatra (Ng, 2004).

While Eschmeyer's Catalog of Fishes 2021 (as of 1 March 2021) claimed 17 species (Pinder et al., 2019) identified 16 numbers of genuine *Tor* species. Rivers in Southeast Asia are home to 13 different *Tor* species. However, only 10 species are recognised as legitimate; the validity of the remaining three species is disputed (Pinder et al., 2019).

Table 2.1: Validity of *Tor* Species Around the World.

No	**Species**	**Eschmeyer's Catalog of Fishes**	**Pinder *et al.* (2019)**	**Distribution**
1	*Tor ater* (Robert, 1999)	Valid	Valid	Laos
2	*Tor barakae (*Arunkumar & Basudha, 2003)	Valid	Valid	India
3	*Tor chelynoides* (McClelland, 1839)	Not Valid Valid as *Naziritor chelynoides* (McClelland, 1839)	Not Valid	India, Nepal
4	*Tor dongnaiensis* (Hoang et al., 2015)	Valid	Valid	Vietnam
5	*Tor douronensis* (Valenciennes, 1842)	Not Valid. Valid as *T. tambra* (Valenciennes, 1842)	Not Valid	Malaysia, Indonesia
6	*Tor hemispinus* (Chen & Chu, 1985)	Not Valid. Valid as *Neolissochilus hemispinus* (Chen and Chu, 1985)	Not Valid	China
7	*Tor khudree* (Sykes, 1839)	Valid	Valid	India
8	*Tor kulkarnii* (Menon, 1992)	Valid	Valid	India
9	*Tor laterivittatus* (Zhou & Cui, 1996)	Valid	Valid	China, Laos
10	*Tor macrolepis* (Heckel, 1838)	Not Valid Valid as *T. putitora* (Hamilton, 1822)	Not Valid	Afghanistan, Bangladesh, Bhutan, India, Myanmar, Nepal, Pakistan
11	*Tor malabaricus* (Jerdon, 1849)	Valid	Valid	India