INTEGRATIVE TAXONOMY OF THE GENUS *BARBODES* (CYPRINIDAE) IN PENINSULAR MALAYSIA WITH A FOCUS ON THE *B. binotatus* SPECIES COMPLEX AND *B. lateristriga* MORPHOTYPES

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABGD	Automatic Barcode Gap Discovery
BI	Bayesian Inference
bp	base pair
BP	Bootstrap Proportion
cm	centimetre
COI	Cytochrome c Oxidase Subunit I
Cyt *b*	Cytochrome *b*
DFA	Discriminant Function Analysis
DNA	Deoxyribonucleic Acid
ML	Maximum Likelihood
mm	millimetre
MP	Maximum Parsimony
n	sample size
OTU	Operational Taxonomic Units
p	P-value
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RP1	First Intron of the Nuclear S7 Ribosomal Protein
rRNA	ribosomal Ribonucleic Acid
SD	Standard Deviation
tRNA	transfer Ribonucleic Acid
UMKL	Zoological Museum of the University Malaya Kuala Lumpur
UMTZC	Universiti Malaysia Terengganu Zoology Collection
USMFC	University Sains Malaysia Fish Collection
ZRC	Lee Kong Chian Natural History Museum of Singapore

LIST OF APPENDICES

GABUNGAN TAKSONOMI GENUS *BARBODES* (CYPRINIDAE) DI SEMENANJUNG MALAYSIA DENGAN TUMPUAN KE ATAS *B. binotatus* SPESIS KOMPLEKS DAN MORFOTIP *B. lateristriga*

ABSTRAK

Kajian ini tertumpu kepada ikan genus *Barbodes* Bleeker, 1859 di Semenanjung Malaysia, yang mana empat spesies seringkali dilaporkan dalam kajian, *B. binotatus*, *B. banksi*, *B. lateristriga* dan *B. dunckeri*. Pemerhatian awal ke atas ikan *Barbodes* di Semenanjung Malaysia telah menemui variasi dalam corak badan. Variasi ini tidak hanya dapat dilihat di antara spesies tetapi juga dilihat dalam spesies, yang mana merumitkan reka bentuk taksonomi spesies di kawasan ini. Ini khususnya untuk dua kumpulan spesies yang dirujuk sebagai (1)"*B. binotatus* spesies kompleks", yang melibatkan tiga spesies sahih: *B. binotatus*, *B. banksi* dan *B. rhombeus*, dan (2)"morfotip *B. lateristriga*". Kajian ini bertujuan untuk menyelesaikan status taksonomi *B. binotatus* spesies kompleks dan morfotip *B. lateristriga* dengan pendekatan morfologi dan molekular dan menyediakan senarai spesies lengkap genus *Barbodes* di Semenanjung Malaysia. Kajian morfologi bagi 106 spesimen dari lokasi berbeza menunjukkan tiga *B. binotatus* spesies kompleks dijumpai di Semenanjung Malaysia: *B. binotatus*, *B. rhombeus* dan *B. banksi*, merujuk kepada saiz tanda hitam pada dasar sirip dorsal. Semua spesimen ini juga, masing-masing dibandingkan dengan *B. binotatus* sebenar dari Java, holotip *B. rhombeus* dari Thailand dan *B. banksi* sebenar dari Sarawak. Berdasarkan pada analisis morfometrik menggunakan ANOVA sehala dan ujian Tukey *post-hoc*, 27 ciri pengukuran morfometrik menunjukkan perbezaan bererti ($p<0.05$) antara *B. binotatus* spesies kompleks. Penyiasatan atas spesies kompleks ini oleh analisis PCA dan DFA mendedahkan ciri-ciri panjang muncung,

panjang prenostril, panjang pangkal ekor, panjang sirip anal ditekan, tinggi sirip anal dan tinggi sirip dorsal sebagai ciri penting dalam membezakan mereka. Kemudian, daripada 24 bilangan meristik, sembilan ciri menunjukkan perbezaan bererti ($p<0.05$) antara spesies kompleks oleh ANOVA sehala dan ujian Tukey *post-hoc*. Hubungan molekular filogenetik *B. binotatus* spesies kompleks dan spesies terdekat dikaitkan di Semenanjung Malaysia dikaji menggunakan tiga penanda molekular (cyt *b*, COI, RP1). Secara genetik di Semenanjung Malaysia, *B. binotatus* spesies kompleks mendedahkan tiga susur galur (kebanyakannya alopatrik) yang mana sepadan dengan priori yang dikenalpasti *B. binotatus*, *B. rhombeus* dan *B. banksi*. Walau bagaimanapun, susur galur *B.* cf. *binotatus* dan *B.* cf. *banksi* berbeza daripada spesimen yang dikumpul daripada atau berdekatan dengan lokasi tip masing-masing dan hanya *B. rhombeus* berkelompok dalam lokasi tip. Untuk morfotip *B. lateristriga*, kajian terkini ke atas 34 individu morfotip *B. lateristriga* mempamerkan empat corak badan berbeza dari beberapa lokasi di Semenanjung Malaysia, dari Perlis di utara ke Johor di selatan: (Corak A-Perlis, Corak B-Perak, Corak C-Kelantan, Terengganu, Pahang, Corak D-Johor). Corak badan mengandungi dua bar menegak, satu jalur melintang pada tengah badan dan satu tanda supra anal pada dasar sirip anal. Berdasarkan pada ANOVA sehala dan ujian Tukey *post-hoc*, 20 ciri pengukuran morfometrik dan lima ciri bilangan meristik mempamerkan perbezaan bererti ($p<0.05$) ke atas morfotip *B. lateristriga*. Merujuk ke atas PCA dan DFA morfotip *B. lateristriga*, analisis ini menunjukkan ciri panjang muncung, panjang pangkal ekor, panjang sirip dorsal ditekan, panjang sirip anal ditekan, tinggi sirip anal, panjang posdorsal dan panjang dari dasar sirip dorsal hingga dasar sirip dada. Perbezaan spesies oleh pendekatan molekular dalam morfotip *B. lateristriga* menggunakan penanda mitokondria cyt *b* ke atas 24 spesimen dan analisis pada jarak genetik menunjukkan nilai terendah (4.53 %) antara *B. lateristriga* untuk

Corak C (Kelantan, Terengganu, Pahang) dengan *B. lateristriga* untuk Corak B (Perak) dan nilai tertinggi (9.58 %) antara *B. lateristriga* untuk Corak D (Johor) dengan *B. lateristriga* untuk Corak A (Perlis). Dalam kajian ini, semakan pada ikan daripada genus Barbodes di Semenanjung Malaysia telah menemui enam spesies yang terdiri daripada *B.* aff. *binotatus*, *B. rhombeus*, *B. sellifer*, *B. zakariaismaili*, empat morfotip *B. lateristriga* dan *B. dunckeri*.

