MORPHOMETRIC, PHYLOGENETIC ANALYSES AND DNA BARCODING OF PANGASIID CATFISHES (TELEOSTEI: PANGASIIDAE) IN PENINSULAR MALAYSIA

HASLAWATI BAHARUDDIN

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

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **HASLAWATI BAHARUDDIN**

Registration/Matric No: **SHC 070079**

Name of Degree: **DOCTOR OF PHILOSOPHY (Ph.D)**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

MORPHOMETRICS, PHYLOGENETIC ANALYSES AND DNA BARCODING OF PANGASIID CATFISHES (TELEOSTEI: PANGASIIDAE) IN PENINSULAR MALAYSIA

Field of Study: **FISHERIES SCIENCE**

I do solemnly and sincerely declare that:
(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:
Designation:

ABSTRACT

Fishes of the family Pangasiidae are medium to large sized catfishes with diverse morphologies and ecologies, ranging from 20-300 cm in length, with most species being more than 50cm long. Pangasiids are generally found in freshwater, however, some species also live in brackish and marine waters. Taxonomic ambiguities arise due to morphological variations between conspecifics found on the Asian Mainland and the Indo-Malayan Archipelago, morphologically disparate life stages and species complexes as well as local scale ecological variations. This work examined the diversity and distribution of pangasiids in Peninsular Malaysia based on past and present collections (n=161 specimens) using 35 morphometric measurements and five meristic counts. Identification keys established were based on seven morphological species from four genera; *Pangasianodon*, *Pangasius*, *Pseudolais* and *Helicophagus*. Four native species were confirmed, together with an introduced aquaculture species from Thailand, *Pangasianodon hypophthalmus.* Three other species: two species previously known only from Indo-China (*Pangasius bocourti* and *Pangasius conchophilus*) and one from Indonesia (*Pangasius djambal*) were also identified. A minimum of three characters: (1) palatal dentition, (2) head shape and (3) gill raker counts, were found to be useful distinguishing features. Previously unnoticed, *Pangasius polyuranodon* can now be positively distinguished from the more abundant *Pseudolais micronemus* by several characters: a distinctive palatal dentition, longer maxillary and mandibular barbels, higher count of anal-fin rays, higher counts of gill rakers on the first gill arch, longer caudal peduncle, wider anterior snout and wider mouth. Clarification on the identity between this two morphologically similar species is important in drafting proper conservation measures, since they have different ecological and biological requirements. Many native pangasiid species are threatened in the wild due to hybridisation and introduction of non-native species, through fishing pressure, habitat degradation and aquaculture practices. This study demonstrates the strong potential of cytochrome C oxidase subunit I (COI) DNA barcoding as an identification tool in detecting potentially threatened as well as invasive pangasiids. A neighbour-joining (NJ) dendogram generated five groups representing four distinct genera. The genus *Pangasius* was further subdivided into two clades, with a mixture of morphospecies: (i) *P. bocourti-P. djambal* and (ii) *P. nasutus-P. conchophilus*. A barcode gap was detected using corrected Kimura-2-Parameter (K2P) genetic distance with higher intraspecific value (5.1%) compared to interspecific distance (3.0%), suggesting the possibility of a species complex, hybridisation or incorrect taxonomic identification, following unclear species delineation for *P. conchophilus*. Despite this complexity, Automatic Barcode Gap Discovery (ABGD) analysis revealed potential barcode gaps for most species pairs. This study also showed that both mitochondrial regions (16S rRNA and COI) can be used for taxonomic purpose. Nevertheless, COI showed better performance in species delineation. The phylogenetic and sequence analyses revealed that closely related species, despite their origin, are important in conservation measures. K2P genetic distance revealed seven molecular groupings, which were congruent with the morphological species. This study clarified and established the diversity of Pangasiidae in Peninsular Malaysia, thus connecting the gap between the two previous pangasiid distributions.

(486 words)

ABSTRAK

Ikan berduri dari keluarga Pangasiidae bersaiz sederhana dan agak besar, dengan kepelbagaian morfologi dan ekologi. Ikan dewasa bersaiz di antara 20-300 cm panjang, kebanyakannya melebihi 50 cm. Pangasiid atau ikan patin biasanya ditemui di habitat air tawar, namun terdapat beberapa spesies yang menghuni persekitaran air payau dan marin. Terdapat kekeliruan taksonomi disebabkan variasi morfologi di antara spesies yang ditemui di Tanah Besar Asia dan Kepulauan Indo-Malaya; perbezaan morfologi pada kitaran hidup dan 'species-complex'; serta variasi ekologi berdasarkan ciri morfologi tempatan. Kajian ini meneliti diversiti dan taburan ikan patin di Semenanjung Malaysia berdasarkan koleksi lepas dan terkini. Sebanyak 161 spesimen diperiksa menggunakan 35 ukuran morfometrik dan lima kiraan meristik. Kekunci pengenalan spesies dibina berdasarkan tujuh spesies dari empat genera; *Pangasianodon, Pangasius, Pseudolais* dan *Helicophagus*. Empat spesies tempatan berjaya dikenalpasti, beserta satu spesies akuakultur yang dibawa masuk dari Thailand, *Pangasianodon hypophthalmus.* Tiga spesies lain yang diketahui hanya berasal dari Indo-China (*Pangasius bocourti* dan *Pangasius conchophilus*) dan Indonesia (*Pangasius djambal*) juga ditemui. Sekurang-kurangnya tiga karakter pengenalpastian utama; (1) penggigian palatal (2) bentuk kepala dan (3) bilangan ruji pada sisir insang pertama. Kajian ini mengesahkan kewujudan *Pangasius polyuranodon* di Semenanjung Malaysia yang sebelumnya dikelirukan dengan *Pseudolais micronemus* dengan ciri-ciri perbezaan berikut; penggigian palatal yang tersendiri, sesungut maksila dan mandibel yang lebih panjang, lebih banyak bilangan ruji sirip anus dan sisir insang pada sisir pertama, kaudal pedunkel lebih panjang serta muncung hadapan dan mulut yang lebih lebar. Kepentingan mengenalpasti identiti kedua-dua spesies yang mempunyai rupa yang hampir sama, namun mempunyai perlakuan biologi dan ekologi yang berbeza, dengan itu memerlukan kaedah pengurusan dan strategi konservasi berlainan. Beberapa spesies dan populasi ikan patin asli dikategorikan sebagai terancam di habitat semulajadinya akibat tekanan antropogenik seperti pertambahan tekanan akibat perikanan, kemerosotan habitat dan amalan akuakultur yang tidak mapan. Kajian ini menunjukkan potensi penggunaan teknik Pengkodan DNA ('DNA barcoding') menggunakan gen Cytochrome Oxidase sub unit I (COI) untuk mengesan ancaman terhadap ikan asli dengan mengenalpasti ikan asing invasif. Filogram 'Neighbour-joining (NJ)' (Parameter-Kimura-2) menghasilkan lima kumpulan mewakili genera berlainan kecuali *Pangasius* yang kemudiannya terbahagi kepada dua kumpulan dengan percampuran morfospesies (i) *P. bocourti-P. djambal* dan (ii) *P. nasutus-P. conchophilus*). Terdapat jurang barkod ('barcode gap') di antara nilai intraspesies yang lebih tinggi (5.1%) dari interspesies (3.0%) dikenalpasti menggunakan K2P jarak genetik. Kemungkinan terdapatnya 'species complex', hibridisasi atau kesalahan identifikasi pada *P. conchophilus*. Namun demikian, analisa 'Automatic Barcode Gap Discovery' (ABGD) menunjukkan potensi penggunaan jurang barkod sebagai kaedah mengenalpasti spesies. Kajian ini membuktikan bahawa kedua-dua jujukan DNA mitokondria Cytochrome Oxidase I (COI) dan 16S ribosomal RNA (rRNA) boleh digunakan untuk tujuan taksonomi, dengan COI menunjukkan prestasi pengenalpastian yang lebih baik. Perhubungan filogenetik dan analisa jujukan DNA menunjukkan spesies ciri genetiknya yang berhubung rapat, penting dalam usaha konservasi. Jarak genetik K2P juga menunjukkan tujuh kumpulan molekular, selari dengan identifikasi morfologi. Kajian ini menjelaskan dan mengesahkan kepelbagaian Pangasiidae di Semenanjung Malaysia, dengan melengkapkan jurang penyambungan di antara kedua-dua taburan spesies ikan Patin.

(479 patah perkataan)

ACKNOWLEDGEMENTS

Alhamdulillah, thank you Allah…

"*Success is journey, not a destination*…" quotation from my schooldays.

I am conveying my deepest gratitude to my supervisors; Prof. Dato' Dr. Mohd. Sofian Azirun, for his counsel, motivation and moral support, especially during my lowest level of motivation. My former supervisor, Prof. Dr. Mohd Zakaria-Ismail, whose intellectual guidance and advice being major contributions to my study, unfortunately have to retire before seeing me completing this journey. My technical mentor, Prof. Dr. Sekaran Muniandy who is always be my saviour with his infinite support, concern and consultation on the technicalities. I am also very much indebted to Dr. Mohamed Rizman-Idid for his patience, guidance and dedication, not to mention long hours of tutoring, arguing and productive discussion. Not forgetting Associate Prof. Dr Ramlah Zainudin (UNIMAS) for being such a dedicated *sifu*; her superb knowledge, intelligence, and time doing the analysis and discussions were much treasured.

I also thank Dr Chris Barlow (ACIAR, Australia), Dr John-James Wilson and Prof Dr Chong Ving Ching (UM), Prof Talib (UKM); Mr Kelvin Lim (Raffles Museum for Biodiversity Research, NUS, Singapore and Pak Rudhy (Dr Rudhy Gustiano) for rare and important Pangasiid references, also Pak Memet (Ahmad Jauhar Arief, LIPI, Bogor) for the warm hospitality during museum visits. Many indebtedness to the colleagues, staff of FRIGL, UM and '*orang kampung*' that help me during fieldworks. Thanks also to Crawford Fund (Australia) for supporting my precious trip to the 4th International Barcode of Life Conference at the University of Adelaide, Australia (2011).

There are also many person in my career that contributed to what I am this far, to whom I would dedicate this success to my late mentor Allahyarham Mohamad Ashhar Othman (may you be forever rewarded for what you left behind), my former bosses, Tuan Haji Hambal Hanafi, Tuan Haji Rosly Hassan, Encik Ismail Awang Kechik whose support and

encouragement remains as countless motivation, not forgetting Encik Zulkafli Abdul Rashid, who opens many opportunities that enabled me to learn and contribute to my working experiences in the Fisheries Department.

I would also like to express my utmost gratefulness to my life partner, Mohd Ruslan Wagiman for his immeasurable support, love, endurance and constant encouragement who has assisted me to overcome many obstacles and challenging moments; including the fieldworks and the final writing processes. Without your patience and unconditional support, this journey would have been impossible to complete. To my precious adorable children; Qurratul Aini, Izzatul Husna, Ahmad Alif Adnin, Ahmad Kamil, Ahmad Adam and Nadhratul Syafiqah ; two of them were born during this study, at all times been the sources of my inspiration, delight and bliss. Thanks for enduring the long hours spent away from you all; in the field, in the lab, at meetings and conferences, in front of the computer with total ignorance, procrastination, lack of attention and affection. My dear children, you have seen me dedicating part of my life into this journey; reminisce as your future inspiration and life endurance, to be a successful professional mu'min fiddunya wal akhirah.

My biggest appreciation for the sincere prayers and devoted support from my parents, Ustaz Haji Baharuddin Imam Awang, mother Hajah Noriyah Razali, also my mother in-law, Hajah Tuminah Abdullah. To my siblings and in-laws for their unremitting assistance they offered me during my hours of need.

Last but not least, to my fellow postgrads from ISB, and Protein Lab (Faculty of Medicine), thanks for sharing this wonderful journey, insightful discussions, assistance and friendships. May Allah guide us to the righteous path and granted us Jannatul Firdaus.

Jazakumullah khairan kathira!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
<	Less than
>	More than
µl	Microlitre
A	Adenosine
C	Cytosine
cm	centimetre
g	Gram
G	Guanine
kb	kilobyte
kg	kilogram
km	kilometre
m	meter
min	minute
mM	mili molar
mm	millimetre
^{o}C	Celsius degree
pmol	Piko mol
rpm	round per minute
s	second
T	Thymine
U	unit
V	Vol

LIST OF SYMBOLS AND ABBREVIATIONS

12 S rRNA	12 S ribosomal ribonucleic acid
16 S rRNA	16 S ribosomal ribonucleic acid
ABGD	Automated Barcoding Gap Discovery
ANOVA	Analysis of Variance
BI	Bayesian Inference
BLAST	Basic Local Alignment Search Tool
COI	Cytochrome Oxidase Subunit I
Cyt b	Cytochrome *b* gene
DNA	Deoxyribo Nucleic Acid
DoSM	Department of Standard Malaysia
EN	Endangered
et al.	et alia (and other people)
FAO	Food and Agriculture Organization United
FISK	Fish invasiveness scoring kit
FRIGL	Fisheries Research Institute, Glami Lemi
GAqP	Good Aquaculture Practice
GM	Geometric Morphometrics
GMO	Genetically Modified Organism
H.	*Helicophagus*
HL	Head length
hLRT	hierarchial likelihood ratio tests
ICZN	International Code of Zoological Nomenclature
IUCN	International Union for Conservation of Nature and Natural Resources
K2P	Kimura-2-Parameter

LIST OF SYMBOLS AND ABBREVIATIONS

LBM	Landmark-Based Morphometric Analysis
MCMCMC	Metropolis-coupled Marker Chain Monte Carlo
MP	Maximum Parsimony
mt *cyt b*	mitochondrial *cytochrome b*
mt DNA	mitochondrial DNA
MT	Moderately Threatened
MYA, Mya	million years ago
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
OTU	Operation Taxonomic Units
P.	*Pangasianodon, Pangasius, Pseudolais*
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
rag 1	recombinant-activating gene 1
RFLP	Restriction Fragment Length Polymorphism
SL	standard length
SSCP	Single Strand Conformation Polymorphism
TBR	Tree-Bisection-Regrafting
TM	Traditional Morphometric
tRNA-Val	Valyl- transfer RNA

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Fishes are aquatic vertebrates with gills developed from birth, not after it, and have limbs in the shape of fins (Nelson, 2006). Fish can be found in any aquatic environment, be it freshwater (e.g. lakes, streams, pools) estuaries or saltwater (oceans and saline, inland lakes) (Moyle & Cech, 2000; Nelson, 2006). Freshwater ecosystems occupy less than 2% of the earth's total surface area and approximately 0.01% of its water in the form of freshwater lakes and rivers, yet is the habitat for 11,952 species (43% of total fish species) (Berra 2001). Fish exhibit the greatest biodiversity among the vertebrates. Among them are the catfishes, which represents 5.5% of all vertebrates and 10.8% of all fishes (Armbruster, 2011).

Catfishes (Teleostei: Siluriformes) are a diverse fish group with wide geographical distribution. They are primary freshwater species except for two families (Ariidae and Plotosidae) (Teugels, 1996; Lundberg & Friel; 2003; Nelson, 2006). Siluriformes is monophyletic (Sullivan, 2006; Lundberg et al., 2007) with approximately 3, 700 known species within 39 families (Eschmeyer & Fong, 2016; Armbruster, 2011).

The catfish species found within the family Pangasiidae are recognised by having a laterally compressed body, two pairs of barbels (maxillary and mandibular), a long anal fin, a short dorsal fin and a small adipose fin with free posterior margin (Teugels, 1996; Gustiano, 2003). Maximum length is about 3 meters with a maximum weight of 300 kg, referring to one of the largest freshwater fish species, *Pangasianodon gigas* (Nelson, 2006).

A total of 28 valid pangasiid species from four genera have been reported throughout Asian river systems (Nelson, 2006; Ferraris, 2007; Eschmeyer & Fong, 2016; Froese & Pauly, 2016) with thirteen species occurring in Indo-China and fifteen species found in the Indo-Malayan Archipelago (Pouyaud et al., 1999; Pouyaud & Teugels, 2000; Ng & Kottelat, 2000; Pouyaud et al., 2002; Gustiano, 2003; Kottelat, 2013).

Several species have great economic importance; some are commercially cultured, while others have good aquaculture potential. Not only popular in South East Asia, the pangasiids are known to be exported and even cultured in the USA and several European countries (FAO, 2009; Więcaszek et al., 2009). Meanwhile, some other native pangasiid species and populations are known for their conservation interests (Gustiano, 2003; Na-Nakorn et al., 2006; Haslawati et al., 2014).

Within Siluriformes, the Pangasiidae has a close relationship to Schilbeidae (Mo, 1991) with which they share many similar characteristics in some of the species (Burgess, 1989; Roberts, 1989). Pangasiidae occurs in Southeast Asia while Schilbeidae is mainly found in the Indian subcontinent and Africa. Both show an allopatric distribution with some overlap (Gustiano, 2003). Osteological evidence (Gustiano, 2003) shows that some synapomorphies are shared with Schilbeidae. Differences in the Weberian apparatus anatomy have classified the two families into distinct families (Burgess, 1989; Roberts, 1989), which is supported with further researches (Mo, 1991; Diogo, 2003; Karinthanyakit & Jondeung, 2012).

In order to resolve the unstable morphological and osteological classifications within both closely related families, a few inferences were done using molecular data (Pouyaud et al., 2000; Gustiano, 2003; Pouyaud et al., 2004; Karinthanyakit & Jondeung, 2012). Variations in morphology and ecology, threatening status of the native species, as well as taxonomic ambiguities between some taxa, have led to studies using molecular genetic markers including the DNA barcoding method.

1.2 Problem Statement

Systematically, the Pangasiidae family is recognised to have four genera (Gustiano, 2003; Ferraris, 2007; Kottelat, 2013). They are *Helicophagus* Bleeker 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, in Cuvier & Valenciennes, 1840, and *Pseudolais*, Vaillant 1902. However, these groupings are still unstable at the genus and species level. In their systematic revision, Roberts & Vidthayanon (1991) recommended only two valid genera; *Pangasius* (nineteen species) and *Helicophagus* (two species). Later, Vidthayanon & Roongthongbaisuree (1993) and Vidthayanon (1993) revealed the existence of two other genera (*Pangasianodon* and *Pteropangasius*) with further support from osteological characteristics.

Molecular analysis using allozymes and the partial cytochrome b gene by Pouyaud et al. (2000), mitochondrial 12 S rRNA partial gene (Gustiano, 2004) and partial 16S rRNA (Na-Nakorn, 2006) have supported the previous classification however without strong support. A recent work using multigene DNA markers (complete mitochondrial cytochrome b, partial 12S rRNA, tRNA-Val, partial 16S rRNA and partial nuclear gene of recombination-activating gene (*rag1*)) has supported the four groupings of genera (Karinthanyakit & Jondeung, 2012). Even though this work is quite comprehensive, there is no attempt to include specimens or samples from Peninsular Malaysia, which is in the middle region on the freshwater faunal diversity distribution for Pangasiids. It should be noted that molecular evidence could be used to support morphological characteristics of such species, but each must be carefully used in conjunction with the other (Scotland et al., 2003; Teletchea et al., 2009). Thus, combining both analyses for taxonomic purpose would provide better understanding on the diversification and evolutionary relationships of some speciose fish groups (Dodson & Lecomte, 2015).