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ABSTRACT

This study focused on the fish genus *Barbodes* Bleeker, 1859 in Peninsular Malaysia, where four species have been often reported in the literatures, *B. binotatus*, *B. banksi*, *B. lateristriga* and *B. dunckeri*. Preliminary observations on the *Barbodes* fishes in Peninsular Malaysia had found variation in body pattern. These variations can not only be seen interspecifically but also be seen intraspecifically, which complicates the taxonomic designation of the species in this region. This is particularly true for two groups of species which are referred to as the (1) "*B. binotatus* species complex", which includes three valid species: *B. binotatus*, *B. banksi* and *B. rhombeus*, and (2) "*B. lateristriga* morphotypes". This study aims to resolve the taxonomic status of *B. binotatus* species complex and *B. lateristriga* morphotypes by morphology and molecular approach and provide a comprehensive species list of genus *Barbodes* in Peninsular Malaysia. Morphology studies of 106 specimens from different localities indicated three types of *Barbodes* species found in Peninsular Malaysia: *B. binotatus*, *B. rhombeus* and *B. banksi* referred to their size of black spot at the dorsal-fin base. All of these specimens also compared with the true *B. binotatus* from Java, holotype of *B. rhombeus* from Thailand and the true *B. banksi* from Sarawak respectively. Based on the analysis of morphometric using one-way ANOVA and post-hoc Tukey test, there are 27 characters of morphometric measurements showed significant difference ($p<0.05$) among the *B. binotatus* species complex. Investigation on this species complex by PCA and DFA analysis revealed the characters of snout length (SnL), prenostril

length (PreNosL), caudal peduncle length (CPL), anal-fin depressed length (ADepL), anal-fin height (AHi) and dorsal-fin height (DHi) as important characters in separation of them. Then, out of the 24 meristic count, nine characters exposed significant different ($p<0.05$) between the species complex by one-way ANOVA and post-hoc Tukey test. Molecular phylogenetic relationship of *B. binotatus* species complex and closely related species in Peninsular Malaysia was investigated by using three molecular markers (cyt *b*, COI, RP1). Genetically in Peninsular Malaysia, *B. binotatus* species complex revealed three genetic lineages (mostly allopatric) which correspond to a priori identified *B. binotatus, B. rhombeus* and *B. banksi*. However, the lineages of *B.* cf. *binotatus* and *B.* cf. *banksi* differ from specimens collected from or nearby their respective type locality and only *B. rhombeus* clustered within type locality. For the *B. lateristriga* morphotypes, current study on 34 individuals of *B. lateristriga* morphotypes exhibited four different body patterns from several localities in Peninsular Malaysia, from Perlis in the north to Johor in the south: (Pattern A-Perlis, Pattern B-Perak, Pattern C-Kelantan, Terengganu, Pahang, Pattern D-Johor). The body pattern consists of two vertical bars, a horizontal stripe on the mid body and a supra anal blotch at anal-fin base. Based on one-way ANOVA and post-hoc Tukey test, 20 characters of morphometric measurement and five characters of meristic count exhibited significant difference ($p<0.05$) on the *B. lateristriga* morphotypes. Based on the PCA and DFA of *B. lateristriga* morphotypes, this analysis revealed the characters of snout length (SnL), caudal peduncle length (CPL), dorsal-fin depressed length (DDepL), anal-fin depressed length (ADepL), anal-fin height (AHi), postdorsal length (PosDL) and length from dorsal-fin base to pectoral-fin base (D-PcL). Molecular species delimitation within *B. lateristriga* morphotypes using mitochondrial marker of cyt *b* on 24 specimens and analysis on genetic distance presented the lowest value (4.53 %) between *B. lateristriga*

for Pattern C (Kelantan, Terengganu, Pahang) with the *B. lateristriga* for Pattern B (Perak) and the highest value (9.58 %) between *B. lateristriga* for Pattern D (Johor) with the *B. lateristriga* for Pattern A (Perlis). In this study, the revision of the fish genus *Barbodes* in Peninsular Malaysia has found six species, namely *B.* aff. *binotatus*, *B. rhombeus, B. sellifer*, *B. zakariaismaili*, four morphotypes of *B. lateristriga* and *B. dunckeri*.

CHAPTER 1
GENERAL INTRODUCTION

1.1 General Background

Malaysia is one of the world's megadiverse countries and is endowed with an aquatic environment and rich resources (Chong et al., 2010). In addition, in Malaysia, a total of 1,951 freshwater and marine fish species are recorded, belonging to 704 genera, 186 families, and 37 orders (Chong et al., 2010). Malaysia's freshwater fish species can be divided into two zoogeographic regions which the first region is the Peninsular Malaysia and the second region is west Malaysia, that is Sabah and Sarawak (Mohsin & Ambak, 1991; Zakaria-Ismail, 1994; Yap, 2002). Diversity of fish life in the freswater ecosystems in Peninsular Malaysia is compratively high (Zakaria-Ismail, 1994; Chow et al., 2014). This is due to the variety of habitats (Zakaria-Ismail & Fatimah, 2007; Zakaria-Ismail & Lim, 1994) and the overlapping of distributional range of the Thailand as well as Indonesian fish elements (Ng & Ng, 1998).

Instead, since Malaysia's independence, the socio-economic growth had increased for more than 60 years and consequently, species enumerating action requires new urgency as extensive landscapes are increasingly modified and damaged by anthropogenic activities. Unfortunately, the reality of taxonomy impediments is known as a major obstacle globally (Ebach & Holdrege, 2005; Kim & Byrne, 2006; de Carvalho et al., 2007; Reddy et al., 2009), and Malaysia has not escaped such difficulty. Most of the researchers in ichthyological study stated that this study is still in a discovery and exploratory stage (Zakaria-Ismail, 1992; Lim et al., 1993; Zakaria-Ismail, 1994; Ahmad & Khairul-Adha, 2005; Chong et al., 2010; Khairul, 2011) and there is an obvious lack of centralized taxonomy governance in Malaysia. Continuously from the not commercialized of the disciplines on fish taxonomy and

systematics, their rigour and progression also are held back by the lack of institutional and funding support in Malaysia (Ng et al., 2017).

In this case, the condition has become worse since researchers only "point-and-click" over websites for fish species identification which purportedly provide the latest taxonomic information and species illustrations (Ng et al., 2019). Hitherto, in Malaysia, the most work in the country has been confined to inventory the aquatic habitats and also increasing fish-based DNA studies by researchers who have less of the basic in taxonomy and systematics (Ng et al., 2019). Hence, taxonomic vandalism arises when researchers apply a mix of synonyms, invalid names, or worse incorrect names into their publications which consequently constitutes misinformation presented in the websites, online database, and doubtful literature sources (Ng et al., 2019).

1.2 Problem Statement

The present study focuses on the genus *Barbodes* Bleeker 1859 (Teleostei: Cyprinidae) which currently includes more than 40 species. Fish of the genus *Barbodes* is a small to medium size (5 to 10 cm) species and handsomely silvery or coppery coloured, distributed in Southeast Asia (Kottelat, 2013). Fish of the genus *Barbodes* exhibits a unique characteristic that is the marking pattern of their body changes ontogenically (Kottelat, 2013; Fahmi-Ahmad et al., 2020). These changes can be seen from inception to maturity.

From the preliminary observations, the fish of the *Barbodes* in Peninsular Malaysia shows large variations in its body pattern, both at the inter-specific and intra-specific levels making the recognition of species difficult. The presence of the intraspecific polymorphisms has complicate taxonomic designations of the fish in this

region, particularly among species with similar morphology. The mechanistic basis of such variations is yet to be known as they could result of genetic or environmental causes.

In this context, some species of *Barbodes* are difficult to identify because of their overall morphological appearance and the presence of local populations with slightly different characters. This is particularly true for two groups of species which are referred to as the "*Barbodes binotatus* species complex" (Bariche, 1998), and "*Barbodes lateristriga* morphotypes" (Tweedie, 1961).

In Peninsular Malaysia, the *Barbodes binotatus* species complex includes three valid species, namely *B. binotatus* (Valenciennes, in Cuvier & Valenciennes 1842), *B. banksi* (Herre, 1940a) and *B. rhombeus* (Kottelat, 2000). These three species have similar black markings on their body, including a mark under the dorsal fin (but the shape and size of this mark are different among the three species making it a criterion for species identification). Other than that, these three species also share a black mark on the caudal peduncle which is variable in size and intensity within and among species; this mark is sometimes absent. Minimizing the taxonomical importance of the marking variation in these fishes, Ng & Tan (1999) considered *B. binotatus* and *B. banksi* as two colour forms of the same species. According to Kottelat (2000), the true *B. binotatus* is restricted to Java (type locality), Bali, Lombok, and highlands of Sumatra whereas *B. rhombeus* (described from the Trat Province, Thailand) is endemic to Indochina, north to Isthmus of Kra. *Barbodes banksi* is known from Kuching, Sarawak (type locality), Malaysia, and Peninsular Malaysia. Regional genetic investigations in Sumatra and Java showed large genetic diversity in *B. binotatus* suggesting the presence of cryptic species (Roesma et al., 2018; Hutama et al., 2017).

Furthermore, in an unpublished academic work, Bariche (1998) used morphometric and meristic characters to study different populations of *B. binotatus* in Java and Sumatra. The author found that the variability observed is of an interspecific level, with the presence of, at least, four distinct species.

The second species in Peninsular Malaysia that is *Barbodes lateristriga* morphotypes documented by Tweedie (1961) have six different forms of body pattern i.e., Johore form, Selangor form, Muar River form, Pahang and Kelantan form, Perak form, Perlis and Kedah form. Generally, its distribution shows regional variation of body pattern based on anterior and posterior of vertical and horizontal bars (Tweedie, 1961; Ng et al., 1999). Thus, a lot of confusion on the identity existed because of the variety of body pattern. In addition, the discrepancy of the available data and the absence of recent documented taxonomic revision on this species cannot answer the query of the variety of body pattern on this species.

Hence, the study of the taxonomy for both groups will provide 1) better morphological and molecular information on how to delimitate and identify the different of '*Barbodes binotatus* species complex'' in Peninsular Malaysia, 2) a current study on '*Barbodes lateristriga* morphotypes' by combination of morphological and molecular in Peninsular Malaysia and 3) a list of species under genus *Barbodes* in Peninsular Malaysia.

1.3 Objectives

To address the issues described above, this study aimed to achieve the following objectives:

i. To resolve taxonomic status of the *Barbodes binotatus* species complex in Peninsular Malaysia based on morphological approach

ii. To investigate the phylogenetic relationships of the *B. binotatus* species complex in Peninsular Malaysia using molecular markers in order to reveal and delimitate species

iii. To determine the taxonomic status of *B. lateristriga* morphotypes in Peninsular Malaysia.

iv. To revise the taxonomy of all species of *Barbodes* in Peninsular Malaysia and provide a comprehensive species list of genus *Barbodes*

1.4 Outline of the Thesis

This thesis contains eight chapters starting with a General Introduction and Literature Review in Chapters 1 and 2, respectively. The present chapter provides a brief overview of the study, problem statement, research objectives and thesis outline.

Chapter 2 presents a broad contextual statement to frame the study in the light of current knowledge and understanding on the freshwater fishes in Peninsular Malaysia and the biogeography of Sundaland. This chapter also reviews some details on the biology of the *Barbodes binotatus* species complex and *B. lateristriga* morphotypes including taxonomy, morphological characters, distribution and present status. Otherwise, this chapter described the tools and methods used to resolve the taxonomy of these species.

In the next four chapters, four investigations are presented, each focusing on specific issues: a morphology-based study of the *Barbodes binotatus* species complex in Peninsular Malaysia in Chapter 3, a phylogenetic study of the *B. binotatus* species complex in Peninsular Malaysia in Chapter 4, a taxanomic status of *B. lateristriga* morphotypes in Peninsular Malaysia in Chapter 5 and a taxonomic revision of the genus *Barbodes* in Peninsular Malaysia in Chapter 6. The research study from Chapter 3 to Chapter 5 covered by molecular and morphological assessments that are supported by statistical analysis to fulfill the objectives of this project.

Chapter 3 evaluates the differences of morphological characters in *Barbodes binotatus* species complex in Peninsular Malaysia based on past and present specimens' collection, using morphometric and meristic counts. The specimens collected in Peninsular Malaysia were also compared with specimens of each valid species of the *B. binotatus* species complex, collected from or nearby their type localities, currently housed in the Zoological Reference Collection of the Lee Kong Chian Natural History Museum, National University of Singapore (ZRC), General Biology Laboratory, Universiti Malaysia Terengganu (UMTZC) and Muzium Zoologi Universiti Malaysia Sarawak (UNIMAS).