Members of the Pangasiids are very similar in appearance to each other. Some may exhibit different morphological resemblances at different life stages (e.g. *P. bedado* Roberts, 1999 was actually a junior synonym to *P. djambal* Bleeker, 1846) (Gustiano et al. 2004); there are also morphological variation between allopatric species that occur between the Asian Mainland and the Indo-Malayan Archipelago (*Helicophagus leptorhyncus* Ng & Kottelat, 2000 vs *H. typus* Bleeker, 1858; *P. bocourti* Sauvage 1880 vs *P. djambal* Bleeker, 1846; *P. conchophilus* Robert & Vidthayanon, 1991 vs *P. nasutus* (Bleeker, 1863); *P. elongatus* Pouyaud, Gustiano & Teugels, 2002 vs *P. mahakamensis* Pouyaud, Gustiano & Teugels, 2002). For these reasons, together with living in various ecological habitats, confusion has arisen in many of the previous classifications. Because some species may be previously unknown or even unnoticed, further taxonomic clarification and supporting evidence is clearly required for proper management and conservation of such species.

Peninsular Malaysia is the location where many Indonesian species have their northernmost distributional limit. A handful of Indian and Indochinese species have their southernmost distributional limit (Zakaria-Ismail, 1994) in Peninsular Malaysia as well. This is a result of landmass changes along the Sunda Shelf during the Pleistocene glacial maxima (Rainboth, 1996; Voris 2000; McConnell, 2004). In Peninsular Malaysia, three species of Pangasiidae have been recorded, namely *P. nasutus, P. micronemus* and *H. waandersii* (Tweedie, 1936; Herre & Myers, 1937; Roberts & Vidthayanon, 1991; Lim & Zakaria-Ismail, 1995). Fowler (1938) listed *P. polyuranodon* as having been found in Peninsular Malaysia, but its occurrence remains doubtful (Lim & Zakaria-Ismail, 1995). In a recent study by Chong et al. (2010), all four species of Pangasiids are listed as being moderately threatened in Malaysia due to a combination of overharvesting and habitat degradation. Therefore, it is of utmost important to positively identify the diversity of existing species and understand their ecology to ensure proper management such that a

conservation strategy can be implemented to maintain a healthy and sustainable population. This will result in protecting the rich fish biodiversity and its genetic resources in the country.

1.3 Significance of the study

Due to heavy exploitation, many pangasiid species are now reported to be rare and under threat of extinction. These species include, for example, the Mekong Giant Catfish, *Pangasianodon gigas,* which is listed as Critically Endangered (Hogan, 2011). The wild populations of *P. hypophthalmus*, despite widely cultivated as aquaculture species and ornamentals, is threatened at all life stages (Van Zalinge et al., 2002; Vidthayanon & Hogan, 2011). Some others are not currently listed as threatened, but are observed to be rare or threatened in the wild (Haslawati et al., 2014).

In Malaysia, the Pangasiid catfishes are among the most popularly consumed freshwater fishes (Annual Fisheries Statistics 2000 - 2013, Department of Fisheries Malaysia). Even though none of the pangasiid species previously reported in Peninsular Malaysia are under threat of extinction or on the endangered list according to IUCN criteria; a lack of comprehensive studies on the population structure or analysis of catch report data on the current Pangasiid status in Malaysia (Peninsular, in particular) have been presumed unknown. Some of the wild populations are highly sought after by local fishermen and may fetch a high price at local markets. *Helicophagus waandersii* and *Pangasius nasutus* were assessed as moderately threatened (MT), which is equal in meaning to endangered (EN) in the IUCN Red List categorisation (Chong et al., 2010). As far as the literature is concerned, no known comprehensive publication on the status of the pangasiid catfish in Peninsular Malaysia exists, which could lead to misleading information. Anthropogenic disturbances such as increased fishing pressure, introduction of new species for aquaculture, and land development (especially for agriculture

purposes) are affecting the natural habitat and thus impacting the wild native fish population. The extent of impacts of introduced species on wild pangasiid populations thus are also unknown.

High economic demands for the pangasiid species have grabbed the interest of breeders and aquaculturists to import them from the Chao Phraya and Mekong rivers and culture the species locally in Peninsular Malaysia. Some of the introduced species may have established themselves in their new environment and this could affect the native populations in some way. Hybridisation between the local, wild pangasiid and the imported *Pangasius* sp. is also being practised by local breeders in order to cater to the high demand for the local species (personal observation; Anuar et al., 2011). Furthermore, in any aquaculture practice, there are high possibilities for escapees and the introduction of species to hybridise with native species (Gross, 1998; McGinnity et al., 1997; Slaney et al., 1996).

The impacts and effects of introduced species to biodiversity conservation are widely known. Through predation, genetic interactions (hybridisation, introgression and other indirect genetic effects), habitat use and modification and transmission of a novel disease (De Silva, 2006; Gozlan et al., 2010). Introduced pangasiids may integrate into ecological systems by commencing its basic biological function; foraging, reproduction and habitat utilisation. Singh et al. (2011) discussed many possible impacts due to the introduction of *P. hypophthalmus* into India, while some pangasiid diseases were reported in Bangladesh (Faruk, 2008), New Zealand (Reed, 2008) and Malaysia (Székely et al., 2009; Siti-Zahrah et al., 2014).

Hence, this study will provide baseline information and clarify the current taxonomic status of pangasius catfishes in Peninsular Malaysia, while providing evidence on possible impacts on the interaction between native and aquaculture species.

Relationships among taxa and evolutionary studies is also a prominent criterion in any biodiversity conservation planning.

1.4 Research Objectives

i) To examine the diversity and distribution of pangasiid species in Peninsular Malaysia based on past and present collections and to establish identification keys for pangasiid species in Peninsular Malaysia based on the evaluation of morphological and meristic characters (Chapter 3)

ii) To re-assess the taxonomic status of *Pangasius polyuranodon* through comparative evaluation with its close morphospecies *Pseudolais micronemus* in Peninsular Malaysia (Chapter 4)

iii) To evaluate the usefulness of the Cytochrome Oxidase I gene in detecting potential invasive Asian Pangasiid catfishes and their possible implications for the native species (Chapter 5)

iv) To appraise the phylogenetic relationship of Peninsular Malaysian pangasiids and other species in the region based on partial sequence of mitochondrial DNA Cytochrome Oxidase subunit I (COI) and 16S ribosomal RNA (rRNA) (Chapter 6)

1.5 Thesis Overview

This thesis contains seven chapters starting with a general introduction and literature review in Chapter 1 and 2 respectively. Chapter 1 (General Introduction) describes the subject of this study (catfishes of the family Pangasiidae), problem statement and significance of this work. Research objectives are listed and explains in further details in this 'Thesis Overview' section.

Chapter 2 reviews details on the research background. Pangasiid diversity and geographical distribution are discussed, with relevance to their zoogeography. Previous studies on pangasiid systematics are presented including morphology and molecular works. DNA-based markers used for fish identifications including Polymerase Chain Reaction (PCR) sequencing methods using mitochondrial DNA (mtDNA) primers are discussed. Implications and importance of correct species identification, complementary of these two methods, are discussed with relevance to biodiversity conservation.

Chapter 3 evaluates the diversity and distribution of pangasiids in Peninsular Malaysia based on past and present collections using morphological and meristic characters following Roberts & Vidthayanon (1991), Vidhayanon (1993) and Gustiano (2003). Even though these authors have presented their comprehensive taxonomic studies on each species of Pangasiid in the family, results and some details were still lacking on the species that occurred in Peninsular Malaysia. It would be interesting to know how pangasiids are naturally distributed in this region because many freshwater fish species have their recent distribution because of the landmark change that occurred during the Pleistocene period. This chapter tries to explain why Roberts & Vidthayanon (1991) have established two identification keys for the Pangasiidae (one for Indo-Chinese and one for Indo-Malaya Archipelago). Examination of specimens collected from Peninsular Malaysia fill the connection gap between these two distributions. Introduced species for aquaculture could also form the additional diversity of pangasiid in Peninsular Malaysia and this is also examined and discussed in this chapter.

Chapter 4 remarks the taxonomic status of *Pangasius polyuranodon* in Peninsular Malaysia. *Pangasius polyuranodon* has a wide distribution in the region, and it was recently divided into three remarkably different species according to their location. In Peninsular Malaysia, this species was previously reported but there is no conclusive prove for its presence. Through comparative evaluation with a morphologically similar species,

Pseudolais micronemus, together with reference to available regional collections from the Raffles Museum for Biodiversity Research, Singapore and the Museum Zoologicum Bogoriense, Bogor, Indonesia, an identification key is established in order to clarify the status and presence of *P. polyuranodon* in Peninsular Malaysia.

Chapter 5 evaluates the usefulness of the partial sequence of mitochondrial DNA (mtDNA) Cytochrome Oxidase sub unit I (COI) gene in detecting potential invasive Asian Pangasiid catfishes and discusses possible threats and implications to the native species. However, using this gene, the recently diverged *Pangasius* (late Pleistocene period – 3.99 Million years before present, MYA) could not be separated clearly using barcoding gaps or phylogenetic trees. Barcoding gaps were successfully detected using Automated Barcode Gap Discovery (ABGD) between intraspecific and interspecific distances. DNA barcoding can be used as a demonstrative tool to identify closely related species thus justifying its application in the biodiversity conservation of these fishes.

Chapter 6 reveals the phylogenetic relationship of the pangasiid catfishes found in Peninsular Malaysia with comparison to the other species in the region based on a partial sequence of mitochondrial (mtDNA) 16S ribosomal RNA (rRNA) and the COI gene. Genetic diversity was illustrated as haplotypes and evolutionary divergence was produced by a phylogenetic tree.

Chapter 7 concludes this study with an evaluation on the pangasiid status in Peninsular Malaysia, focusing on the conservation status of certain species of interest. The importance of correct taxonomic identification and potential threat to the survival of native species are discussed. Further work and future recommendations on the assessments of the potential impact of introduced species to the native species, either on ecological or sociological impacts.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The Asian catfish Pangasiidae family consists of riverine catfishes that generally inhabit freshwater environments, though some species may be found in brackish and marine habitats. They are widely distributed, ranging from the Indian subcontinent to Indo-China until Indo-Malayan Archipelago (Roberts & Vidthayanon, 1991) and China (Yang et al., 2007). Four genera are recognised; *Helicophagus* Bleeker 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, 1840, and *Pseudolais*, Vaillant 1902 (see also Kottelat, 2013). A total of 28 species are recognised with diverse morphology and ecological variations. Pangasiidae can be morphologically identified by a laterally compressed body, two pairs of barbels, a short dorsal fin with two hard spines, a well-developed adipose fin, a long anal-fin and a strong pectoral spine (Teugels, 1996; Gustiano, 2003) (Figure 2.1).

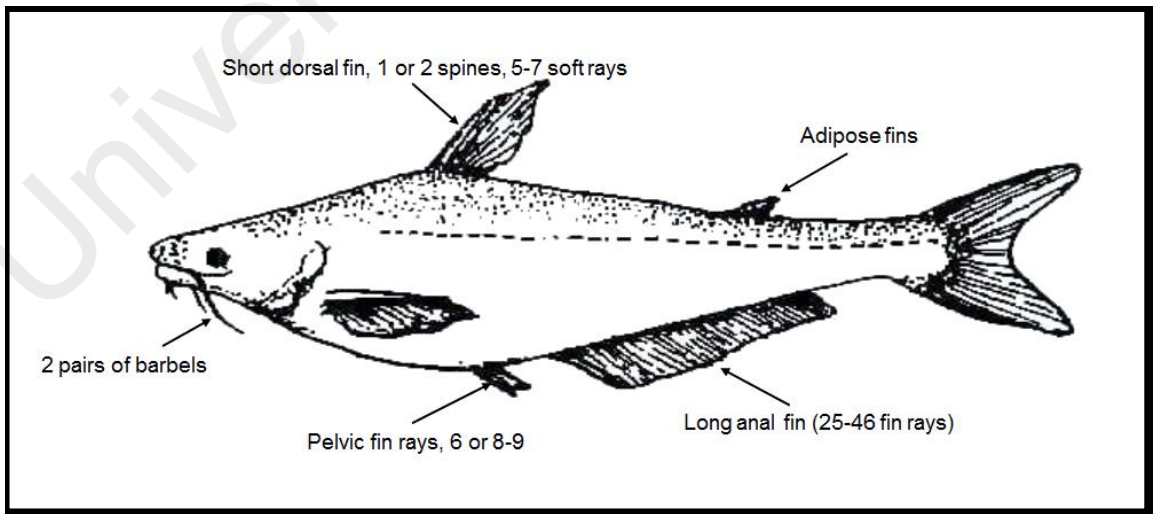


Figure 2.1 Morphological characteristics of a pangasiid catfish (modified from Rainboth, 1996).

The pangasiids are a significant component of fisheries in the Southeast Asian region. Some species are highly valued in aquaculture, such as *P. hypophthalmus* and *P. bocourti*. The juveniles of *P. hypophthalmus* are sold as ornamental fish (Armbruster, 2011) while other pangasiids are regarded as important conservation targets due to their threatened status (Sriphairoj et al., 2010).

This chapter is an overview on studies on the Asian pangasiid catfish. Variations in morphology and ecology, threatened status of native species, as well as taxonomic ambiguities between some taxa have led many studies to resolve the pangasiid taxonomical status. Useful molecular markers used for species identification were revised and finally the significance of this study to develop a conservation plan for sustainable pangasiid fisheries is contextualised.

2.2 Zoogeography and Distribution of the Asian Pangasiid

Zoogeography is the study of patterns and processes related to the geographic distribution of animal species. Southeast Asia has been a focus of zoogeographic interest since the foundation of the biogeographic concept by Alfred Russel Wallace in 1876. Wallace (1863) suggested the geological history of formation of all western islands between the Indonesian islands of Bali and Lombok to a land mass named Sundaland, which was connected to mainland Southeast Asia until the Eocene period (54-33 MYA) (Hall, 1998). This land mass formation explains the modern distribution of species, including freshwater fish. Periodic sea-level changes in the Pleistocene glacial maxima (1.6 – 0.1 MYA) caused part of the Sundaland rivers to be drowned as the Sunda Shelf submerged with rising sea levels (Rainboth, 1996; Voris, 2000). Major current river system extended and drained into this shelf during Pleistocene glacial maxima (Figure 2.2). A hypothetical boundary was situated between the Oriental and Australian faunas, later called Wallace's Line.

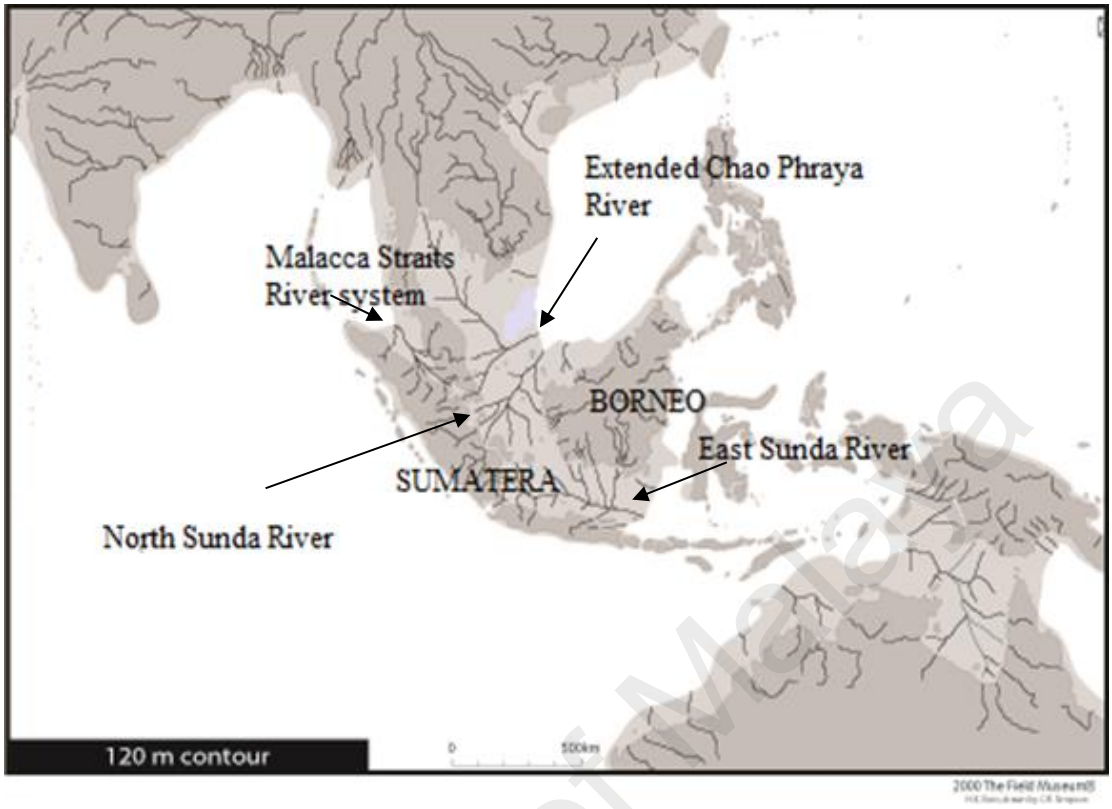


Figure 2.2: Map of Southeast Asia with river systems in the Sunda Shelf (light grey shading) during the Pleistocene glacial maxima when sea level was 120 m below the present level (Modified from Voris, 2000).

Distribution patterns of Southeast Asian freshwater fish can be divided into five zoogeographic regions, as suggested by Zakaria-Ismail (1994) (Figure 2.3). These are the Salween basin (in Burma), the Indo-Chinese peninsula (which includes the Mekong, Chao Phraya and Mae Khlong rivers of Thailand and Cambodia), the Malay Peninsula, the Indo-Malayan archipelago and Mindanao (in the Philippines).

The Malay Peninsula has become an area of overlapping species between the Indian, Indo-Chinese and Indonesian fishes (Zakaria-Ismail, 1994). Located at the southern half of the Malay Peninsula, lies a piece of land called Peninsular Malaysia, where many Indonesian species have their northernmost distributional limit. A handful of Indian and Indochinese species have their southernmost distributional limit along the Malay Peninsula as well (Zakaria-Ismail, 1994), resulting from the previous landmark change on the Sunda

Shelf during the Pleistocene glacial maxima (Rainboth, 1996; Voris, 2000; McConnell, 2004). On this Sunda Shelf, there were previously several very large river systems, like the largest rivers of today's mainland Southeast Asia. Those were the Malacca Straits River system, Siam River system, North Sunda River system and East Sunda River system (Figure 2.2 – Voris, 2000, Figure 2.4 – Rainboth, 1991).