Chapter 4 describes the investigation on the phylogenetic relationships among the *Barbodes binotatus* species complex using mitochondrial DNA (cytochrome *b* and cytochrome oxidase I [COI]) and nuclear DNA (RP1) sequences. The molecular data were utilized to disentangle the interspecific and intraspecific genetic variations in order to have a better estimate on the number of species within the *B. binotatus* species complex in Peninsular Malaysia. An addition, this chapter had been published as "To lump, to split or to maintain? Molecular taxonomy of the spotted barb *Barbodes*

binotatus (Cyprinidae) and closely related species in Peninsular Malaysia", DOI: 10.1111/jfb.14754 (Ahmad Sobri et al., 2021).

Chapter 5 focused on the taxonomic status of *Barbodes lateristriga* morphotypes by morphology and molecular approach. In this chapter, the specimens of this species were divided by body pattern and also evaluate the morphology characters based on morphometric and meristic counts. Then, this chapter continuously on delimitation species by using mitochondrial marker (cyt *b*) and genetic distance analysis.

During the course of this study, there are two new species described in this genus. Hence, Chapter 6 provides a comprehensive species list on the taxonomy for all species of *Barbodes* in Peninsular Malaysia. This chapter focused on the material examined, comparison material examined, diagnosis characters, descriptions of characters, colouration in live and in preservation of fish specimen, habitat, and distribution of each species. Then, this chapter highlighted the characters used to differentiate between species under genus *Barbodes*.

Chapter 7 summarizes the key findings obtained from the above-mentioned chapters and Chapter 8 concludes the finding of research study and provides orientation for future studies.

CHAPTER 2
LITERATURE REVIEW

2.1 Diversity of Freshwater Fishes in Peninsular Malaysia

Based on species community similarity, the distribution of Southeast Asian freshwater fishes can be divided into five zoogeographic regions: 1) the Salween basin in Myanmar; 2) the Indo-Chinese Peninsula which includes the Mekong, Chao Phraya and Mae Khlong rivers of Thailand and Cambodia; 3) the Malay Peninsula which includes the southern Thailand, Peninsular Malaysia, and Singapore; 4) the Indo-Malayan Archipelago; and 5) Mindanao (in the Philippines) (Zakaria-Ismail, 1994).

Ambak et al., (2012) stated that Malaysia is well known to be among the mega biodiverse regions on Earth, both for flora and fauna. This region possesses a large range of ecosystems housing various life forms, especially aquatic ecosystems including their fish fauna. Primary freshwater fishes are only found in lakes and rivers where the salinity is less than 0.05% (Gene et al., 2009). The local diversity and abundance of freshwater fishes are influenced by abiotic factors (such as temperature, salinity, and pH), biotic factors (including food availability, competition with other fish species) and historical factors (evolution and geology) (Moyle & Cech, 2003).

In Peninsular Malaysia, Zakaria-Ismail et al., (2019) listed 289 native fish species inhabits the freshwater ecosystems. In this publication, the authors stated that the diversity of freshwater fishes in Peninsular Malaysia is comparatively high due to the variety of available freshwater habitats such as standing waters (lakes, ponds, swamps, and marshes), running waters (river, streams, brooks, and creeks) and the overlapping of the distributional range of the Thailand and Indonesian fish elements.

Within Peninsular Malaysia, there are several inventory studies for freshwater fishes, confined to single drainage such as the Endau drainage (Lim et al., 1990; Ng & Tan, 1999; Zakaria-Ismail & Fatimah, 2007; Chow et al., 2014), Pahang drainage (Zakaria-Ismail, 1984; Zakaria-Ismail, 1993; Khan et al., 1996; Lee & Zakaria-Ismail, 1997; Khaironizam et al., 2009; Rashid et al., 2015; Rashid et al., 2018; Mohd-Azham & Singh, 2019), Terengganu drainage (Cramphorn, 1983; Kottelat et al., 1992), Kelantan drainage (Hashim et al., 2015; Alias et al., 2019), Muda drainage (Sah et al., 2012; Lee et al., 2013), Perak drainage (Hashim et al., 2012; Ahmad et al., 2018; Ng et al., 2019), Bernam drainage (Ng et al., 1994), Selangor drainage (Zakaria-Ismail & Fatimah, 2002), Klang drainage (Bishop, 1973; Fatinizzati et al., 2018) and Muar drainage (Jeffrine et al., 2004; Halim et al., 2018). Few inventory studies focusing on specific habitats such as lakes (Kamaruddin et al., 2011; Shafiq et al., 2014; Fahmi-Ahmad et al., 2015; Sah et al., 2016), peat swamp forest (Mizuno & Furtado, 1982; Ng et al., 1994; Shah et al., 2006; Ahmad et al., 2011; Ahmad & Samat, 2015; Sule et al., 2016; Amal et al., 2020), agriculture-converted areas (Aqmal-Naser & Ahmad, 2018), and insular freshwater systems (Ahmad & Lim, 2006; Azmir & Samat, 2010; Tan et al., 2015; Aqmal-Naser et al., 2018; Aqmal-Naser et al., 2019).

In addition, Zakaria-Ismail et al., (2019) reported that the freshwater fish in Peninsular Malaysia consisted of 14 orders which included Myliobatiformes, Anguilliformes, Osteoglossiformes, Clupeiformes, Cypriniformes, Siluriformes, Sygnathiformes, Gobiiformes, Synbranchiformes, Anabantiformes, Pleuronectiformes, Beloniformes, Tetraodontiformes and Perciformes. Based on the listed above, order Cypriniformes dominated the freshwater fish species followed by Siluriformes, Anabantiformes and Synbranchiformes which represented 47%, 23%, 14% and 5% respectively. Other fish orders are represented by less than 5%.

2.1.1 Cyprinid Fishes in Peninsular Malaysia

Cyprinidae is one of the families of the order Cypriniformes and superorder Ostariophysi. This family has the particularity to be one of the three largest fish families with more than 1,700 valid species (the two other families are the Cichlidae and Gobiidae) (Nelson et al., 2016). Cyprinid fishes are commonly known as minnows, carps, barb and loaches. Within the family Cyprinidae, there are currently 11 recognized subfamilies, namely Acrossocheilinae, Barbinae, Cyprininae, Labeoninae, Poropuntiinae, Probarbinae, Schizothoracinae, Schizopygopsinae, Smiliogastrinae, Spinibarbinae and Torinae (Tan & Armbruster, 2018).

Cyprinids are distributed worldwide excepted in South America, Australia, and Antarctica (Mayden, 1991) and they include many culturally, economically, and scientifically important species. Some members of this family are also important as ornamental and food fishes (Nelson et al., 2016), and in some part they are scientifically used as bio-indicators to monitor water quality (Ho & Tan, 1997; Zakaria-Ismail & Fatimah, 2002).

Most of the cyprinid species are primarily lives in freshwater habitats and few of them are able to tolerate brackish waters (Nelson et al., 2016). Currently, there are about 157 valid genera of Cyprinidae consisting about 1,780 valid species recorded in the freshwater ecosystems throughout the world (Fricke et al., 2022). However, the number is far from complete as many species are still undescribed (Tan & Armbruster, 2018).

The family Cyprinidae is characterized by pharyngeal teeth in one to three rows, but never more than eight teeth in any row; the presence of a Weberian organ near the anterior part of the vertebrate column; the lips usually thin; barbels may

present or absent; upper jaw usually protrusible; always toothless; adipose fin absent and head almost always scaleless. Compared to other families, cyprinid fishes are not only the most diversified in Southeast Asian river systems, contributing to more than 40% of the ichthyofaunal composition, but also the most abundant (Rainboth 1991; Zakaria-Ismail, 1994).

Despite a large amount of data on the systematics and taxonomy of cyprinid fishes in Peninsular Malaysia, the number of species and their correct identity still need further investigations (Ismail, 1989; Lim & Tan, 2002). Currently, in Peninsular Malaysia, Zakaria et al., (2019) recorded highest species under Cyprinidae followed by Danionidae and Osphronemidae that listed 59, 32 and 30 species respectively. For decades, the list of fish species recorded from Peninsular Malaysia, especially for the cyprinids, was strictly based on previous taxonomic works whereas several groups required obvious taxonomical revisions. This led to the situation where the species names used are incorrect and the taxonomic status of several species is not resolved with the presence of "cryptic" species. Lim et al., (1993) listed some cyprinid genera that, according to them, need to be revised: *Barbodes*, *Hypsibarbus*, *Labiobarbus*, *Neolissochilus*, *Poropuntius*, *Puntioplites*, *Puntius*, *Rasbora* and *Tor*. Therefore, Intan-Faraha (2019) and Muhammad-Rasul (2018) recently revised the genera *Puntioplites* and *Poropuntius*, respectively, using morphological and molecular approaches.

2.1.2 Taxonomic Studies of Cyprinid Fishes in Peninsular Malaysia

Conway et al., (2010) stated that the family Cyprinidae is a good example of the complexity of Sundaland freshwater fishes in taxonomy and systematics. The systematics of this large family has been controversial for more than a century and the systematics and taxonomy of many species of Cyprinidae in Peninsular Malaysia are poorly understood (Ismail, 1989; Lim & Tan, 2002).

Kottelat (1998) discussed the taxonomic status of *Barbodes binotatus*, one of the most widely distributed and abundant freshwater fishes in Southeast Asia, which is known for its morphological variation. This species was known from a few scattered localities in Java, Sumatra, Borneo, Malaysia, Thailand, and Vietnam; despite showing significant morphological variability within and among populations, all these populations were kept under a single name, *B. binotatus*.

Kottelat (1998) noted *Barbodes binotatus* as a distinctive species having different habitat preferences where different morphs can occur in sympatry. In this context, this present study aimed to solve the taxonomy of this species, *B. binotatus*, which likely forms a species complex, using two different approaches, morphology and genetics, and a large taxonomic sampling throughout the entire Peninsular Malaysia along with specimens from the type localities of nominal species related to *B. binotatus*.

Esa et al., (2012) investigated the phylogenetic relationships of 21 species of Cyprinidae inferred from the mitochondrial COI gene. The authors mentioned that the phylogenetic, systematics and taxonomic studies of Cyprinids in Malaysia, particularly among the native taxa are still highly fragmented and poorly resolved. This study

suggested the need for a taxonomic revision of the genus *Barbonymus* because *B. schwanenfeldii* and *B. gonionotus* do not form a monophyletic group.

According to Muhammad-Rasul et al., (2018), three nominal species of the genus *Poropuntius* (i.e., *P. deauratus* [Valenciennes in Cuvier & Valenciennes 1842], *P. normani* [Smith, 1931], and *P. smedleyi* [de Beaufort, 1933]) have been reported from Peninsular Malaysia's freshwater ecosystems. Nevertheless, the valid species of *Poropuntius* is still unknown because of the low morphological differentiation among these species that has led to identification confusion in this genus. Hence, Muhammad-Rasul et al., (2018) reviewed the taxonomic status of *Poropuntius* using morphological and molecular approaches and the results strongly supported the presence of only one species in Peninsular Malaysia, *P. normani* (*P. smedleyi* described from Johor, is a junior synonym of *P. normani* and the presence of *P. deauratus* in Peninsular Malaysia is doubtful; all evidence suggests that *P. deauratus* is endemic of Vietnam).

The cyprinid genus *Tor* represents a group of large-bodied freshwater fishes whose taxonomy and systematics are also poorly known (Walton et al., 2017). *Tor* represents a suitable model for the application of an integrative taxonomic approach combining morphology, genetics, and historical records to examine species identities, where one or more of these fundamental approaches may have been lacking in the past. Hence, Walton et al., (2017) provided comprehensive evidence for discussing the classification and conservation of mahseer species in Malaysia.

2.2 Paleogeology of Sundaland and Biogeography

The Sundaland is the region of Southeast Asia comprising exposed landmasses Indochina, the Thai-Malay Peninsula, Sumatra, Java, Borneo, and the currently submerged shallow marine shelf (the 'Sunda Shelf') between these islands/landmasses (Hall & Morley, 2004). This marine shelf was exposed during the Pleistocene sea level low stand, connecting all landmasses previously listed.

The Sundaland region is delimitated to the south and west by the deep waters of the Indian Ocean, and comprised of the small island chains west of Sumatra such as the Mentawai Islands (Bird et al., 2005). To the east, Sundaland is bordered by the so-called Wallace Line that marks the limit running between Bali and Lombok in the south, Borneo, and Celebes/Sulu archipelago on the equator and between Palawan and the rest of the Philippines in the north (Lohman et al., 2011) (Figure 2.1).