Peninsular Malaysian freshwater faunal distributions were affected by the first two river systems. The Perak River fauna was related to the fauna of north Sumatra. The Malacca Straits River system was a single river that drained north to the Andaman Sea from tributaries of today's major Sumatran rivers. There were large rivers along the west coast of Peninsular Malaysia (which includes Sungai Perak, Sungai Bernam, Sungai Muar and Sungai Lenek) as suggested from present day land-erosion contours (McConnell, 2004; Rainboth, 1996; Voris, 2000). The fauna of the lower Mekong and the eastern Malay Peninsula resemble the Chao Phraya and Meklong of Central Thailand (Rainboth, 1991). The Extended Chao Phraya River system, which is part of the Siam River system, was the main riverbed located in the Gulf of Thailand. The major drainage in eastern Peninsular Malaysia is the Pahang River, whereas the Kelantan River is the major drainage to the north.

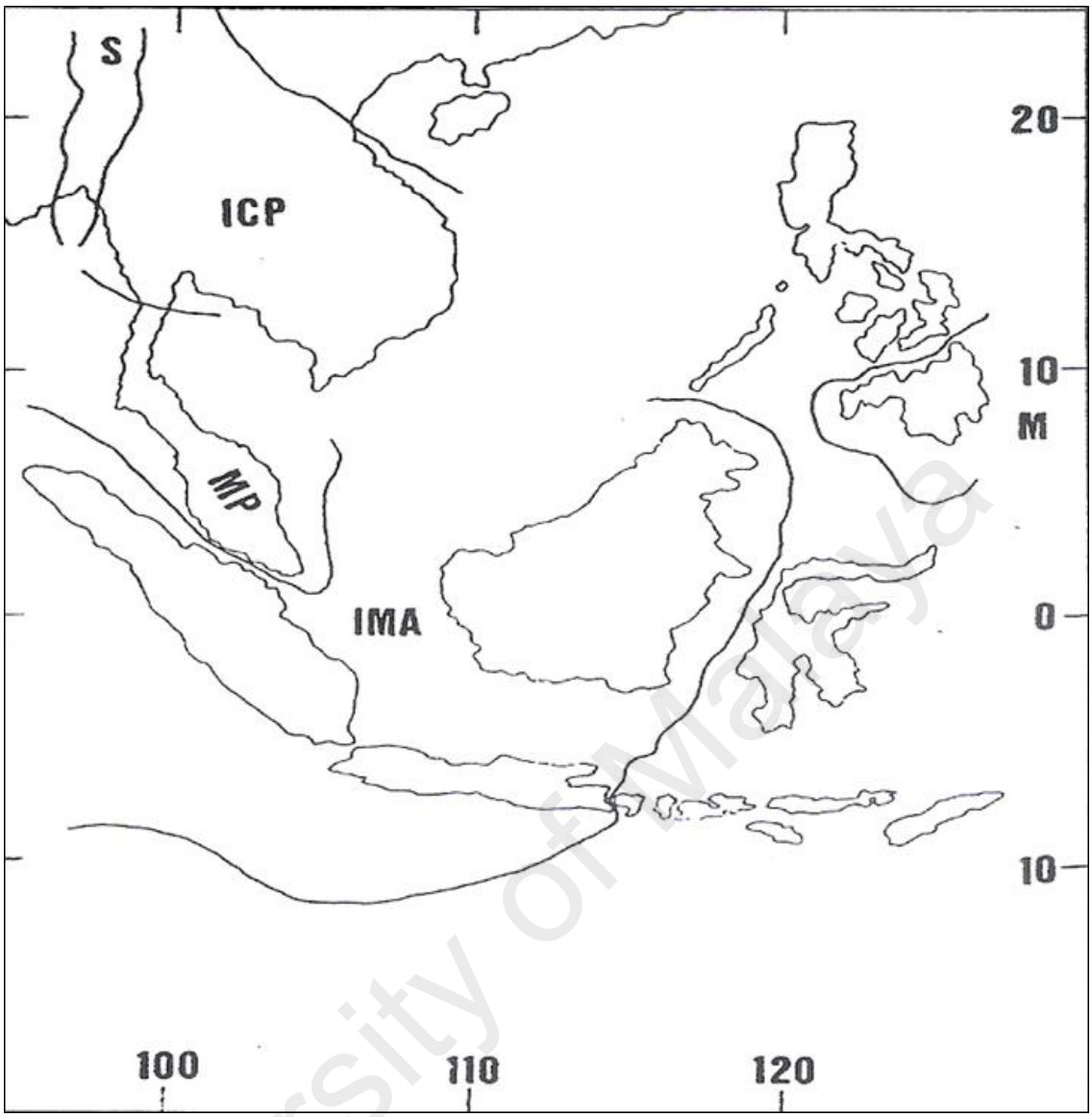


Figure 2.3: Major zoogeographic regions of freshwater fishes in Southeast Asia. S = Salween basin, ICP = Indo-Chinese peninsula; MP = Malay Peninsula; IMA = Indo-Malayan archipelago; M = Mindanao (after Zakaria-Ismail, 1994).

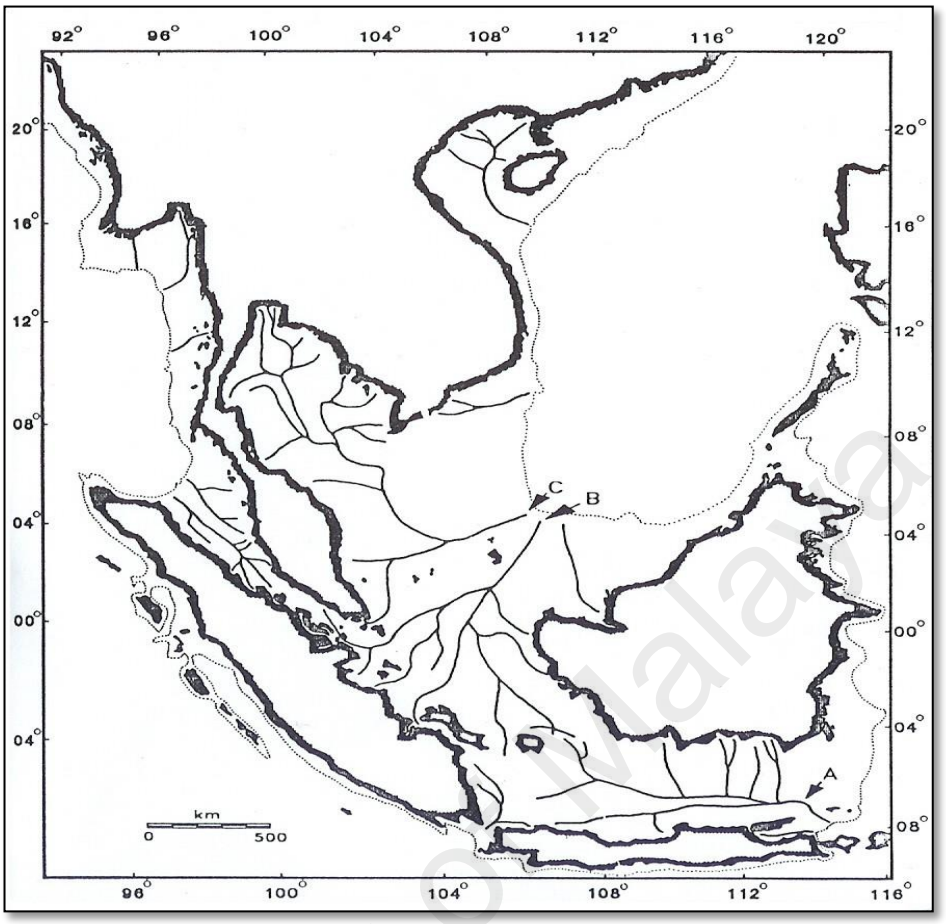


Figure 2.4: Pleistocene River System (Rainboth, 1991). A: East Sunda River. B: North Sunda River. C: Chao Phraya River System

A few researchers (Rainboth, 1991; Dodson et al., 1995; Ferdous, 2013 and Dodson & Lecomte, 2015), while reporting the similarities and distribution of freshwater fish species in Southeast Asia, noticed the relationships of major drainages that suggests that there was a connection among rivers in the past (Figure 2.4). This may be linked to the period when Sunda Shelf was exposed and interconnected as a large islands in the Pleistocene epoch. The Siam River system that now drain into Gulf of Thailand, enabled the freshwater faunal exchanges before the isolation of South East Asia (Ferdous, 2013).

As for pangasiid species, the distribution pattern is also congruent with the geological history of the Southeast Asian region (Gustiano, 2003) as well as divergence time. These patterns were also showed in the cyprinid, *Barbodes gonionotus* (Rainboth,

1991), and the bagrids, *Hemibagrus nemurus* (Dodson et al., 1995; Dodson & Lecomte, 2015) and *Mystus gulio* (Ferdous, 2013). The Salween basin served as the eastern barrier for several fish genera that are widely distributed in India, and is the western limit for Southeast Asian genera which is also observed in pangasiid. Two species known to occur in India's subcontinent, Pakistan and India (including Myanmar) are *Pangasius pangasius* and *P. myanmar* which represent the western limit of Southeast Asian pangasiid and they are slightly different due to evolution caused by vicariant speciation followed by adaptive radiation (Gustiano, 2003). Interestingly, this is compatible with the distributions and ecology of *Mystus gulio*, a brackish and marine bagrid species, between Southern Asian region (Bangladesh and India) and the Indonesian populations. Ferdous (2013) suggested that the average of the split probably around 1.6 MYA due to uplifting process of Sunda Shelf, contribute to its limited dispersal as reflected by the basal split, however there is no clear evidence for the formation of cryptic species within *M. gulio*.

Freshwater fish distribution and faunal similarities in Southeast Asia can be seen at the intraspecific level as demonstrated by Dodson et al. (1995) for the bagrid catfish *Mystus* (*Hemibagrus*) *nemurus*. This suggests that faunal exchange between the extended Chao Phraya River basin and other Sundaic river basins occurred prior to or early in the Pleistocene (Dodson et al., 1995; McConnell, 2004). Ferdous (2013) showed that the divergence time for separation of *Mystus singaringan* of the Indonesian and Thailand populations were estimated at 6.7 MYA. This can also be seen in pangasiid speciation, marked by explosive radiation (splitting of the most basal lineage of *Pangasianodon*) in the late Miocene (6.75 MYA) (Karinthanyakit and Jondeung, 2012) to early Pleistocene epochs (*Helicophagus leptorhyncus* and *H. typus* separation). Intense radiation of *Pangasius* species began at 3.99 MYA and extended to 1.59 MYA, which corresponds to the late Pleistocene. The oldest pangasiid ancestor is characterised by a fossil of

Cetopangasius chaetobranchus, which is dated from the middle or late Miocene (5-10 MYA) (Roberts & Jumnongthai, 1999) and possibly dispersed thereafter.

The presence of endemic species, the majority of which occur in Kalimantan or Borneo, suggests that the area is the centre of pangasiid speciation. Five of the endemics (*P. kinabatanganensis*, *P. sabahanensis*, *P. rheophilus*, *P. nieuwenhuisii* and *P. mahakamensis*) occurs in the rivers in the north to eastern Borneo area (Figure 2.5, Table 2.1), as clearly corresponds to the formation of the Borneo island in the Miocene to early Pliocene 15 MYA to 5 MYA. The Borneo island, which emerged from the north and east region and developed into the present large island with the formation of land areas including central mountains on the Sarawak-Kalimantan border which becomes wider and higher to the east, thus representing new barriers to faunal dispersal (Hall, 1998; 2009).

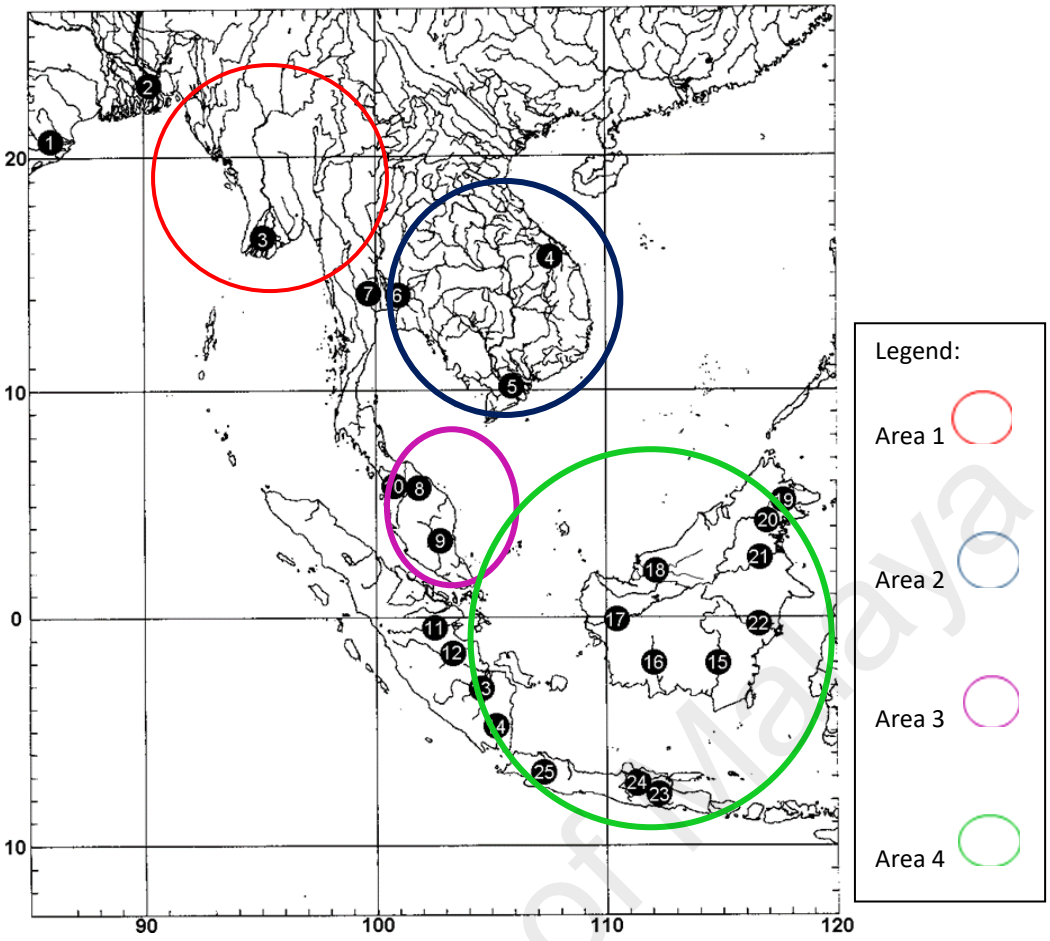


Figure 2.5: Asian pangasiid catfish distribution, showing main river basins (Gustiano, 2003). 1) South Asia – three species, (one endemic); 2) Mainland Asia – 13 species, (2 endemics); 3) Malay Peninsula – four species; 4) Indo-Malayan Archipelago – 15 species, (7 endemics).

The average time of pangasiid divergence occured between 13 MYA to 11 MYA (Pouyaud et al., 2000) corresponding to the time of the presence of the fossil genus *Cetopangasius* (5-10 MYA) (Roberts & Jumnongthai, 1999). This is also observed by Dodson & Lecomte (2013) while discussing the endemism of *Hemibagrus baramensis* and *Hemibagrus fortis* in North Borneo.

Based on a divergence time assessment, the hypothesis of Vidthayanon (1993) regarding the dispersal of pangasiids can not only be explained by the recent connections of river systems but took place since the Paleogene, as was proven by the fossil records found in Sumatra (Sanders, 1934 in Vidthayanon, 1993; India (Romer, 1947) and Thailand

(Roberts and Jumnongthai, 1999). The highest species diversity in the Mekong and Chao Phraya river basins (Asian mainland) and the islands of western Indonesia (Kapuas and Mahakam in Kalimantan) may play a major role in terms of refuge zones during marine transgressions that resulted in vicariant speciation (Roberts, 1989; Kottelatt, 1995). This scenario is illustrated by the allopatric species distribution of *H. leptorhyncus* and *H. waandersii*, *P. conchophilus* and *P. nasutus*, *P. bocourti* and *P. djambal* or between population groups of *P. kunyit* and *P. polyuranodon* (Gustiano, 2003) (Figure 2.5). Table 1 lists the pangasiid distribution according to river and basins, based on literature and museum records.

From this evidence, it is obvious that there is no study of the Peninsular Malaysian species' distribution. Records were only for the Malay Peninsula (three rivers included: the Tapi River in South Thailand, the Pahang River in the SouthEast Peninsular Malaysia and the Perak River, North East of Peninsular Malaysia). The records of pangasiid species diversity in Peninsular Malaysia, such as *P. nasutus*, *P. micronemus* and *H. waandersii* (Tweedie, 1936; Herre & Myers, 1937; Roberts & Vidthayanon, 1991; Lim & Zakaria-Ismail, 1995) (Figure 2.3) may shed new light on pangasiid distribution and its divergence from the species that occurred in the Asian mainland and the Indo-Malayan archipelago. This could be related to the phylogenetic hypotheses on the geological events that influence the Indochinese and Indo-Malayan speciation and dispersal (Dodson & Lecomte, 2015) as observed in the silurids (Bornbusch & Lundberg, 1989; Ng, 2003; 2004) and the bagrids (Ng, 2002; Ferdous, 2013).

Table 2.1: The pangasiid distribution according to area and river basins. This list is based on literatures and museum records. Numbers on superscript shows literature cited. [1]Smith (1931); [2]Lim & Zakaria-Ismail (1995); [3]Inger & Chin (1962). [4]Yang et al. (2007). For river numbers, refer Figure 2.5.