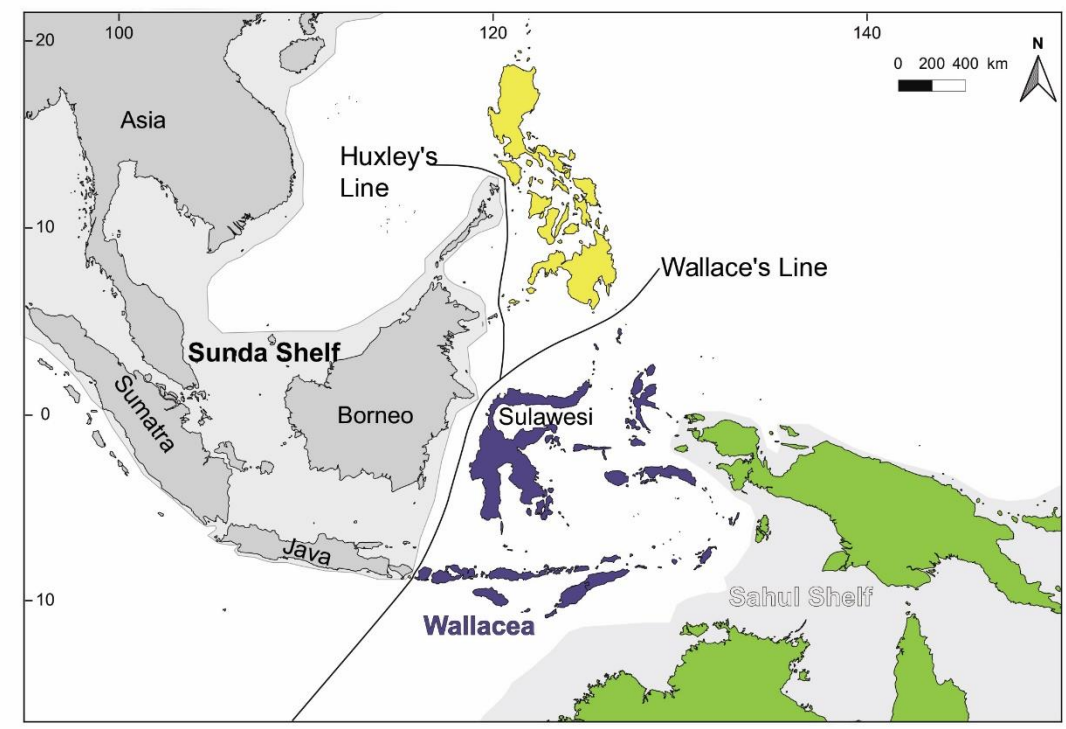

Figure 2.1 Shaded area represents continental shelves of Sunda. Map modified from Beron (2015)

The Sundaland had undergone several landforms changes due to eustatic sea level variations during the Pleistocene (2.5 million years ago to 12 thousand years ago) (Gradstein et al., 2004). During the glacial periods (also kown as ice ages), seawater levels were up to 200 meters lower than today, exposing most of the Sunda Shelf, connecting the landmasses and river systems that were once separated by marine environments. Thus, allowing the expansion of the distribution of both freshwater and terrestrial organisms from one landmass to another (Molengraaff & Weber, 1919; Darlington, 1957).

The biodiversity in Sundaland also originates from the complex geological history of the region, related to major tectonic changes in the distribution of land and sea during the last 50 million years (Hall, 2012). Subsequent from the aggregation processes, the Southeast Asian (SEA) region is considered to be one of the most

diverse regions in the World (Metcalfe et al., 2001). Mittermeier et al., (2005) explained that over 50% of the world's plant species and an estimated of 42% of terrestrial vertebrate species are endemic to only 34 biodiversity hotspots, four of these hotspots are located in SEA: Indo-Burma, Philippines, Sundaland and Wallacea. Sundaland is not only currently ranked among the most important in terms of species richness and endemism, but also ranked as the most threatened by human activities (Hoffman et al., 2010).

Myers et al., (2000) found that the Sundaland contains endemic plants and vertebrates amounting to at least 2% of the World total species but retained only 7.8% of its primary vegetation. In addition, these authors stated that the Sundaland is listed in the top-three of the eight hottest hotspots when considering the following factors: endemic plants, endemic vertebrates, endemic plants/area ratio (species per 100 km^2), endemic vertebrates/area ratio (species per 100 km^2) and remaining primary vegetation as % of original extent.

Thus, these extraordinary biodiversity species richness of Sundaland region may serve as a natural laboratory for biogeographical, ecological, evolutionary, and taxonomic research (Ng, 1991; Schilthuizen et al., 1999; Salzburger, 2005).

2.2.1 Paleo River Systems of Sundaland

The importance of fluctuating sea levels over geological time has long been considered vital to understand the distribution of both aquatic and terrestrial organisms in the Sundaland (Wallace, 1881; Molengraaff & Weber, 1919; Darlington, 1957). Many of the biogeographic works recognized the importance of the exposure of the Sunda shelf in forming land bridges and connecting river systems during the Pleistocene (e.g., Inger & Chin, 1962; Morley & Flenley, 1987; Heaney, 1991; Rainboth, 1991; Dodson et al., 1995; How & Kitchener, 1997).

Voris (2000) explained that the sea level was 120 meters below present level several times during the Pleistocene (the last cycle occurred about 17 thousand years ago), at that time the paleo river system of the Sunda Shelf was the most extensive. This paleo river system was specific to the Sundaland and the largest Southeast Asian rivers outside the Sundaland such as the Salween and Mekong, did not expressively expand even when sea level was 120 m lower than today (Voris, 2000).

On the other hand, three or four large river systems were formed when the Sunda Shelf was exposed: the Malacca Straits River system, Siam River system, North Sunda River system and East Sunda River system. (Voris, 2000).

Due to the size and interconnectedness of these four major systems (Malacca Straits River system, Siam River system, North Sunda River system and East Sunda River system), they played a major role in shaping the distribution and relationships of the present-day freshwater fauna (Rainboth, 1991; Dodson et al., 1995) (Figure 2.2).