No	Area/region	Species number	River (number)	Endemic species	Species name
1	South Asia India and Myanmar	3	(1, 2) Krishna & Godavari		*Pangasius pangasius*
			(3) Irrawaddi	*Pangasius myanmar*	*P. pangasius*
2	Indo-China	13	(4) Hue		*Pseudolais micronemus, P. macronema, P. krempfi,*
			(5) Mekong	*Pangasianodon gigas* *Pangasianodon mekongensis*	*Helicophagus leptorhyncus, Pangasianodon hypophthalmus, P. bocourti, P. conchophilus, P. elongates, P. larnaudii, P. krempfi, P. macronema, P. sanitwongsei, Pseudolais. micronemus, P. pleurotaenia*
	Thailand		(6) Chao Phraya (7) Maeklong		All species from (5) Mekong River Basin except *P. gigas* and *P. krempfi, Pseudolais pleurotaenia, Pangasianodon hypophthalmus*
3	Malay Peninsula	4	(10) Tapi, South Thailand		*Pseudolais pleurotaenia*[1]
			(8) Pahang		*Helicophagus waandersii*[2]*, Pseudolais micronemus, P. nasutus*
			(9) Perak		*P. micronemus*
4	Indo-Malayan Archipelago (including Borneo)	15	(12) Batang Hari		*H. typus, H. waandersii, P. micronemus, P. djambal, P. kunyit, Pangasius nasutus, P. polyuranodon*
			(11) Indragiri		*P. micronemus, P. djambal, P. kunyit, P. nasutus, P. polyuranodon*
			(13) Musi		*H. typus, H. waandersii, P. micronemus, P. djambal, P. kunyit, P.nasutus, P. polyuranodon*

Table 2.1: Continued

No	Area/region	Species number	River (number)	Endemic species	Species name
4	Indo-Malayan Archipelago (including Borneo)		(14) Way Rarem		*P. micronemus, P. polyuranodon*
			(15) Barito		*H. typus, P. micronemus, P.nasutus, P. polyuranodon, P. djambal, P. macronema, P. kunyit*
			(16) Kahayan		*P. nasutus*
			(17) Kapuas		*H. typus, P. micronemus, P. nasutus, P. polyuranodon, P. kunyit*
			(18) Batang Rajang, Sarawak, Malaysia	*P. lithostoma, P. humeralis*	*P. micronemus, P. nasutus, P. polyuranodon*
			(19) Kinabatangan, Sabah, Malaysia	*P. kinabatanganensis, P. sabahensis*	*P. micronemus*[3], *P. polyuranodon*[3]
			(20) Berau	*P. rheophilus*	
			(21) Kayan	*P. rheophilus*	
			(22) Mahakam	*P. nieuwenhuisii, P. mahakamensis*	*P. micronemus, P. kunyit*
			(23) Brantas		*P. djambal, P. micronemus*
			(24) Solo		*P. djambal, P. micronemus*
			(25) Ciliwung		*P. djambal*
5	China		upper reach of Mekong (5) Lancangjiang River Basin		*P. djambal, P. micronema (micronemus), P. sanitwongsei*[4]

2.3 Systematics Studies of the Pangasiid Catfishes

The catfishes (Actinopterygii: Teleostei: Siluriformes) form a significant part of fisheries, and are economically important in aquaculture with numerous attractive species commercially available as ornamental fish. Catfish are widely distributed from the Americas (North, Central and South), Africa, Eurasia, Southeast Asia, Japan and Australasia (Teugels, 1996); occur on all continents including historically in Antarctica, only as fossils (Armbruster, 2011). They primarily occur in freshwater environments except for Ariidae and Plotosidae, which are mainly marine species. They exhibit various morphological sizes and features (Figure 2.6) (Burgess, 1989).

Judging from the literature, among other families in the order Siluriformes, the family Pangasiidae and Schilbeidae have always been subject to taxonomic confusion. Even the nomenclature is unstable. Kottelat (2013) corrected the usage of Schilbidae by Ferraris (2007) and confirmed it as Schilbeidae, adhering to International Code of Zoological Nomenclature (ICZN) (Code art. 29.3.3). Schilbeidae is regarded to be polyphyletic according to morphological (Mo, 1991) and molecular data (Hardman, 2005; Sullivan et al. 2008). Previously, a few species are placed in the Pangasiidae family (Hora, 1938; Jayaram, 1977; Berg, 1947; Roberts, 1989), while some of the currently known pangasiid species were included in the Schilbeidae (Hora & Gupta, 1941; Smith, 1945; Inger & Chin, 1962).

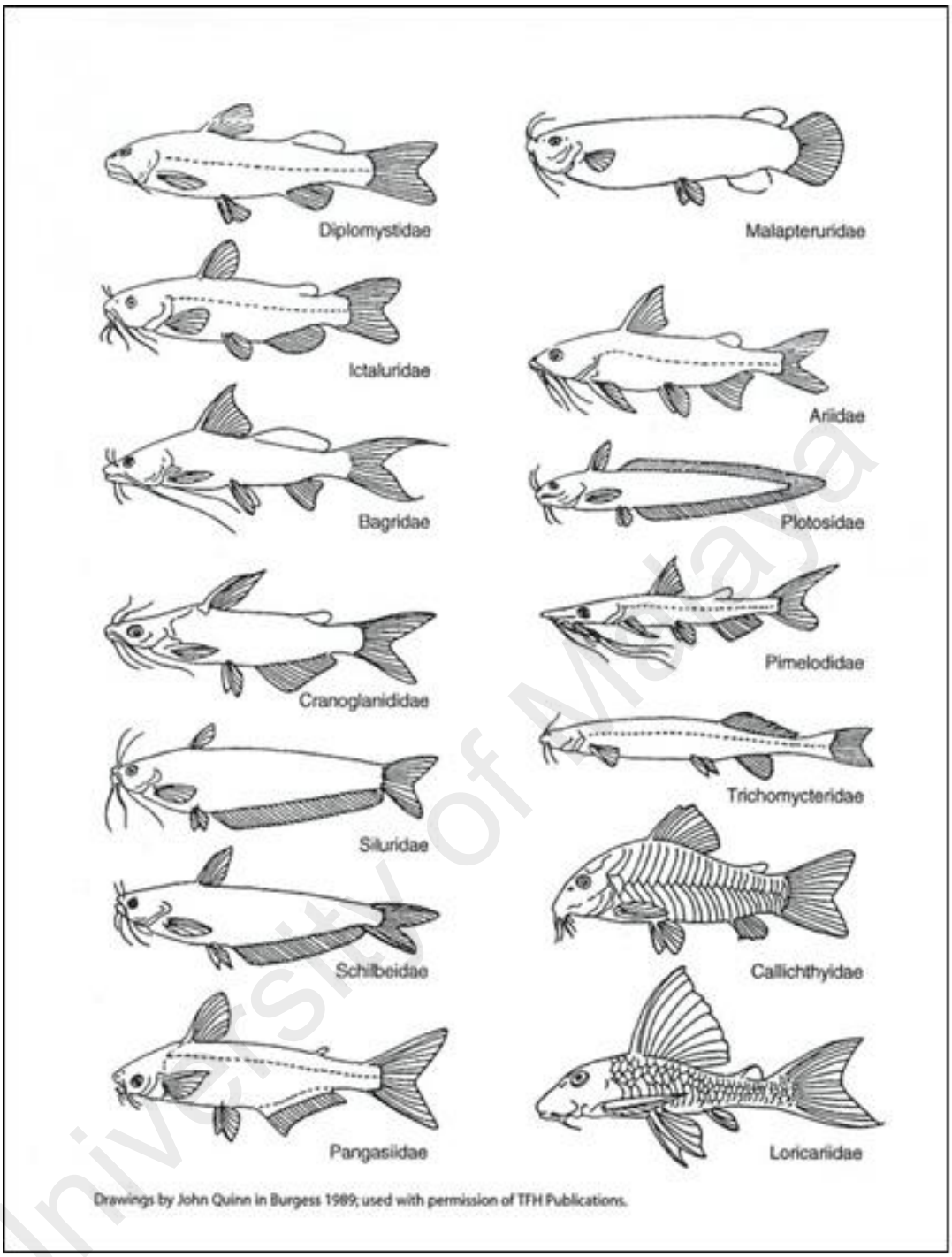


Figure 2.6: Selected catfishes, showing some of the shared characteristics (Burgess, 1989).

The most important distinguishing character for Siluriformes is based on the anatomy of the Weberian Apparatus (Tilak, 1964; Greenwood et al., 1966; Chardon, 1968), which distinctly separates these two families. In addition, Roberts & Vidthayanon (1991) suggested the main axis of the olfactory lamellae, which is longitudinal, compared to transverse in Schilbeidae as another useful character, especially when comparing *Laides*

(Schilbeidae) and *Pangasius* (Pangasiidae). Hora and Gupta (1941) listed the specimens found along the Malay Peninsula that are placed in four genera of closely alike catfishes in the family Schilbeidae which include: *Helicophagus* Bleeker, *Laides* Jordan, *Pangasius* Cuvier & Valenciennes and *Pseudotropius* Bleeker. Previous observations (Burgess, 1989; Roberts, 1989) mentioned that *Laides* formerly belongs to Pangasiidae on the basis of keeled belly as the diagnostic character of the genus *Pteropangasius* Fowler 1937. However, Zakaria-Ismail (1992) agreed to follow classification by Roberts & Vidthayanon (1991) given that there is not enough information to define this genus and using this character is unnatural.

In their systematic revision, Roberts & Vidthayanon (1991) recommended two valid genera: *Pangasius* (19 species) and *Helicophagus* (2 species). Later, Vidthayanon & Roongthongbaisuree (1993) and Vidthayanon (1993) revealed the other two genera (*Pangasianodon* and *Pteropangasius*) with further support using osteological characteristics. Gustiano (2003) validated the classification through an extensive study with many more specimens for many species and more variables (35 morphometric characters). Additionally, they described six new valid species: *Pangasius kunyit* Pouyaud, Teugels and Legendre 1999 (Pouyaud et al., 1999), *P. rheophilus* Pouyaud and Teugels 2000 (Pouyaud et al., 2000), *P. elongatus* Pouyaud, Gustiano & Teugels, 2002 (Pouyaud et al., 2002), *P. mahakamensis* Pouyaud, Gustiano & Teugels, 2002 (Pouyaud et al., 2002), *P. sabahensis* Pouyaud, Gustiano & Teugels, 2002 (Gustiano et al., 2003) and *P. mekongensis* Gustiano, Teugels & Pouyaud, 2003 (Gustiano et al., 2003). Another species, *P. bedado*, which was first described by Roberts (1999), was then confirmed to be the junior synonym to *P. djambal* (Gustiano et al., 2004). With another new *Helicophagus* species described from Indochina, *H. leptorhynchus* (Ng & Kottelat, 2000), the number of valid species of the family Pangasiidae increased to 28, arising from four genera (Gustiano, 2003; Kottelat, 2013).

Molecular systematic studies tried to support the previous classification and inferred phylogenetic relationships of this catfish by using allozyme and partial cytochrome b gene sequence of mitochondrial DNA (mtDNA) (Pouyaud et al., 2000), partial 12S rDNA mtDNA (Figure 2.7) (Gustiano et al., 2004) and partial 16S rRNA mtDNA gene (Na-Nakorn et al., 2006). Dendogram for allozyme and cytochrome b data validate the genera *Helicophagus* and *Pangasianodon* but could not clarify the status of the other pangasiid genera of *Pteropangasius* (*Pseudolais*) and *Pangasius*. *Pteropangasius pleurotaenia* and '*micronema*' (*P. micronemus*) were suggested to be distinct groups. Poor phylogenetic resolution between species in the genus *Pangasius* do not allow the possibility of finding subgenera in the two earlier genetic works (Pouyaud et al., 2000; Gustiano et al., 2004). Interestingly, both works suggested and validated closely related species, *P.djambal* vs *P. bocourti* and *P. nasutus* vs *P. conchophilus* with allopatric distribution between continental Asia and the Sunda region. In partial 12S rDNA study, monophyletic status of the three genera (*Helicophagus*, *Pangasianodon* and *Pseudolais* (*Pteropangasius*) were suggested, as well as species complexes of *P. kunyit* and *P. polyuranodon* as shown in modified phylogram adapted from Gustiano et al. (2004) (Figure 2.7).

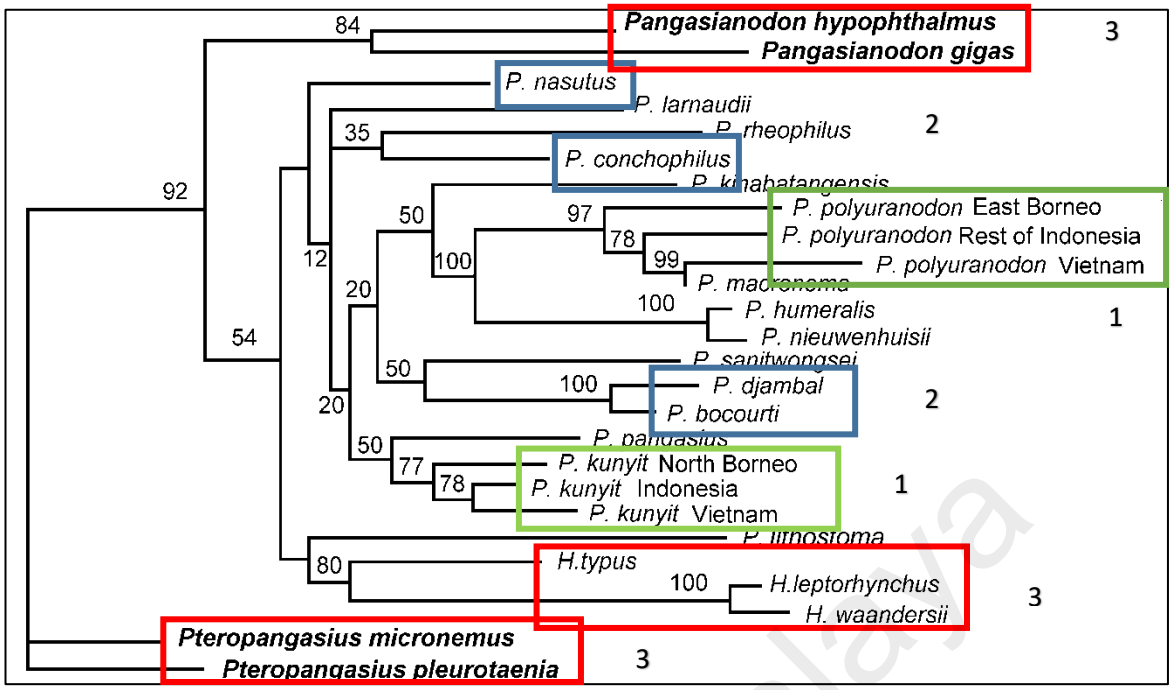


Figure 2.7: Modified phylogram of partial 12S rDNA mtDNA (Gustiano et al., 2004) showing relationships between pangasiid and its distributions 1) species complexes (*P polyuranodon, P. kunyit*); 2) allopatric species (*P. nasutus* vs *P. conchophilus, P. djambal* vs *P. bocourti*); 3) monophyletic status of *Pangasianodon, Helicophagus* and *Pteropangasius (Pseudolais).*

Later studies Na-Nakorn et al. (2006) were focused on resolving the classification using phylogenetics, however, phylogenetics studies within pangasiids are still lacking. The genetic diversity of several pangasiid species were evaluated with another molecular marker; the mitochondrial 16s rRNA partial gene, in order to examine the relationships of nine pangasiid species from the Mekong and Chao Phraya river basins. Rooting the tree to *Helicophagus*, resulted in unresolved relationships between the other three genera.

Na-Nakorn et al. (2006) also discussed the implications of studying genetic diversity, whereby the unexpected high haplotype variation of the critically endangered population of *Pangasianodon gigas* as compared to other closely related counterparts (non-endangered) could reflect the historical population size of *P. gigas*. The genetic signature observed could reflect the past population status but not the current situation. This study also raised attention to other *P. gigas* relatives for conservation measures whereby current

levels of genetic diversity should be properly maintained. Breeding programmes for restocking (and aquaculture) should particularly be designed to reduce inbreeding risks.

The drawback of this study, clearly shows that using a single non-coding genetic marker is inadequate to resolve the phylogeny as well as to clarify the genetic variation. Further assessment on combination of coding and non-coding loci was suggested (Na-Nakorn et al. 2006). Even though the intraspecific genetic diversity of some species were revealed, they only evaluate the species that occur in the Mekong and cannot reveal the true status of pangasiids throughout the region. It is also interesting to note that the species identified as *H. waandersii* in their study could actually be a newly described species, *H. leptorhyncus*, according to Ng & Kottelat (2000).

A recent study by Karinthanyakit & Jondeung (2012) attempted to further resolve the relationships between pangasiid and schilbeid species in Thailand using multiple loci (mt cyt b, 12s rRNA, valyl-transfer (t) RNA-Val and 16s rRNA genes, and nuclear recombination-activating gene 1 (*rag 1*). The reconstructed phylogeny successfully distinguished between Schilbeidae and Pangasiidae, and resolved more internal branches compared to previous molecular studies. However, resolution of the three genera (*Pangasius, Pseudolais* and *Helicophagus)* are still unresolved, especially for *Pangasius*. More comprehensive taxon sampling and more individuals throughout their distribution is suggested. Furthermore, since this study was also meant to discern the divergence times of species, it is much better to include more samples from the Indo-Malayan archipelago i.e. *P. djambal*, which has an allopatric distribution to the Indo-Chinese species, *P. bocourti.* Another species from the genus *Helicophagus*, *H. waandersii* could also be analysed as in Pouyaud et al. (2004), which showed a different rate of divergence between the three *Helicophagus* species.

All these studies so far never attempted to discuss how pangasiid distribution is connected between the Indo-Chinese species to the Indo-Malayan archipelago. There must

be some evidence on this kind of species distribution pattern due to interesting findings that were derived from a few species which previously had wide distributions throughout Southeast Asia: *P. polyuranodon* complex, *P. kunyit* complex (in the genus *Pangasius*), *Pseudolais* (*P. micronemus*) as well as *Helicophagus*.

The *P. polyuranodon* complex was initially known to be well distributed throughout the region. Some morphological variations have been observed which then diverged from *P. polyuranodon* and another two new species, with three different geographical distributions (Pouyaud et al., 2002). Haslawati et al. (2014) (Chapter 4) denotes a new distribution of *P. polyuranodon* from Pahang River in Peninsular Malaysia, which were only previously found in major drainages in Sumatra and Borneo (Kalimantan and Batang Rajang, Sarawak). Another two newly described species, *P. elongatus* (confined to the lower reaches of the Chao Phraya, Bangpakong and Mekong) and *P. mahakamensis* (endemic to Mahakam River in Kalimantan) were previously noticed to have variation in anal-fin ray numbers (Roberts & Vidthayanon, 1991). In this case, perhaps *P. polyuranodon* would be the intermediate species, with the character derived from longer predorsal length (more than 30.1% SL) for these new species.

Whereas for *Pseudolais*, *P. pleurotaenia* occurs throughout the IndoChina mainland to the southernmost limit of Tapi river basin in Malay Peninsula and *Pseudolais micronemus* is well distributed throughout the Southeast Asian region (Gustiano, 2003). This genus may have existed during Miocene glaciation while the Asian mainland was still connected to the Sundaland when evaluated through the evolutionary divergence mechanism (Pouyaud et al. 2004). Interestingly, ecological characteristics like plant-eating habits may indicate high survival (thus explaining their wide distribution) and a possible close relationship between *P. micronemus* and *P. polyuranodon* (Roberts & Vidthayanon, 1991) as clarified by Haslawati et al., (2014).

The *Helicophagus* species, *H. leptorhynchus*, which is distributed from the Asian mainland and may have dispersed through the Malay Peninsula (Pahang River) and South Sumatra (Musi River) as *H. waandersii* and from South Sumatra to Central Kalimantan (Barito and Kapuas River) to be identified as *H. typus*. The intermediate species between the two distribution areas is possibly *H. waandersii*.

Higher biodiversity of pangasiid species recorded from rivers located in the East Coast of Peninsular Malaysia, compared to the rivers in the west coast, is strongly supported by the past river connection influenced by the Siam River System. Dispersal of pangasiids that occured during the late Miocene corresponds to the emergence of the oldest pangasiid fossil found in north central Thailand (Roberts & Jumnongthai, 1999).