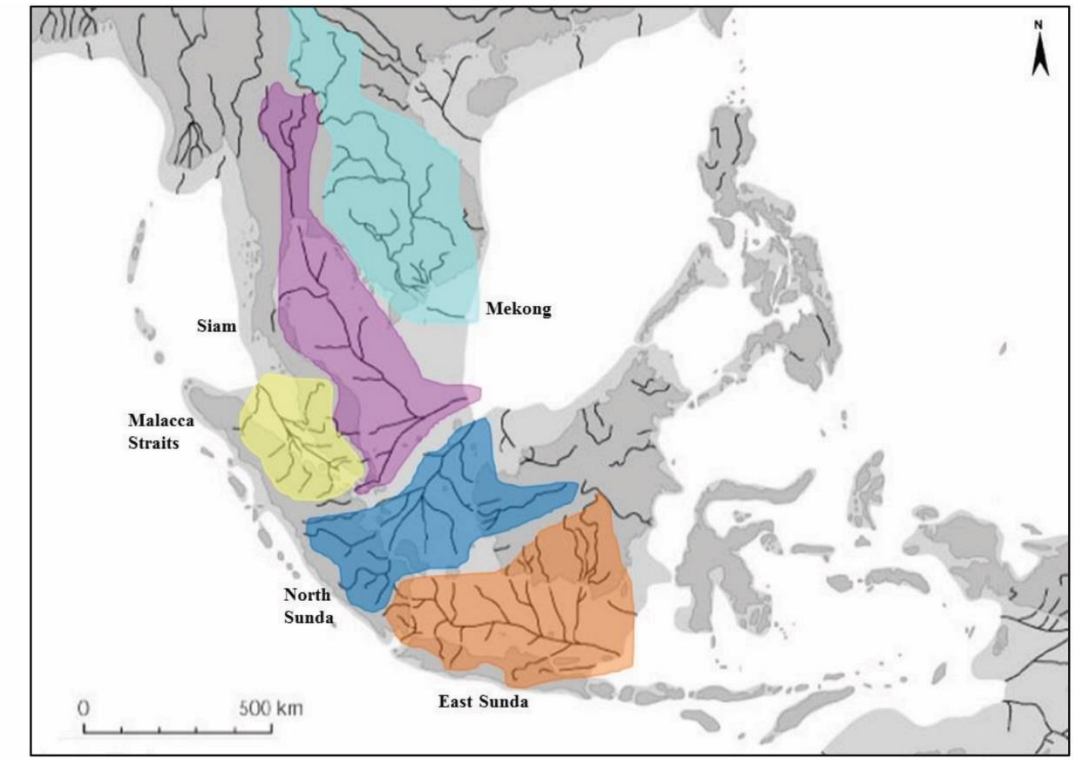

Figure 2.2 Map showing paleo river system illustrating depth contours at 120 m below present level (adapted from Samsudin, 2017)

2.3 Fishes of the Genus *Barbodes*

Genus *Barbodes* was described by Bleeker, 1859 with the type species is *Barbus maculatus*, Valenciennes, in Cuvier & Valenciennes (1842). Before Kottelat (1999) placed it in the genus *Barbodes*, this genus classified in the genus *Barbus* Valenciennes, in Cuvier & Valenciennes (1842), the catch-all genus *Puntius* (Weber & de Beaufort, 1916; Smith, 1945; Kottelat et al., 1993) and genus *Systomus* (Rainboth, 1996).

In fish, distinct markings are often used as taxonomic characteristics to identify the fish species (Pethiyagoda et al., 2012; Kottelat, 2013). Therefore Kottelat (2013) stated that genus *Barbodes* Bleeker has a prominent character that the body colour

pattern changes ontogenically and this character was used to differentiate them from other genera formerly assigned in *Puntius* in Southeast Asia.

The species of genus *Barbodes* known from its geographical ranges in Southeast Asia included *B. aurotaeniatus* (type locality: Vietnam: Thu-dau-mot), *B. banksi* (type locality: Malaysia: Borneo:Malaysia), *B. binotatus* (type locality: Java, Indonesia), *B. bunau* (type locality: Kalimantan Timur, Indonesia), *B. dunckeri* (type locality: Bukit Timah, Singapura), *B. everetti* (type locality: Malaysia: Borneo: Sarawak), *B. kuchingensis* (type locality: Malaysia: Borneo: Sarawak), *B. lateristriga* (type locality: Indonesia: Java), *B. microps* (type locality: Indonesia: Java), *B. rhombeus* (type locality: Thailand: Trat Province), *B. sealei* (type locality: Malaysia: Borneo: Sabah), *B. semifasciolatus* (type locality: China) and *B. xouthos* (type locality: Borneo: Brunei Darussalam) (Kottelat, 2013).

Others than that, majority the species of genus *Barbodes* known from Philippines: *B. amarus*, *B. bantolanensis*, *B. baoulan*, *B. cataractae*, *B. clemensi*, *B. disa*, *B. flavifuscus*, *B. hemictenus*, *B. herrei*, *B. ivis*, *B. joaquinae*, *B. katolo*, *B. lanaoensis*, *B. lindog*, *B. manalak*, *B. manguaoensis*, *B. montanoi*, *B. pachycheilus*, *B. palaemophagus*, *B. palata*, *B. palavanensis*, *B. quinquemaculatus*, *B. resimus*, *B. sirang*, *B. tras*, *B. truncatulus*, *B. tumba* and *B. umalii* (Kottelat, 2013).

Based on the listed species under genus *Barbodes* above, it can be seen that this genus has large distribution in the Philippines. For instance, Lake Lanao in Mindanao Islands was considered as an important biodiversity site in the Philippines and considered as one of the 15 ancient lakes in the world (Abdulmalik-Labe & Quilang, 2019). Consequently, the lake was once home to 20 ancestral cyprinid species belonging to the genera *Cephalakompsus*, *Mandibularca*, *Ospatulus*, and *Barbodes*

(Abdulmalik-Labe & Quilang, 2019). This endemic fauna played an important role in the development of species flock concept that had been widely cited in discussions on evolutionary rates (Herre, 1933; Myers, 1960). However, because of overexploitation and the introduction of the predatory fishes *Glossogobius* spp. and *Giuris margaritaceus* (Kornfield, 1982), most of those species are critically endangered or extinct. Consequently, only two endemic species remain in the waters of Lake Lanao, namely *Barbodes lindog* and *B. tumba* (Ismail et al., 2014).

2.4 *Barbodes binotatus* Species Complex

A "species complex" comprises several populations that show little morphological differentiation among them, leading to the situation that intra- and inter-specific boundaries are blur. In other words, it is difficult to identify species from populations in a "species complex" because they exhibit only slight or incomplete morphological and/or genetic differentiation, thus, interspecific boundaries could not be confidently established (Fahmi-Ahmad et al., 2020)

The above situation applies to *Barbodes binotatus*. Bariche (1998) conducted a morphometric analysis on several populations of *B. binotatus* of Java, Lombok, and Sumatra. This author found significant variation among his sampling, however, populations or species cannot be fully separated into discrete subgroups based on morphology. Bariche (1998) concluded that the difference in the body pattern among these populations may be a better taxonomic character and the author suggested the presence of four putative species: the "real" *Barbodes binotatus* (or *Barbodes binotatus sensu stricto*) and three other undescribed species.

Extending the conclusions of Bariche (1998), outside the Javanese-Sumatran region, Kottelat (2000) proposed that what is called *B. binotatus* in Southeast Asia is likely a complex of species: the "real" *B. binotatus* seems to be distributed only in Java (type locality), Bali, Lombok, and highlands of Sumatra. Furthermore, other valid species show strong morphological similarities with *B. binotatus* or some of its populations: *B. banksi* (Herre, 1940a) and *B. rhombeus*. Kottelat, (2000) created the "*Barbodes binotatus* species complex" for these three species because these three species shared a roughly common marking pattern. In Peninsular Malaysia these three valid species occured.