2.4 Morphometrics in fish taxonomy

Traditionally, fish species identification is based on external morphological characteristics. Body shape and formation, size, colouration, fin type and positions and also various relative meristic counts such as scale counts and numbers of fin rays and gill rakers (Strauss & Bond, 1990). Occasionally, otoliths are also used for identification of fossils or predator-prey relationships (stomach contents analysis) when no other features are available (Pierce & Boyle, 1991; Granadeiro & Silva, 2000).

The morphometric character set is a complete set of measurements and varies from one organism to another (Sneath & Sokal, 1973). Hubbs & Lagler (1964) suggested the general method for fish measurement, however, to overcome disadvantages due to several differences in body form, *truss* protocol was described (Strauss & Bookstein, 1982). The truss consists of points identified on a morphological feature basis by dividing the body into functional units called anatomical landmarks. This set of distance characters is assumed to be homologous among species (Bookstein et al., 1985; Strauss, 1987).

This landmark-based geometric morphometrics created a revolution called Geometric Morphometrics (GM), which analyses shape variation in coordinate systems (Bookstein, 1991; Adams et al., 2004). Landmarks are defined as homologous points of which a biological form has its geometrical information (Bookstein, 1991; Gunz et al., 2004) and is size independent (Pierce et al., 2008). GM is a powerful approach for studying morphological variation and covariation of biological organisms with advantages that include more statistical power and improved visualisation (Ferdous, 2013).

Armbruster (2012) presented a standardised measurements and meristic counts for Cypriniformes for the All Cypriniformes Species Inventory data measurements. This work suggests three morphometric schemes i.e. Geometric Morphometric Landmarks (GM), Landmark-Based Morphometric Analysis (LBM) and Traditional Morphometrics (TM). GM and LBM shared some landmarks, and some others shared with TM. LBM is based on a partial truss configuration (Strauss & Bookstein, 1982) and can be used on photographs and lengths are calculated using Pythagorean theorem (Armbruster, 2012). This method can reduce distortions due to the shape of many cyprinids which are laterally compressed. Measurements of homologous landmarks can now be taken using LBM and these are comparable among taxa, the shortcoming of using TM as showed in most recent cyprinid descriptions (which are based on Hubbs & Lagler, 1964).

However, the application of GM for species identification is not fully developed yet (Ferdous, 2013), though proven to discriminate between closely related species and populations (Albertson & Kocher, 2001; Costa & Cataudella, 2006). Differences in morphotypes of adipose fins of *Mystus* do not display any distinct phenetic groupings, thus further analysis could be combined with other approaches such as molecular phylogeny and traditional morphometrics (Ferdous, 2013) for inferring phylogenetic relationships.

Morphometrics can also be used for evolutionary studies. The advantage of using morphometric data is that the inferred evolutionary relationships are based on body form

rather than body-size differences (Strauss & Bond, 1990). In this case, GM has potential use in phylogenetic investigation (Monteiro & Abe, 1999; Pierce et al., 2008; Ferdous, 2013).

However, due to some limitations of using morphological characters for species identification; especially for identifying species with intraspecific variations, specimens that lost some of their morphological features also difficulties in identification specimens at early life stages (Strauss & Bond, 1990), makes the development of molecular methods as supporting evidence all the more important (Teletchea, 2009).

Teletchea (2009) has reviewed various protein analyses used for fish identifications which are based on the separation and characterisation. Some methods are useful but not for routine analysis, especially for fish identifications. Proteins lose its biological activity after death, and most of the proteins are heat labile, thus determination by these techniques are not available (Asensio, 2007; Teletchea, 2009). Furthermore, in immunoassay application, antibodies are useful for fresh samples only (Ansfield et al., 2000). These shortcomings have made DNA-based identification methods to be the alternative techniques and it is becoming a popular diagnostic tools for fish species identifications.

2.5 DNA-Based Markers for Species Identification

The introduction of Polymerase Chain Reaction (PCR) amplification technique has facilitated the application of DNA-based methods. This method has many advantages; (1) only small fragments of DNA are needed for PCR amplification, (2) DNA is present in almost all cells of an organism, therefore it can be easily retrieved from any substrate, (3) DNA provides more information than protein, due to degeneracy of the genetic code and presence of many non-coding regions (Lockley & Bardsley 2000; Teletchea et al., 2005). Among many methods listed and reviewed by Teletchea (2009), PCR-sequencing gained popularity recently with the reduction of price, time and technical matters (Teletchea,

2009). It, moreover, produces the most information (Lockley & Bardsley, 2000; Asensio, 2007). Depending on the study objectives, other DNA-based methods that were routinely employed include PCR-RFLP (Restriction Fragment Length Polymorphism) (Lin & Hwang, 2007; Hsieh et al., 2007; Wong, 2011), real-time PCR (also known as quantitative PCR or qPCR) (Loh et al., 2014), PCR-SSCP (Single Strand Conformation Polymorphism) (Weder et al., 2001; Sridphairoj et al., 2010), and DNA microarray (Kochzius et al., 2008, 2010, Teletchea et al., 2008).

2.6 DNA Markers and Barcoding in Fish Studies

Development of accurate, reliable technologies for the rapid screening of DNA sequence variation is one of the remarkable achievements in modern biology. Detection and identification of animal species using the mitochondrial DNA (mtDNA) sequencing method, together with bioinformatics, were reviewed by Yang et al. (2014).

The mitochondrion (plural mitochondria) exists in cells of most eukaryotic species (Henze & Martin, 2003). In mammalian cells, the mitochondrial genome consists of a double-stranded DNA molecule approximately 16 kb in length. The genome comprises of 37 genes, encodes for 13 essential oxidative phosphorylation subunit proteins; seven subunit of Complex I (ND1-6 and ND4L), one subunit of Complex III (Cytb) three subunits of Complex IV (COI-COIII), two subunits of Complex V (ATPase 6 and 8), encodes two rRNAs (12S rRNA and 16 rRNA) as well as 22 tRNAs (Anderson et al., 1981; Chan et al., 2006).

The advantages of using mtDNA over nuclear DNA include: (1) high copies of mtDNA as a small closed circular DNA with size range of 15-20 kb, which could be easily amplified, (2) maternal inherited in most animal species, haploid and does not undergo

recombination (Giles et al., 1980; Jenuth et al., 1997) finally (3) faster evolution rate than nuclear DNA, hence has good characteristics to differentiate between closely related species.

Nevertheless, mitochondrial markers also have limitations. Usually, a short DNA sequence or only partial mtDNA gene is used (especially in COI (barcoding applications). Therefore some information may be lost and thus will affect the construction of reliable phylogenetic trees, if OTU (Operation Taxonomic Units) number is large enough (Nei, 1987; Naylor et al., 1995; Nei & Kumar, 2000). The advantage of maternal inheritance, however, made it unsuitable for many genetic studies due to non-Mendelian inheritance. Detecting hybrids is also a constraint for this reason. High mutation rates and back mutation can happen and will also reflect the evolutionary history. Fishes are known for sometimes having gene introgression at mtDNA markers (Campton, 1987; Avise, 1994; Hebert et al., 2003). Such studies should be organised in a special mode to cover, firstly, different levels of variability (sex, ontogenetic, intra-, interpopulation) and, secondly, to maximize representation of a different species or other OTU in the taxa that are analysed.

Most of the previous studies showed that mtDNA gene has a great utility in discriminating fish species. Due to the lack of genetic recombination, they are also powerful tools in population genetics and evolutionary biology (Moritz et al., 1987; Avise, 1992; Castro et al., 1998; Ward et al., 2005). All mtDNA is inherited as a single unit called haplotypes, thus, evolutionary history (relationships between mtDNA) from different individuals can be inferred by the gene tree produced.

Both mitochondrial and nuclear gene sequences have been employed to construct deep-level phylogenetic relationships in order to recover clades at different taxonomic levels. However, combining different genes with different rates of evolution would make phylogenetic inferences more chaotic. Combining genes with similar evolution rates (no

significant value for partition homogeneity test) would give higher resolutions (Ramlah, 2009).

According to Teletchea (2007), in the last decade (1997-2007) the most studied mtDNA gene was cytochrome b, followed by 16s rRNA (small subunit of ribosomal RNA) before the rise of 'the barcoding of life campaign' in 2005. These mtDNA genes have potential usage in many biological studies employed in forensic applications, systematic studies (phylogenetic relationship/evolutionary history), as well as ecological applications.

The highly conserved gene of ribosomal rRNA (mtDNA 12S rRNA and 16s rRNA) can be developed to be a species-specific primer to identify mammals, birds and shrimp (Mitsuhiro et al., 2004; Bataille et al., 1999; Wang et al., 2009; Gupta et al., 2008; Rojas et al., 2008; Pascoal et al., 2008; Gharrett et al., 2001; Martin et al., 2007; Saini et al., 2007; Martin et al., 2007; Barr et al., 2006; Kitano et al., 2007; Pook et al., 2005; López-Calleja et al., 2004; Medina et al., 2001;Simons et al., 1998; Zhang et al., 1994). A recent study by Yang et al. (2014) developed universal primers to identify a wide range of species, (11 species) including fish, amphibians, and mammals with 100% match to BLAST search results in the GENBANK database. These primers specifically hit mitochondrial genomes rather than nuclear genes, accurately identifying species without misidentification (Yang et al., 2014).

12S rDNA is highly conserved and used to study the genetic diversity of higher taxonomic levels such as phyla, while 16S rDNA is applied for the middle taxonomic levels (family levels or genera) (Gerber et al., 2001). Average sequence divergence for 16S were higher in 12S rRNA genes of Chinese antelopes (9.9% vs 6.3%) (Lei et al., 2003) meaning that the former gene possesses better power in species differentiation. Khan et al. (2008) evaluated the utility of 12S rRNA in comparison to 16S rRNA for identification of oryx species, with two closely related taxa (addax and roan). Phylogenetic analysis of 12S failed to differenciate oryx with addax, which was successfully done by 16S. However, taking

fish as examples, both genes were proven suitable for identification of fish at genus level, but faster evolving DNA regions are more recommended for confirmation of closely related fish species (Cawthorn et al., 2012). Pouyaud et al. (2004) have identified groupings of pangasiid species according to their genera, however this could not resolve separation at species level (see section 2.3, Figure 2.7) with 12 S rRNA. Particular usage of these genes have been discussed earlier (section 2.3).

When comparing between 16S rRNA and Cyt b gene, the former represents a slowly evolved conserved region that exhibits useful variation to resolve phylogenetic questions among distantly related species (Meyer, 1994). They are 'non-protein coding' genes which means that genes are not translated into protein but the structures of transcribed RNA product forms a functional unit in protein synthesis. Mutations in this gene can include insertion/deletion which are useful for producing changes to sequence lengths and substitution mutational events (Adamson, 2010). On the other hand, cyt b has a moderate evolutionary rate and is known as a powerful marker for resolving genetic diversity at lower taxonomic levels including families, genera and species as shown in Ferdous (2013); Karinthanyakit & Jondeung (2012); Adamson (2010); Ryan & Esa (2006); Farias et al. (2001); Johns & Avise, (1998); Zardoya & Meyer, (1996). The 16S rRNA gene produces more species-specific mutation sites compared to cytochrome b, even though it is observed to have lower sequence diversity inter and intraspecies diversity in endangered Pecoran (Guha et al., 2006). This gene was also used to investigate the levels of genetic diversity in the Mekong giant catfish species and phylogenetic relationships between its closely related species (Na-Nakorn et al., 2006), Mahseer species (Nguyen et al., 2006; 2008), Adamson (2010) and Karinthanyakit & Jondeung (2012).

Vences et al. (2005) compared the performance of mtDNA 16s rRNA over COI in identification of amphibian species. They found that 16S fragment is superior to the latter gene, in terms of universality of priming sites and species identification. 16S priming sites

were highly conserved, while COI showed high variability. Interspecific divergences at larval stages were suitable for species differentiation (1-17%). This study suggested the use of 16S mtDNA as a barcoding marker in vertebrates, complementary to COI. In another trial to combine DNA barcoding and microarrays using three mt genes (16S rRNA, cyt b and COI) for 50 European marine species, 16 S failed to discriminate closely related flatfish and gurnard species. However, 16 S is more suitable for the oligonucleotide probe design based on DNA microarrays, compared to cyt b and COI (Kochzius et al., 2010).

The 16s rRNA gene and protein coding cytochrome c oxidase sub unit I (COI) have been widely used for inferring phylogenies in fish (Brown 1985; Bermingham and Lessios, 1993; Santos et al., 2003; Vinson et al., 2004; Ward et al., 2005; Lakra et al., 2009, 2010; Adamson et al., 2010); Pacific Abalone (An et al., 2005) and sea cucumbers (Byrne et al., 2010).

The protein-coding cytochrome oxidase I gene is also known as the animal barcoding gene. The term 'DNA barcoding' was initially proposed by Hebert et al. (2003) in order to produce a standard protocol in species identification in a global perspective. The principle of DNA barcoding is based on the sequencing of a short segment of a uniform mt genome of a target specimen and compared to the existing barcode database for species identification (Hebert et al., 2003).

It is interesting to note that a barcoding gene might not be effective in evolutionary studies (phylogenetic reconstruction), due to the short sequence, but such information is still useful in comparing closely related organisms (Ward et al., 2005). Most significant applications of DNA barcoding is that this method is considered a rapid, accurate, and informative compared to the conventional method. This is crucial in a few situations, whereby morphological-based identification is not possible, such as in forensic applications (Dawnay et al, 2007, Tan et al., 2010), identifications of fraud or mislabelled food products (Botti et al., 2010; Lowenstein et al., 2009; 2010; Wong et al., 2011,

Carvalho et al., 2011; Hanner et al., 2011, Yancy et al., 2008); fish systematics (Hebert et al., 2003; Bhattacharjee et al., 2012; Serrao et al., 2014; Ward et al., 2005; Hubert et al., 2008; Chu et al., 2013; Mohd-Shamsuddin et al., 2011; Malakar et al., 2012; Esa et al., 2008; Kartavtsev et al., 2008; Khedkar et al., 2014; Aquino et al., 2011; Kochzius et al., 2010; Khare et al., 2014) as well as ecological applications (Valentini et al., 2009 Ficetola et al., 2008; Taberlet et al., 2007).

Nevertheless, despite many applications of molecular tools in species discrimination for fisheries managers, ecologists and retailers, still, morphological analysis from an expert taxonomist is largely recommended (Ward et al., 2005). Many barcoding publications emphasise the need for incorporating morphological analysis (Bhattacharjee et al., 2012; Hubert et al., 2008; Aquino et al, 2011; DeSalle, 2005, 2006). Thus, many recent systematics studies have merged morphological analysis with the molecular approach to reconcile taxonomic problems in various fish taxa (Song et al., 2013; Laskar et al., 2013; Fazimah, 2013; Ferdous, 2013).

2.7 Implication in Fisheries Management

Towards sustainable fisheries management, assessment of biodiversity is considered crucial before outlining any strategic planning. Precise knowledge on species identification (taxonomy), biology and ecology is a strong drive to successful conservation planning. In order to keep a balance between the economic importance of aquaculture and wild stock protection, a systematic approach should be considered. Knowing the genetic status of species that have an ambiguous taxonomy is crucial and there is a state of urgency for better control of the introduction of exotic species.

Monitoring of wild and hatchery stock can now be facilitated using molecular techniques. Genetic variation can be analysed and negative impacts can be reduced. Genetic studies suggested by Vrijenhoek (1998) could contribute to systematic management planning. The points raised were resolving taxonomically difficult taxa, captive breeding programmes must be properly designed, natural breeding systems must be well understood, species diversity within and among population must be measured, gene flow management must be implemented and finally factors that contribute to stock fitness should be clearly considered. Undoubtedly morphological and genetic evidence could provide baseline information on the status of any species under study and therefore could facilitate fisheries management program.

CHAPTER 3: TAXONOMIC EVALUATION OF THE FISHES FROM THE FAMILY PANGASIIDAE (TELEOSTEI: SILURIFORMES) IN PENINSULAR MALAYSIA

3.1 Introduction

The catfish species of the family Pangasiidae are recognised to have a laterally compressed body, two pairs of barbels, a long anal fin, a short dorsal fin and a small adipose fin with free posterior margin (Teugels, 1996; Gustiano, 2003). They are medium to large-sized catfishes with diverse morphologies and ecologies. Adults range from 20 to 300 cm in length, but most species are more than 50 cm. Maximum length is about 3 meters with a maximum weight of 300 kg, referring to one of the largest freshwater fish species, *Pangasianodon gigas* (Nelson, 2006).

Pangasiids are generally found in freshwater areas; however, some species can also be found in brackish and marine environments (Roberts & Vidthayanon, 1991) A few species are important in the aquaculture and the aquarium industry, while several others are under threat of extinction (Roberts & Vidthayanon, 1991; Gustiano, 2003; Haslawati et al., 2014).

They are widely distributed throughout Asia, from Pakistan to Indochina and the Indonesian Archipelago (Roberts & Vidthayanon, 1991) and extending to the Lanchangjiang River Basin, China (Yang et al., 2007). Until recently 28 valid Pangasiidae species have been reported throughout the Asian river systems, with 13 species occurring in Indo-China and Thailand and 15 species found in the Indonesian Archipelago (Indonesia and Borneo) (Pouyaud et al., 1999; Pouyaud & Teugels, 2000; Ng & Kottelat, 2000; Pouyaud et al., 2002; Gustiano, 2003). This shows more species diversity when compared to the first systematic work of Roberts & Vidthayanon (1991) (11 species in Thailand and 10 – Indonesia). As for species distribution; one species belongs to the

Indian subcontinent (*P. pangasius*), two species are known from Myanmar (*P. pangasius* and *P. myanmar*) and 13 from Indo-China (*Pangasianodon gigas, P. hypophthalmus, Pangasius bocourti, P. conchophilus, P. elongates, P. krempfi, P. larnaudii, P. macronema, P. mekongensis, Pseudolais pleurotaenia, P. sanitwongsei, P. micronemus* and *Helicophagus leptorhyncus*). The other 15 other pangasiid species occur in the Indonesian Archipelago which includes Sumatra, Java and Kalimantan/Borneo (*P. djambal, P. humeralis, P. kinabatanganensis, P. kunyit, P. lithostoma, P. macronema, P. mahakamensis, P. nieuwenhuisii, P. nasutus, P. polyuranodon, P. reophilus, P. sabahensis, Pseudolais micronemus, H. typus* and *H. waandersii*) (Chapter 2 – Figure 2.5, Table 2.1 as adapted from Gustiano (2003)).

Peninsular Malaysia, located at the southern tip of Malay Paninsula, is the land where many Indonesian species have their northernmost distributional limit while a handful of Indian and Indochinese species have their southernmost distributional limit (Zakaria-Ismail, 1994). The previous landmark change on the Sunda Shelf during the Pleistocene glacial maxima (Rainboth, 1996; Voris, 2000; McConnell, 2004) have classified the pattern of freshwater fish distribution in Southeast Asia into seven (Kottelatt, 1989) or five zoogeographic regions (Zakaria-Ismail, 1994) including the Malay Peninsula.