2.4.1 Taxonomy and Nomenclature

The cyprinid species *Barbodes binotatus* was described by Valenciennes, in Cuvier & Valenciennes (1842) from Java, as *Barbus binotatus*. In the same publication but subsequently to the description of *Barbus binotatus*, Valenciennes described another species, *Barbus maculatus* (pages 195-196 whereas the description of *B. binotatus* was page 168). Bleeker (1855) synonymized *Barbus binotatus* with *Barbus maculatus* and acted as the first reviser to treat *B. maculatus* as a simultaneous subjective synonym of *B. binotatus* due to the description of the former species based on the drawing by Kuhl and van Hasselt in van Hasselt (1823) (this drawing was reproduced by Roberts [1993]). The drawing by Kuhl and van Hasselt could possibly be part of RMNH 2455, syntypes of *B. binotatus* (Roberts, 1993; Kottelat, 2013). Bleeker (1855) gave the precedence of species name to *B. binotatus*.

Prior to Cuvier & Valenciennes (1842), Kuhl & van Hasselt, in van Hasselt (1823) mentioned the species name *Barbus maculatus* in a van Hasselt's letter to the Director of the Rijksmuseum van Natuurlijke Histoire (RMNH) in Leiden (Alfred,

1961). However, the name *Barbus maculatus* by Kuhl & van Hasselt, in van Hasselt (1823) is unavailable because no type specimen was preserved (Kottelat, 1987).

Described in the genus *Barbus*, *B. binotatus* also was placed under the genus *Puntius* (Weber & Beaufort, 1916; Smith, 1945; Kottelat et al., 1993). Several studies have shown that the Southeast Asian species that were historically placed under the genus *Puntius* do not form a monophyletic group (Rainboth, 1996; Bariche, 1998; Fang et al., 2009; Yang et al., 2010; Pethiyagoda et al., 2012). One group of species of "*Puntius*", containing all species of the "*B. binotatus* complex group", has been transferred into the genus *Barbodes* with *Barbus maculatus* as its type species (Kottelat, 1999, 2000, 2013; Pethiyagoda et al., 2012).

2.4.2 Current Status of the *Barbodes binotatus* Species Complex

The latest study in Peninsular Malaysia aiming to decipher the species-group taxonomic complexity of *Barbodes binotatus* and the saddle barb, *B. banksi*, was Fahmi-Ahmad et al., (2020). These authors addressed through a traditional morphometric and landmark-based geometric morphometric method for the taxonomic status of these two species. The landmark-based geometric morphometric revealed a highly similar body shape in both species. However, both species can be distinguished to each other by morphological characters generally located in the anterior part of the body, including the cephalic region of the fish. The authors concluded that combining conventional morphometric with the more advanced landmark-based geometric morphometric proved to be useful in clarifying species complex in *B. binotatus* and *B. banksi* and the marking pattern of blotch under dorsal fin was still the most reliable character to be used in the field to distinguish and identify species.

Another study on this species complex in Peninsular Malaysia was done by Kamaruddin (2012). The author investigated the phylogenetic and molecular taxonomy of the barb fish, *Puntius* spp (including *Barbodes binotatus* and *B. banksi*), by applying a molecular method using three mitochondrial molecular markers (COI gene, 16S rRNA gene and D-loop gene). At the time of this study, *B. binotatus* and *B. banksi* were still classified into the genus *Puntius* before their transfers into the genus *Barbodes* (Kottelat, 2013). By using the D-loop gene, a more detailed study of the population genetics of *B. binotatus* and *B. banksi* were examined. Kamaruddin (2012) also revealed two major divergences between genetic variations in the *B. binotatus* and *B. banksi* group and also involved the taxonomic status of the western and northern versus eastern populations of *B. binotatus*.

As mentioned above, the true *Barbodes binotatus* is restricted to Java (type locality), Bali, Lombok, and highlands of Sumatra (Kottelat, 2000). As reference to this present study in Peninsular Malaysia, there are other previous findings on *Puntius binotatus* (now *Barbodes binotatus*) from Gunung Tujuh Lake in Sumatra (Karlina et al., 2016) and West Sumatra (Roesma et al., 2018). Both investigations used molecular method based on cytochrome *b* (cyt *b*) and cytochrome oxidase I (COI) gene respectively. Roesma et al., (2018) recorded new species and subspecies of *Barbodes binotatus* and *B. banksi* and the results of their study further revealed that the Bukit Barisan Mountain range in Sumatra Island contributed to genetic diversity, evolutionary process, and speciation mechanism of freshwater fish in Sumatra.

In summary, based on the information from the previous studies especially in Peninsular Malaysia, the taxonomic status of *Barbodes binotatus* species complex is still unconfirmed due to the variety in their unique marking pattern. Hence, the

combination of morphology and molecular methods is useful to achieve the objectives of this study.

2.5 *Barbodes lateristriga* Morphotypes

The cyprinid species *Barbodes lateristriga* was described by Cuvier & Valenciennes (1842) from Java, as *Barbus lateristriga*. Distribution of this species included Peninsular Thailand, Malay Peninsula, Sumatra, Borneo, Java, Banka, Billiton, Singkep (Roberts, 1989). In Peninsular Malaysia, Tweedie (1961) as a first researcher work on this species covered the range of localities from Perlis in the north to southern Johor in the south.

Tweedie (1961) noted that this species appeared to be constant as regard to morphology on the pattern of black markings that varies in a way and shows an interesting regional differentiation. The pattern consists of two vertical bars and a mid-lateral longitudinal stripe (Tweedie, 1961; Alfred, 1966; Roberts, 1989; Kottelat et al., 1993). Tweedie (1961) also documented that *Barbodes lateristriga* have six different forms of body pattern i.e., 1) Johore form, 2) Selangor form, 3) Muar River form, 4) Pahang and Kelantan form, 5) Perak form, 6) Perlis and Kedah form.

Peninsular Malaysia also recorded that *Barbodes lateristriga* as an island species because it founded from Pulau Langkawi (Ahmad & Lim, 2006; Azmir & Samat, 2010; Samat et al., 2012) and Pulau Tioman (Ng. et al., 1999; Tan et al., 2015) in which the islands located about 30 km off Kuala Perlis on the West Malaysian mainland (Ahmad & Lim, 2006) and located about 45 km off the east coast of Peninsular Malaysia (Tan et al., 2015) respectively. In addition, *B. laterisitriga* noted as primary freshwater fish for both islands mentioned above (Ng. et al., 1999; Ahmad