During the Pleistocene epoch, the land mass of the Sunda Shelf had several very large river systems, namely the Malacca Straits River system, Siam River system, North Sunda River system, and East Sunda River system (Chapter 2- Figure 2.1) (Voris, 2000). Peninsular Malaysian freshwater faunal distribution were affected by the first two river systems; the Malacca Straits River system and the Siam River system. As suggested from the present day contours, the Malacca Straits River system, which includes Sungai Perak, Sungai Bernam, Sungai Muar and Sungai Lenek (large rivers along the west coast of Peninsular Malaysia) were derived from a single river that drained north to Andaman Sea.

The Siam River system or the influenced contributions from Sungai Endau, Sungai Pahang, Sungai Terengganu and Sungai Kelantan in the East Coast of Peninsular Malaysia, running north from Sungai Kampar (Sumatra) through the Singapore straits, joined by the Johore River which then joined the Gulf of Thailand river branches (Rainboth, 1996; Voris, 2000; McConnell 2004). Thus, the present day Malay Peninsula is influenced by the overlapping freshwater species from India, Indo-China and Indonesia (Ismail, 1989).

Four pangasiid species were reported from the Malay Peninsula, (*P. nasutus, P. micronemus, Helicophagus waandersii*) with *P. pleurotania* are found in the Tapi river basin in southern Thailand. While the first three species mentioned could be found in the Pahang River, there is only one species from Perak River (*P. micronemus*) (Gustiano, 2003). However, in Peninsular Malaysia (in this case, Tapi River was excluded since it is located in Thailand) only three species; *Pangasius nasutus, P. micronemus* and *Helicophagus waandersii* were recorded (Tweedie, 1936; Herre & Myers, 1937; Roberts & Vidthayanon, 1991; Lim & Zakaria-Ismail, 1995). Records for *Pangasius pangasius* (Herre & Myers, 1937) were revised and then clarified as *P. micronemus* while the holotype of *P. ponderosus* from Tasik Chenderoh, Perak (Chandra Dam as in Herre & Myers, 1937; Ismail, 1989) is actually a synonym of *P. nasutus* (Roberts & Vidthayanon 1991, Gustiano 2003). The status of *P. polyuranodon*, which was mentioned in Fowler (1938), was confirmed as valid by a recent work (Haslawati et al., 2014) (Chapter 4). Nevertheless, further study is needed to clarify the fishery status before any suitable management plan for all these native species could be proposed.

Because pangasiid catfish are among the most popularly consumed freshwater fish (Annual Fisheries Statistics, Department of Fisheries), the high aquaculture demand increased for some species while wild Pangasiids are sought for their exotic value and have a high price. Unsustainable exploitation has created conservation interest as the wild

catch have been reported to decrease on a yearly basis (Annual Fisheries Statistics, Department of Fisheries, 2000-2014). Other anthropogenic disturbances such as increased fishing pressure and land development are affecting the natural habitat thus decreasing the wild fish population. The extent of impacts of introduced species on natural population and the potential threats posed by such introductions are also unknown.

Even though none of the pangasiid species previously reported in Peninsular Malaysia is under the extinct or endangered list in the IUCN criteria, but due to lack of available studies on the population structure or catch report, data on the current Pangasiid status in Malaysia (Peninsular, in particular) remain unknown. In a recent study, all the four species of Pangasiids are listed as moderately threatened in Malaysia because of the combination factor of overharvesting and habitat degradation (Chong et al., 2010).

Therefore, it is crucial to evaluate the current status of pangasiids in Peninsular Malaysia. Since this area covers the Thai and Indonesian faunal overlaps (Zakaria-Ismail & Sabariah, 1995) while Roberts & Vidthayanon (1991) recognised the taxonomic separation of Pangasiidae between these two regions of the Asian Mainland and Indonesian Archipelago, it is interesting to know how these separation occurred and related to each other.

This chapter presents the pangasiid catfishes found in Peninsular Malaysia, from previous and present collections; their morphological characteristics and distinguishable features. Clarification of the taxonomic status of the Pangasiidae family in Peninsular Malaysia is presented and taxonomic keys to facilitate species identification will be further derived. Their distributions will be reviewed.

3.2 Materials and Methods

3.2.1 Field Work

3.2.1.1 Fish collection

Fresh samples were taken from two main rivers in Peninsular Malaysia, the Pahang River and Perak River which are known to have native pangasiids. Sampling locations are shown in figures 3.1, 3.2 and Table 3.1. Local fishing gear such as gill nets, drift gill nets, long-lines and others were used for collecting samples.

(a) Gill nets and trammel nets:

Gill nets of various mesh sizes were used. Nets were mostly set parallel to river banks, in some instances were set across the river. The nets were left *in situ* and checked twice a day (early morning and late evening).

(b) Drift gill nets:

Nets with various mesh sizes. The net was set across the middle of the river with one end tied to a small boat. Both the net and the boat were allowed to drift downstream for some distance before checking for the fish caught.

(c) Rod and line, longlines and traditional method:

Hooks were baited with earthworms, small fish, fish meat or fig fruit. Fish caught were immediately identified (tentatively) whenever possible. Some of the specimens of *Pseudolais micronemus* were caught using a traditional method by baiting a small piece of sandwich white bread attached to an empty mineral water bottle (traditionally the old folks used a coconut fruit). The bottle was thrown upstream in the river, and left to drift freely until a fish was caught. Fish samples were preserved in 10 % formalin and transported to the laboratory for confirmation of identification.

Specimens were identified and described on site based on Roberts & Vidthayanon (1991) and doubtful specimens were brought back for further examination. Fresh specimens were immediately fixed in 10% formalin for at least seven days. In the

laboratory, the specimens were rinsed with tap water and soaked for another seven more days before transferring into 70% denatured ethyl alcohol. Specimens were identified and a catalogue number was given for each specimen or a lot consisting of more than one specimens.

The present Malaysian specimens are now housed at the Fisheries Research Institute (Freshwater Division), Glami Lemi, (FRIGL), Jelebu, Negeri Sembilan, Malaysia.

3.2.1.2 Sampling location

(a) The Pahang River

Khan et al. (1996), divided fish distributions in Peninsular Malaysia into three division i.e. North-west, North-east and Central Division, and Southern Divisions. The most diverse and abundant fish community occurs in the North-east and Central Division where the Pahang River Basin is situated. The water quality in the Pahang River is slightly alkaline compared to the other two areas that are mostly acidic with pH about 5.5 – 6.0 (Khan et al., 1996).

The Pahang River Basin is located between 101° 20’ and 103° 38’ E and 2° 28’ and 4° 47’ N with a catchment area of 29,300 km2. Sampling covered the three sections of the river (upper, middle and lower) based on map-analysed distance, accessibility and the logistic support availability (Khan et al., 1996).

(a) The Perak River

The Perak River is the second longest river in Peninsular Malaysia, after the Pahang River in Pahang. The river originated from the mountainous Perak-Kelantan-Thailand border of the Belum Forest Reserve, flows south to Teluk Intan, further bends westward into the Straits of Malacca. It flows over 400 km in a 15,000 km2 catchment that covers 70% of the state lands. Some of the branches of the river are the Bidor River

and the Kinta River. The Temengor Dam has created a large man-made lake at Banding near Grik. A number of towns are on the banks of the river including the royal town of Kuala Kangsar.

The study location is at a stretch of Perak River, near a small town called Manong, 21 km from Kuala Kangsar (4.59 N 100.88 E) which is known to have active fishery activities (Figure 3.2). This place is famous for many indigenous freshwater fishes.

Table 3.1: Sampling locations of pangasiids in two main rivers of Peninsular Malaysia

No	Location (district, state)	River basin	GPS Coordinates
1	Paloh Hinai, Pekan, Pahang	Lower reach, Pahang River	3° 30’ 35”N ; 103° 08’ 32”E
2	Lubok Paku, Maran, Pahang	Middle reach, Pahang River	3° 28’ 59” N; 103˚ 48’ 56”E
3	Chenor, Maran, Pahang	Middle reach, Pahang River	3° 28’ 53”N ; 102° 36’ 28”E
4	Kuala Mai, Temerloh, Pahang	Middle reach, Pahang River	3° 46’ 37”N ; 102° 22’ 6.6”E
5	Ulu Tembeling, Jerantut, Pahang.	Upper reach, Pahang River	4° 22’ 14”N ; 102° 42’ 48”E
6	Manong, Kuala Kangsar, Perak	Perak River	4° 35’ 39”N ; 100° 52’ 54”E

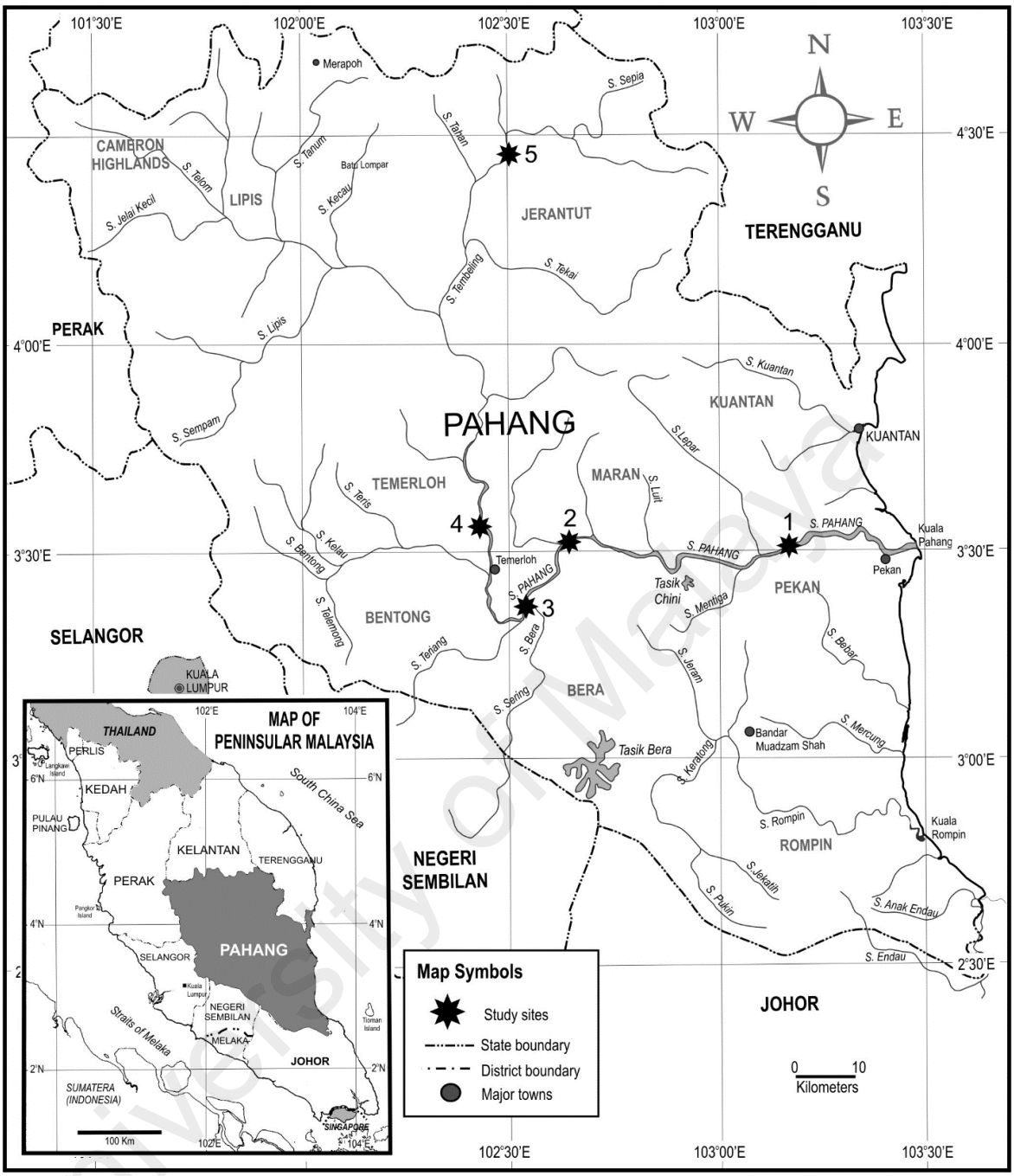


Figure 3.1: Map of Peninsular Malaysia, showing Pahang state, main river basin and its tributaries. Study sites and collection samples are presented as (✸) Numbers refer to the following locations: (1) Paloh Hinai, Pekan, Pahang; (2) Lubok Paku, Maran, Pahang; (3) Chenor, Maran, Pahang; (4) Kuala Mai, Temerloh, Pahang; (5) Ulu Tembeling, Jerantut, Pahang.

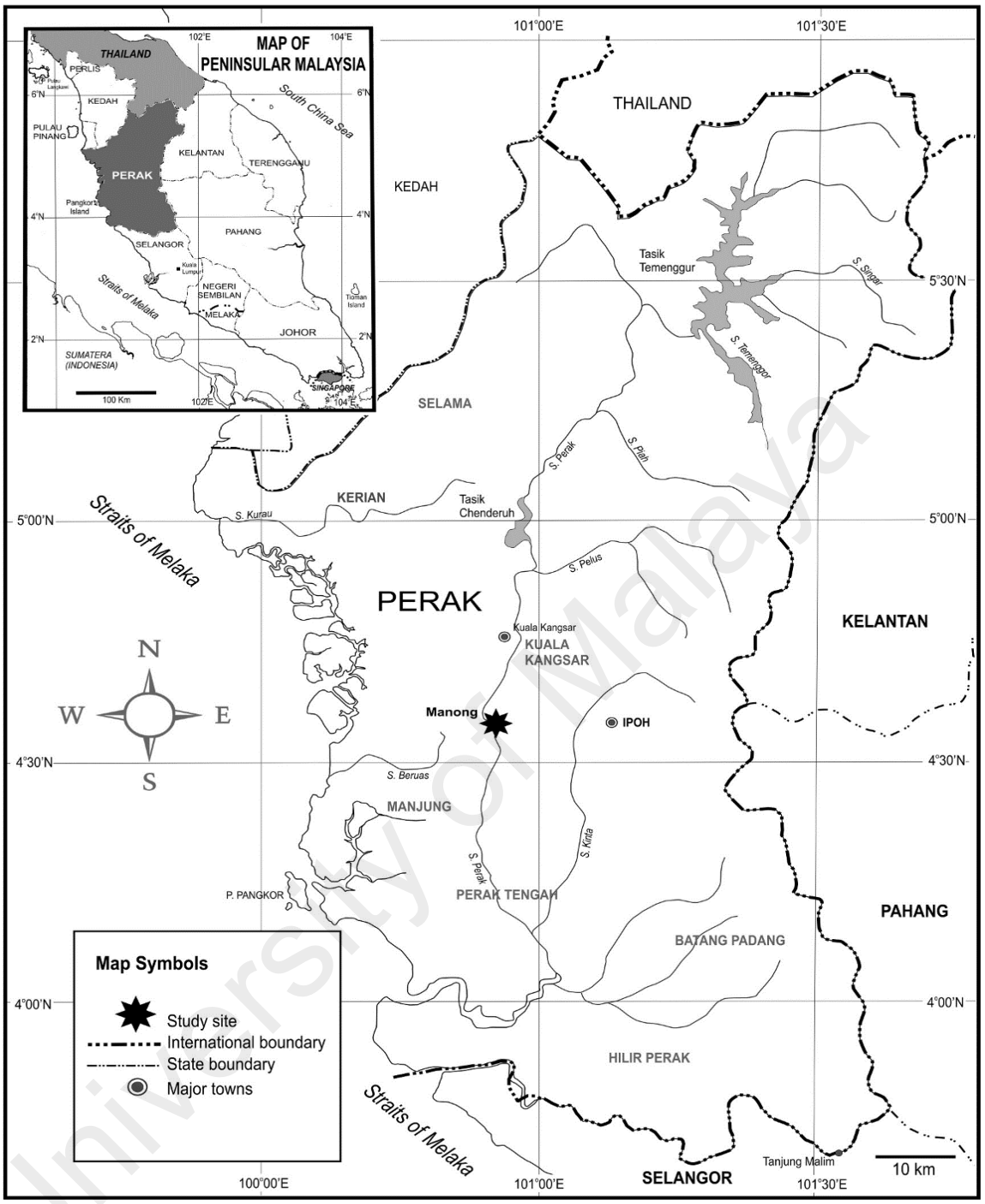


Figure 3.2: Map of Peninsular Malaysia, showing Perak state, main river basin and its tributaries. Study site and collection samples is presented as (✸).

3.2.2 Other Materials Used

Specimens from previous collections of Peninsular Malaysia located at the Universiti Malaya, Kuala Lumpur, Malaysia (BIRCUM and UMKL), Fisheries Research Institute (Freshwater Division), Glami Lemi, Jelebu, Malaysia (FRIGL), the Raffles

Museum for Biodiversity Research, National University of Singapore (ZRC and NMS) also collections of the Asian Wetland Bureau (AWB) (now known as Wetlands International) were examined. A specimen of *P. polyuranodon* and two other specimens of *P. micronemus* from Museum Zoologicum Bogoriense (MZB) served as comparative specimens (will be further discussed in Chapter 4).

3.3 Taxonomic Work

3.3.1 Measurements and Counts

External morphology of the preserved specimens were examined meristically and morphometrically. Morphometric measurements and meristic counts were taken following the standard procedure of Hubbs and Lagler (1964) and Roberts & Vidthayanon (1991) with some additional measurements following Pouyaud et al. (1999). Meristic counts include anal-fin rays, pelvic-fin rays, gill rakers and the number of swim bladder chambers as suggested by Roberts & Vidthayanon (1991).

Morphometric measurements were taken in a straight line (Figure 3.3 and Table 3.1), from point to point, using dial callipers. Recorded to the nearest 0.1 mm, on the left side of the body, following Gustiano (2003, 2009) and Pouyaud et al. (1999). All measurements except standard length were expressed as proportional values of reference length. Measurements on the body including head length were conveyed as a percentage of the standard length (% SL). Other measurements on the head were conveyed as a percentage of the head length (% HL).

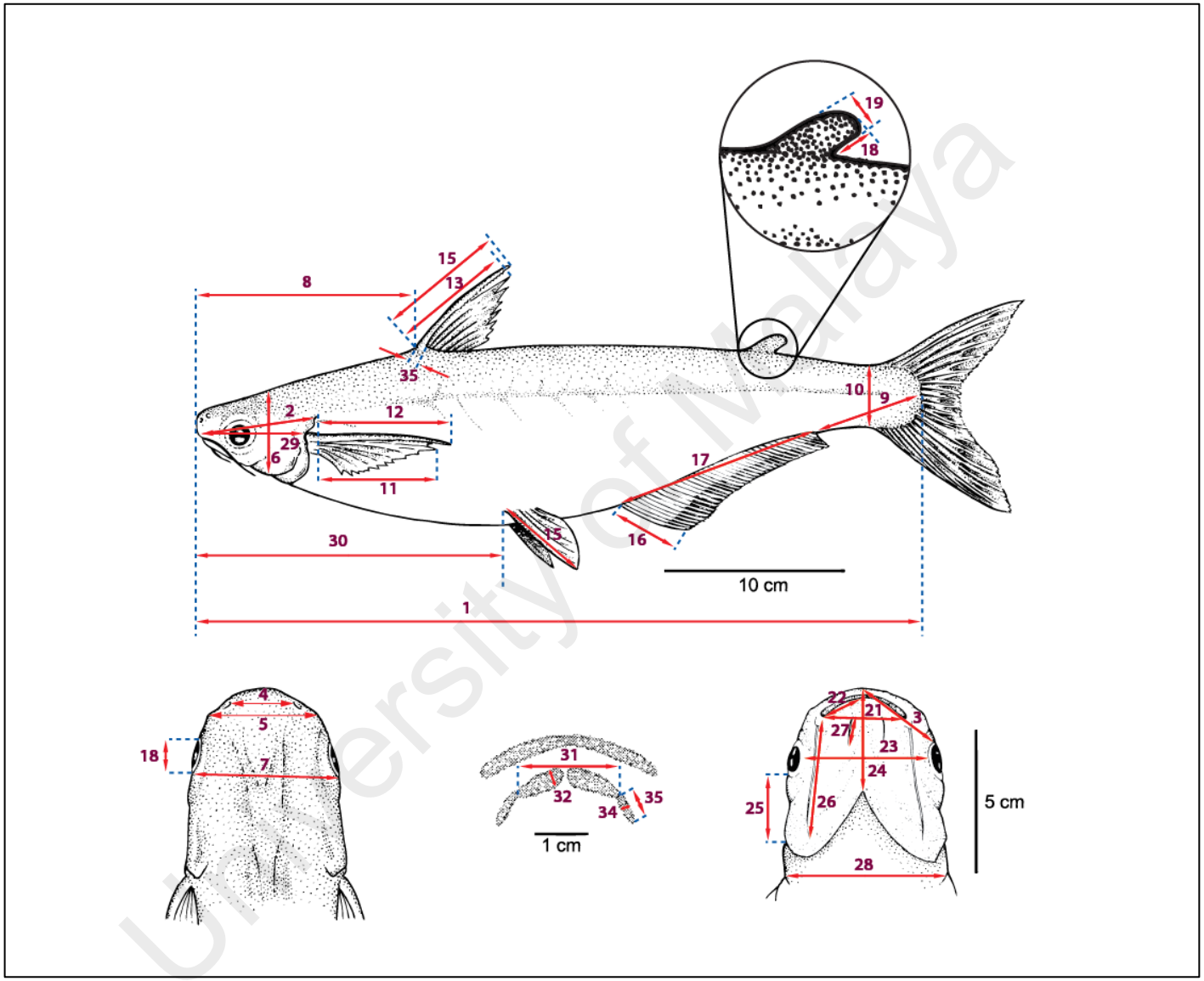


Figure 3.3: Measurements taken on *Pangasius* specimens (from Gustiano 2003). Detailed measurements are shown in Table 3.1**.**

Table 3.2: Abbreviations, measurement characters and its descriptions in morphometric analyses.

No	Abbreviations	Measurement characters	Measurement descriptions
1	SL	Standard length	The distance from tip of snout to caudal peduncle
2	HL	Head length	The distance from tip of snout to posterior border of operculum
3	SNL	Snout length	The distance from tip of snout to anterior border of the eye
4	ASW	Anterior snout width	Taken between the anterior nostrils
5	PSW	Posterior snout width	Taken between the posterior nostrils
6	HD	Head depth	Taken at the level of the posterior eye border
7	HW	Head width	Vertical axis measured along the eye margin
8	PdL	Predorsal length	Distance from tip of the snout to the base of first dorsal spine
9	CPL	Caudal peduncle length	From base of last anal-fin to the middle of caudal peduncle
10	CPD	Caudal peduncle depth	Minimum body depth
11	PFL	Pectoral-fin length	From base of pectoral spine to tip of fin
12	PSL	Pectoral-spine length	From base of pectoral spine to its tip
13	DFL	Dorsal-fin length	From base of first dorsal spine to tip of fin

Table 3.2: (continued).

No	Abbreviations	Measurement characters	Measurement descriptions
14	DSW	Dorsal-spine width	Taken at base of second dorsal spine
15	PFL	Pelvic-fin length	From base of pelvic fin to tip of fin
16	AFH	Anal-fin height	From base of anal-fin to tip of longest ray
17	AFL	Anal-fin length	From base of first ray to base of last anal ray
18	AdiFH	Adipose-fin height	From base of adipose fin to its tip
19	AdiFW	Adipose-fin width	Maximal width of adipose fin
20	ED	Eye diameter	Maximal orbital eye diameter
21	MW	Mouth width	Mouth width
22	LJL	Lower jaw length	From tip of snout to corner of mouth
23	IOD	Interorbital distance	Taken between the eyes, least distance
24	DSI	Distance from snout to isthmus	From tip of snout to isthmus with mouth closed
25	POL	Post Ocular length	From posterior eye border to posterior border of operculum

Table 3.2: (continued).

No	Abbreviations	Measurement characters	Measurement descriptions
26	MxBL	Maxillary barbel length	Length of maxillary barbel
27	MnBL	Mandibular barbel length	Length of mandibular barbel
28	BW	Body width	From left to right scapular excrescent bones close to pectoral-spine base
29	PrePecTL	Prepectoral length	From tip of snout to pectoral-spine base
30	PrePelVL	Prepelvic length	From tip of snout to the first pelvic fin ray base
31	VMW	Vomerine width	Vomerine width
32	VML	Vomerine length	Vomerine length
33	PL	Palatine length	Palatine length
34	PW	Palatine width	Palatine width
35	DSW	Dorsal- spine width	Taken at base of second dorsal spine

3.3.2 Statistical Procedures

Univariate analysis including sample size, mean and standard deviation, minimum and maximum values were analysed using Analysis of Variance (ANOVA). Variables with significant difference were selected as distinguishable characters.

The Principal Component Analysis (PCA) using correlated matrices were calculated using covariate adjustments (Meng & Stoker, 1984; Froese, 1989) to reduce the effect of non-normality of the measurements. All measurements were log-transformed before running PCA on the covariance matrix (Bookstein et al., 1985) and then visualise using scatter plots.

All measurements were corrected for length (Froese, 1989), since differences in body shape are more important than the body size as observed by Pimentel (1979). He detected unusual distributions and error that introduced by the expression of measurements as ratios of body length. Therefore, Froese (1989) suggested to adjust all measurements for covariate to normalise the multivariate and linear relationship to the overall mean standard length using this formula:

$$AM = OM - [RC^* (SL - MSL)]$$

Where;

AM = adjusted measurement for covariate

OM = original measurement

RC = regression coefficient between character and standard length

SL = standard length

MSL = overall mean standard length

Multivariate analysis were done using PCA to discriminate and identify characters that contribute to the species discrimination. All statistical analyses were performed using STATISTICA version 10.0 (Stat Soft, Inc., USA).

3.4 Results and Discussion

3.4.1 Fishes of the family Pangasiidae

Seven morphospecies of Pangasiids from all four genera; *Pangasionodon*, *Pangasius, Pseudolais* and *Helicophagus* exist in Peninsular Malaysia. Based on the analysis of 35 measurements and five meristic counts as well as the species diagnose, the identification key and description are given below. Table 3.3 shows the measurements and meristic counts for all the specimens of Pangasiids collected also from previous collections. Drawings for the seven species collected were provided and distribution maps from current and past studies also included.

Table 3.3: Morphometric and meristic measurements of seven morphospecies of the fishes of the Pangasiidae family from Peninsular Malaysia, including one species identified as *Pangasius* hybrid. Mean and standard deviation are given, with their range values (in brackets). All measurements are in millimetres.

Characters	***Helicophagus waandersii*** **(n = 25) 107.5-409.0**	***Pangasianodon hypophthalmus*** **(n=4) 297.0-432.0**	***Pangasius nasutus*** **(n=33) 103.8-694.0**	***Pangasius conchophilus*** **(n=5) 261.0-479.0**
IN % STANDARD LENGTH				
Head length	20.2±1.4 (18.0-24.1)	24.9±1.0 (23.6-25.8)	24.1±1.7 (18.7-28.0)	23.9±1.4 (22.4-25.5)
Head depth	9.2±1.1(7.6-13.2)	10.1±1.0 (9.3-10.9)	10.6±1.1 (7.7-12.4)	10.5±0.5 (9.7-11.0)
Head width	9.3±0.7(7.8-10.4)	15.5±0.6 (14.9-16.0)	14.2±1.2 (11.1-16.7)	15.2±0.7 (14.1-16.0)
Caudal peduncle length	17.6±1.4(15.0-20.8)	15.8±0.7 (15.1-16.6)	16.6±2.9 (8.7-20.9)	16.9±1.2 (15.1-18.4)
Caudal peduncle depth	7.4±0.7(6.2-9.2)	8.8±0.7(8.2-9.8)	7.2±1.1 (5.4-11.9)	7.2±0.5 (6.6-7.8)
Pectoral-spine length	15.0±1.4(13.1-19.2)	16.3±2.1 (13.7-18.2)	15.3±1.6 (11.7-18.9)	14.4±1.3 (13.1-15.7)
Pectoral-fin length	14.9±1.5(12.4-17.6)	17.4±1.7 (16.1-19.8)	16.1±2.5 (9.2-20.3)	16.4±1.4 (15.0-18.0)
Dorsal-spine length	15.2±2.4(7.6-20.0)	19.6±1.1 (18.9-20.9)	17.0±1.8 (14.0-20.8)	16.2±1.6 (14.0-17.8)
Dorsal-fin length	17.1±2.0(12.8-20.1)	20.6±4.9 (15.6-25.5)	19.6±2.1 (15.3-23.5)	19.1±2.1 (16.9-21.1)
Pelvic-fin length	9.7±1.2(7.09-13.2)	13.7±2.6 (11.1-16.9)	11.6±1.7 (7.4-15.5)	12.5±1.2 (11.4-14.1)
Anal-fin height	11.2±1.5(7.0-15.0)	13.1±3.5 (8.4-16.6)	13.2±1.3 (11.2-15.9)	12.8±2.5 (8.9-15.1)
Anal-fin length	31.0±4.2(16.4-35.4)	29.7±1.1 (28.7-31.0)	25.3±5.4 (20.9-52.8)	24.2±2.4 (22.1-28.2)

Table 3.3: Continued

Characters	*Helicophagus waandersii*	*Pangasianodon hypophthalmus*	*Pangasius nasutus*	*Pangasius conchophilus*
	(n = 25) 107.5-409.0	(n=4) 297.0-432.0	(n=33) 103.8-694.0	(n=5) 261.0-479.0
IN % STANDARD LENGTH				
Adipose-fin height	4.3±1.1(2.6-6.7)	3.7±0.2 (3.4-3.8)	4.0±1.4 (0.4-8.3)	5.2±1.7 (3.1-7.7)
Adipose-fin width	1.6±0.3(1.1-2.0)	2.0±1.1 (0.4-2.8)	2.2±0.4 (1.7-3.0)	2.1±0.4 (1.7-2.6)
Interorbital distance	8.3±1.9(4.9-15.5)	13.5±0.6 (13.0-14.3)	13.6±1.1 (10.3-15.4)	13.4±0.3 (13.0-13.8)
Body width	11.8±1.7(9.4-19.0)	16.1±0.8 (15.0-17.0)	17.7±2.5 (8.6-25.3)	17.3±1.4 (15.2-18.8)
Predorsal length	36.2±2.0(33.6-44.3)	39.8±1.2 (38.9-41.5)	38.5±3.0 (26.8-42.0)	39.4±1.8 (37.6-42.4)
Prepectoral length	18.1±2.3(8.4-21.5)	17.9±6.2 (11.7-23.8)	23.8±5.9 (9.4-49.4)	21.4±0.8 (20.2-22.4)
Prepelvic length	40.4±2.8(34.6-50.6)	44.0±2.9 (40.2-47.1)	47.6±3.3 (36.8-52.7)	48.5±1.6 (46.5-50.2)
IN % HEAD LENGTH				
Snout length	29.8±2.6(23.5-34.6)	37.2±6.6 (28.3-43.7)	37.5±4.8 (19.6-47.2)	37.1±3.2 (33.0-40.3)
Anterior snout width	14.9±1.9(10.5-17.8)	28.6±1.0 (27.8-29.7)	30.0±3.0 (16.5-34.1)	31.7±6.6 (25.1-40.8)
Posterior snout width	31.0±2.8(24.8-36.2)	37.0±1.8 (35.6-39.0)	38.5±4.0 (33.1-51.5)	39.0±8.0 (31.1-50.2)
Eye diameter	23.9±5.2(15.0-35.5)	12.6±1.6 (11.0-14.6)	12.3±1.7 (9.9-17.2)	8.5±2.3 (6.2-11.6)
Mouth width	21.2±2.2(17.6-27.0)	39.1±2.2 (36.6-41.8)	41.0±3.4 (35.3-49.6	38.7±3.4 (34.4-42.8)

Table 3.3: Continued

Characters	*Helicophagus waandersii*	*Pangasianodon hypophthalmus*	*Pangasius nasutus*	*Pangasius conchophilus*
	(n = 25) 107.5-409.0	(n=4) 297.0-432.0	(n=33) 103.8-694.0	(n=5) 261.0-479.0
IN % HEAD LENGTH				
Lower jaw length	15.7±3.0(10.9-23.4)	20.9±0.6 (20.3-21.6)	27.3±5.3 (18.4-49.1)	24.1±4.2 (18.7-29.1)
Distance from snout to isthmus	54.1±6.4(40.2-63.6)	46.6±3.8 (41.9-51.2)	52.0±3.1 (47.2-59.3)	51.9±3.5 (48.6-57.6)
Post Ocular length	46.0±11.2(32.3-85.9)	40.2±3.5 (35.3-43.7)	47.2±6.3 (35.0-60.4)	52.0±9.1 (41.9-62.0)
Vomerine width	1.3±0.2(1.2-1.5)	na	7.6±8.3 (1.5-23.5)	16.9
Vomerine length	0.4±0.2(0.1-0.6)	na	3.4±4.4 (0.0-13.3)	8.6
Palatine length	0.8	na	3.5±4.4 (0.4-12.2)	8.6
Palatine width	0.6	na	1.3±1.4 (0.1-3.9)	4.1
Dorsal-spine width	10.1±2.5(5.6-14.6)	6.6±2.0 (4.5-8.5)	10.1±3.0 (6.4-18.0)	9.2±1.4 (7.6-10.2)
Maxillary barbel length	71.9±13.2(38.6-91.5)	30.9±8.2 (21.6-37.4)	47.8±10.3 (15.6-66.2)	41.0±5.7 (33.1-47.9)
Mandibulary barbel length	63.6±15.5(37.1-92.9)	21.5±5.1 (15.7-25.5)	38.0±8.8 (20.4-70.9)	24.9±2.6 (21.7-27.7)

Table 3.3: Continued

MERISTIC COUNTS	***Helicophagus waandersii***	***Pangasianodon hypophthalmus***	***Pangasius nasutus***	***Pangasius conchophilus***
Anal-fin rays	32-38	30-32	20-34	23-30
Pelvic-fin rays	5-7	7	6	6-7
Pectoral-fin rays	8-11	7-11	7 -12	11
Number of gill rakers on the first gill arch	7-14	30-36	11-24	13-21

Table 3.3: Continued

Characters	*Pangasius polyuranodon* (n=3) 223.0-395.0	*Pseudolais micronemus* (n=82) 72.0-406	*Pangasius* hybrid (n=4) 385.0-429.0	*Pangasius djambal* (n=3) 375.0-495.0	*Pangasius bocourti* (n=2) 460.0-500.0
IN % STANDARD LENGTH					
Head length	17.9±1.0 (17.3-19.1)	19.2 ± 1.8 (15.0-24.1)	92.1±2.7 (89.0-95.2)	96.1±7.7 (89.9-104.7)	21.5±0.4 (21.2-21.8)
Head depth	40.0±1.5 (38.3-41.2)	11.5±1.4 (7.7-15.3)	12.3±2.4 (10.5-15.8)	10.6±0.7 (9.9-11.2)	10.3±0.5 (9.9-10.6)
Head width	13.4±0.4 (13.1-13.7)	13.9±1.1 (10.3-17.1)	14.1±0.5 (13.4-14.6)	70.0±8.2 (62.4-78.7)	74.8±1.4 (73.8-75.8)
Caudal peduncle length	27.1±1.0 (26.0-28.0)	18.4±1.6 (13.9-21.7)	16.7±0.6 (16.3-17.3)	16.6±0.8 (15.8-17.4)	16.4±2.0 (15.0-17.9)
Caudal peduncle depth	21.8±2.3 (19.8-24.3)	7.7±0.5 (6.1-9.1)	7.7±0.5 (7.0-8.2)	7.9±0.2 (7.7-8.1)	8.2±0.4 (7.9-8.5)
Pectoral spine length	16.4	15.1±2.3 (7.1-18.2)	14.4±0.6 (13.8-14.9)	16.2±2.5 (13.9-18.8)	10.9±0.7 (10.5-11.3)
Pectoral- fin length	18.2±0.6 (17.6-18.6)	16.1±1.9 (10.5-20.2)	16.4±1.6 (14.1-17.6)	17.0±2.9 (14.1-19.9)	15.0±0.7 (14.5-15.5)
Dorsal spine length	15.8±1.0 (14.9-16.9)	14.9±1.7 (10.8-19.4)	18.5±3.7 (14.3-23.0)	16.7±3.4 (12.9-19.2)	15.7±4.0 (12.9-18.5)
Dorsal- fin length	16.7±2.1 (14.9-18.9)	16.6±1.7 (11.9-22.2)	19.5±3.1 (15.5-22.6)	21.2±2.2 (19.1-23.4)	17.7±1.0 (17.0-18.4)
Pelvic- fin length	10.4±1.2 (9.1-11.6)	10.7±0.9 (9.0-14.8)	12.5±1.1 (11.3-13.8)	12.8±0.9 (11.8-13.4)	11.4±0.5 (11.1-11.7)
Anal- fin height	9.6±1.4 (8.5-11.1)	9.5±1.5 (6.6-16.6)	13.5±1.1 (11.9-14.6)	13.3±0.9 (12.8-14.4)	12.6±1.5 (11.5-13.6)

Table 3.3: Continued

Characters	*Pangasius polyuranodon* (n=3) 223.0-395.0	*Pseudolais micronemus* (n=82) 72.0-406	*Pangasius* hybrid (n=4) 385.0-429.0	*Pangasius djambal* (n=3) 375.0-495.0	*Pangasius bocourti* (n=2) 460.0-500.0
IN % STANDARD LENGTH					
Anal-fin length	32.1±0.4 (31.8-32.6)	27.8±2.9 (13.6-33.6)	26.5±2.1 (23.8-28.9)	27.2±1.9 (25.1-28.7)	25.9±1.3 (24.9-26.8)
Adipose-fin height	3.0±0.6 (2.7-3.7)	2.8±1.0 (0.4-6.4)	4.4±0.9 (3.3-5.6)	4.1±0.3 (3.8-4.3)	4.2±0.2 (4.1-4.4)
Adipose-fin width	0.8±0.5 (0.3-1.1)	3.2±0.9 (0.4-4.9)	2.0±0.1 (1.9-2.2)	2.2±0.1 (2.1-2.3)	3.0±0.1 (2.9-3.1)
Interorbital distance	10.6± (10.4-11.0)	10.3±1.1 (7.4-14.0)	13.7±1.3 (12.7-15.6)	13.8±1.1 (12.5-14.5)	13.7±0.6 (13.3-14.1)
Body width	14.5±0.9 (13.7-15.4)	15.5±1.4 (11.2-18.9)	16.9±0.4 (16.5-17.4)	18.2±1.0 (17.3-19.3)	17.8±0.9 (17.2-18.4)
Predorsal length	27.1±1.0 (26.0-28.0)	30.8±4.3 (12.2-41.2)	37.3±1.3 (35.5-38.5)	38.2±0.3 (37.9-38.5)	35.8±3.4 (33.4-38.2)
Prepectoral length	18.1±1.1 (17.0-19.2)	17.3±3.9 (6.0-24.5)	11.8±0.5 (11.5-12.5)	17.7±6.1 (10.8-22.2)	19.8±1.4 (18.8-20.8)
Prepelvic length	37.3±0.7 (36.5-37.7)	42.7±5.2 (6.4-51.7)	46.6±1.1 (45.2-47.9)	44.7±8.7 (34.8-51.1)	35.8±19.0 (22.3-49.2)
IN % HEAD LENGTH					
Snout length	40.0±1.5 (38.3-41.2)	21.5±5.1 (15.7-25.5)	39.1±2.8 (35.9-42.5)	41.7±5.1 (38.0-47.4)	44.5±2.2 (42.9-46.0)
Anterior snout width	38.4±2.6 (35.5-40.6)	35.2±2.5 (27.6-41.0)	27.0±1.2 (25.7-28.6)	31.1±5.5 (25.0-35.5)	34.8±0.5 (34.4-35.2)
Posterior snout width	49.4±1.3 (48.1-50.7)	46.5±3.8 (32.3-53.6)	35.6±1.8 (33.8-38.0)	41.3±7.7 (32.9-47.7)	46.5±1.7 (45.4-47.7)

Table 3.3: Continued

Characters	***Pangasius polyuranodon***	***Pseudolais micronemus***	***Pangasius* hybrid**	***Pangasius djambal***	***Pangasius bocourti***
	(n=3) 223.0-395.0	**(n=82) 72.0-406**	**(n=4) 385.0-429.0**	**(n=3) 375.0-495.0**	**(n=2) 460.0-500.0**
IN % HEAD LENGTH					
Eye diameter	24.2±3.1 (22.4-27.7)	25.9±3.6 (14.8-35.9)	11.7±0.6 (11.0-12.5)	13.3±0.8 (12.7-14.2)	13.5±1.4 (12.5-14.5)
Mouth width	50.8±7.7 (45.2-59.7)	44.4±4.2 (32.2-54.8)	39.7±7.3 (32.8-49.9)	45.0±6.2 (38.9-51.2)	41.2±1.9 (39.8-42.6)
Lower jaw length	30.0±3.4 (26.1-32.2)	28.1±5.1 (7.6-41.8)	27.5±4.1 (25.2-33.5)	30.8±5.5 (24.5-34.5)	27.0±7.2 (21.9-32.0)
Distance from snout to isthmus	58.2±3.8 (53.9-61.3)	59.3±3.6 (49.3-67.6)	54.0±5.0 (47.7-59.7)	55.1±1.8 (53.0-56.5)	50.0±0.7 (49.4-50.5)
Post Ocular length	38.8±9.2 (32.2-49.3)	40.7±9.9 (22.8-61.9)	49.6±9.0 (43.5-63.0)	41.3±2.2 (39.0-43.5)	38.3±2.1 (36.8-39.8)
Vomerine width	24.4±0.5 (23.8-24.8)	8.0±8.6 (0.2-29.6)	na	na	2.2
Vomerine length	28.4±9.4 (19.1-37.8)	1.7±2.0 (0.2-8.9)	na	na	0.9
Palatine length	19.2±6.3 (13.5-25.9)	3.2±4.1 (0.5-13.7)	na	na	1.3
Palatine width	12.2±11.8 (4.8-25.9)	1.3±1.6 (0.2-5.5)	na	na	0.6

Table 3.3: Continued

MERISTIC COUNTS	*Pangasius polyuranodon*	*Pseudolais micronemus*	*Pangasius hybrid*	*Pangasius djambal*	*Pangasius bocourti*
Anal-fin rays	38-40	21-40	28	28-30	26-31
Pelvic fin rays	6	6-7	5-7	6-7	6
Pectoral fin rays	13	10-16	10-11	8-10	7-10
Number of gill rakers on the first gill arch	22-28	12-24	19-23	24-35	40-46

Key to Genera

1a - Pelvic-fin rays 8-9, long predorsal length (> 37% SL) with slender dorsal spine width (3.5-5% HL) …….*Pangasianodon*

1b – Pelvic-fin rays 6 ……..…….……2

2a - Anterior part of snout, slender (<16.5% HL), mouth narrow, width lesser than 30% HL… …….....*Helicophagus*

2b - Anterior part of snout, robust (>16.5% HL), mouth wide, width more than 30% HL ……..…... 3

3a - Minute pair of barbels with mean maxillary 28.0±7.0 and mandibulary 13.2±5.7% HL from genus *Pangasius*, minute adipose fin …. *Pseudolais*

3b - Relatively long maxillary barbel, robust dorsal and pectoral fin, robust adipose fin ……..…….……*Pangasius*

Key to species of Pangasiidae in Peninsular Malaysia

1a - Premaxillary teeth in two quadratic patches, clearly separate at midline; palatal dentition consisting of two round or oval quadratic patches, snout elongate……..……..……*Helicophagus waandersii*

1b - Premaxillary teeth thin, undivided; palatal dentition consisting of 1,2, 3 or 4 tooth patches, if two patches the size never oval or round; snout broadly rounded, truncate or slightly elongate with wide mouth……..…….2

2a - Palatal dentition consisting of two palatal tooth bands, tooth band of upper jaw entirely covered when mouth is closed, swimbladder with one lobe……..…*Pangasianodon hypophthalmus*

2b - Palatal dentition with three or four tooth bands, swimbladder with more than one lobe……..……..……...…….3

3a - Palatal teeth with two palatine and two vomerine patches; mandibular barbel short, never reaching more than eye diameter (behind the eyes); maxillary barbel extending to or beyond posterior border of eye; swimbladder with three lobes..….....…….....*Pseudolais micronemus*

3b - Palatal teeth with median vomerine and lateral palatine tooth bands; swimbladder with two lobes……...…..……..……...4

4a - Eye diameter 16.0-30.3% HL, short head (width 9.7-13.9%, length 16.1-20.9% SL)…...…….……...…..…..……..…….……..…..……...5

4b - Eye diameter less than 22.8% HL, broad, rounded head (width 11.9-20.6% SL, length 21.9-28.8% SL)…….……..……...7

5a - Predorsal length short, 25.1-31.2% SL, short snout (21.0-43.7% SL), large median vomerine toothplates .....…..…….……..……..*Pangasius polyuranodon*

5b - Predorsal length longer 36.1-42.8% SL, head large and robust (length 23.8-28.8% SL), with pointed and projecting snout…….……6

6a - Strongly projecting and rounded snout with smaller eyes (41-49 x SL); inferior mouth; tooth band of upper jaw entirely exposed when mouth closed.....*P. nasutus*

6b - Less strongly projecting snout; subterminal mouth; large eye diameter (in specimens below 300 mm)……..……*P. conchophilus*

7a - Gill rakers on the first gill arch, 24 - 35…..……..*P. djambal*

7b - Gill rakers on the first gill arch, 40 – 46 ……...*P. bocourti*

Helicophagus Bleeker, 1858

Helicophagus Bleeker, 1858b:45 (type species *Helicophagus typus* Bleeker, 1858, by monotypy); Günther, 1864: 64; Weber & De Beaufort, 1913: 251; Hardenberg, 1948: Burgess, 1989: 105; Roberts & Vidthayanon, 1991: 138; Kottelat et al., 1993: 100; Rainboth, 1996: 152; Gustiano, 2003:34.

Diagnosis:

The genus *Helicophagus* differs from all other pangasiid genera with an elongate snout, a slender anterior part of snout length (< 16.5% HL); a narrow mouth (< 35% HL); short and a large premaxillary toothplate. It differs from from other species exists in Peninsular Malaysia, except for *P. conchophilus*, by the mollusc feeding preference.

Helicophagus waandersii Bleeker, 1858

(Figure 3.4)

Helicophagus waandersii Bleeker, 1858b: 175 (type locality Musi River, Palembang, Sumatra, Indonesia); Weber De Beaufort, 1913: 253, Figure 102; Roberts & Vidthayanon, 1991: 140, figures 1p, 24; Kottelat et al., 1993: 72, pl.35; Lim & Zakaria-Ismail, 1995: 37, Figure 1; Tan & Ng, 2000: 287.

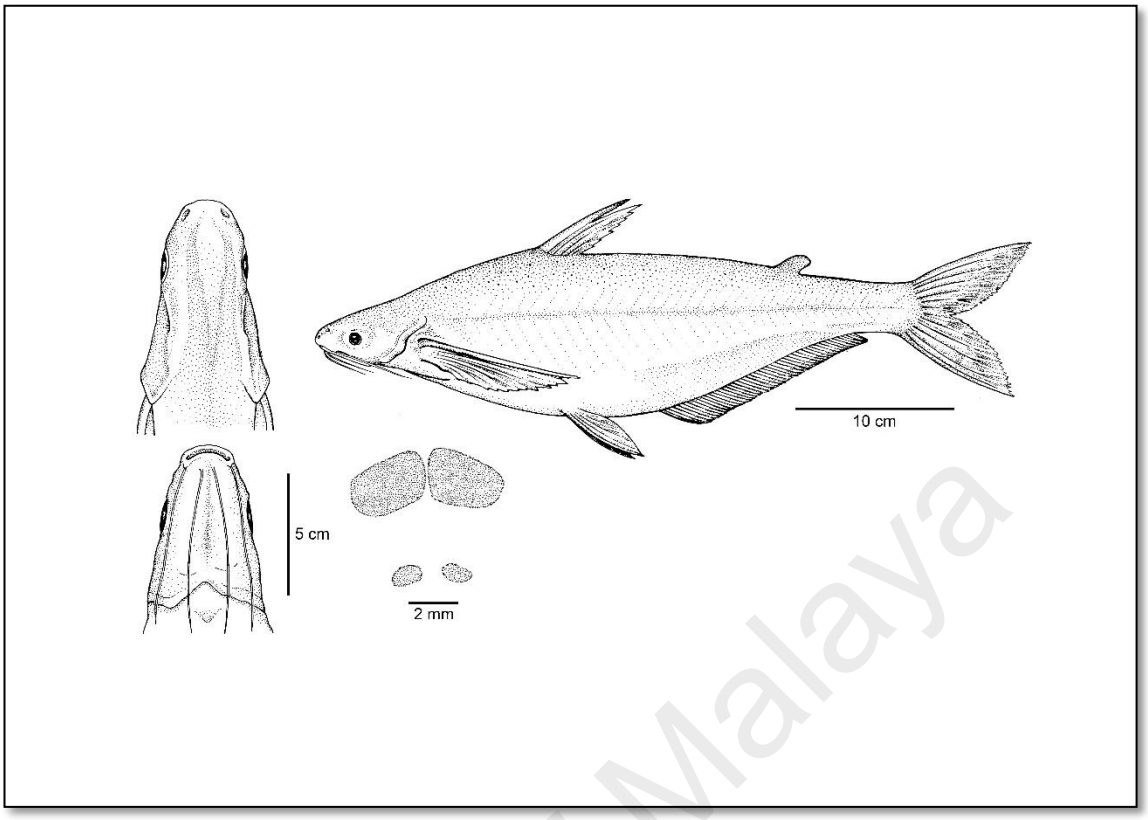


Figure 3.4: *Helicophagus waandersii* Bleeker, 1858. Lateral view of specimen (FRIGL LP2, 409 mm SL) with dorsal and ventral head features. Palatal dentition shown below figure.

Material examined

FRIGL 1024-1028 (5 ex.), 164.00-198.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 25 August 2004.

FRIGL 2002-2003 (2 ex.), 128.22-134.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 27 July 2005.

FRIGL 2007-2010 (4 ex.), 107.84-181.77 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 28 July 2005.

FRIGL 2011-2012, FRIGL 2026 (3 ex.), 113.53-120.15 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 29 July 2005.

FRIGL 2027, 1 ex., FRIGL 2030, 1 ex., FRIGL 2032, 1 ex., 107.48 – 129.06 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 20 Sept. 2005.

FRIGL LP2-LP4, 3 ex., 129.54-409.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. M. Zafri-Hasan, 22 July 2005.

BIRCUM 2266 (1-2) (AWB 34), 2 ex. 136.61-145.90 mm SL, Peninsular Malaysia: Pahang River Basin; Lower Pahang – P. Merah, 6-8 April 1993.

BIRCUM 1945 (AWB 20) 335.00 mm SL. Peninsular Malaysia: Pahang River Drainage; Sg Tembeling near Jeram Pai, 19 Feb 1993.

BIRCUM 2048 (AWB 3) 300.00 mm SL. Peninsular Malaysia: Pahang River Drainage; Sg Pahang near Kg Perian and Jerantut Ferry, 3 Nov 1992.

ZRC 11906 280.00 mm SL. Peninsular Malaysia with no exact locality, C. F. Lim, no date, possibly in the 1970s.

Description:

This is the only species from the genus *Helicophagus* identified in Peninsular Malaysia. This hard-to-get species commands a very high price in the market. It is an esteemed food fish to the locals of the Pahang River Basin (Lim & Zakaria-Ismail, 1995).

Specimens in this study resembled wider range of anal-fin rays (32-38) compared to 35-36 in Lim & Zakaria-Ismail (1995) but still in range of 38-42 as defined in Roberts & Vidthayanon (1991).

Distribution:

Occurs in Pahang River Basin only (Lim & Zakaria-Ismail, 1995). From this study, specimens were collected from the lower and middle reach of Pahang River, with the upper reach (Sg. Tembeling) from previous collections (Figure 3.5).

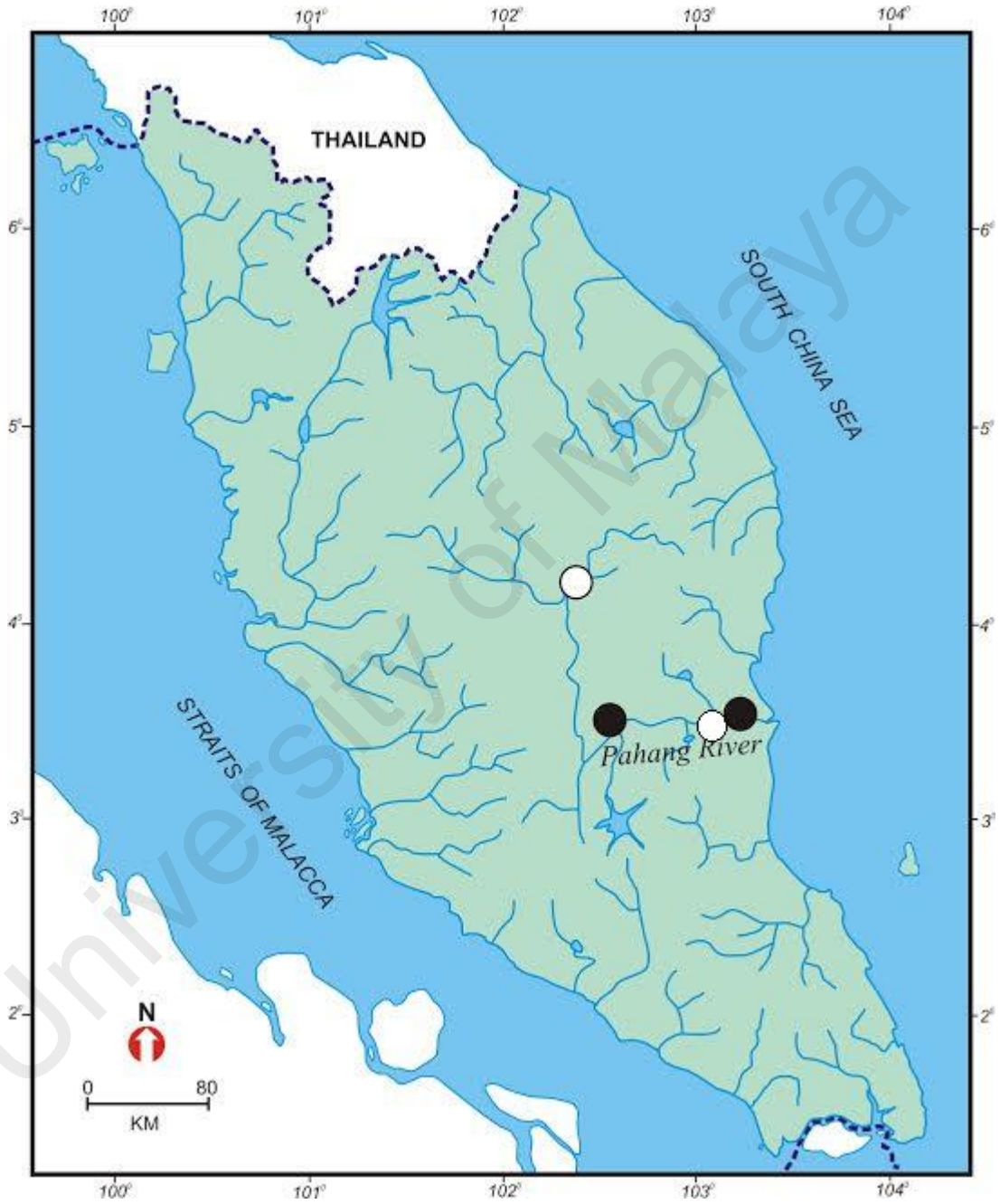


Figure 3.5: Map of Peninsular Malaysia, showing current (●) and historical record of *Helicophagus waandersii* (○)

Pangasianodon Chevey, 1930

Pangasianodon Chevey, 1930: 536, Fig. 1, 2 (type *Pangasianodon gigas* Chevey, 1930; no type designated); Smith, 1945: 372; Rainboth, 1996: 153.

Pangasius (Pangasianodon) Roberts & Vidthayanon, 1991: 102; Vidthayanon, 1993: 160. Gustiano, 2003: 51.

***Pangasianodon hypophthtalmus* (Sauvage, 1878)**

(Figure 3.6)

Pangasianodon gigas Chevey, 1930: 536, Figure 1, 2 (type locality Cambodia; no type designated) Smith, 1945: 372; Burgess, 1989: 105; Rainboth, 1996: 153.

Pangasius paucidens Fang & Chaux, 1949: 344, Figure 6 (type locality Cambodia).

Pangasius (*Pangasianodon*) *gigas*: Roberts & Vidthayanon, 1991: 102; Vidthayanon, 1993: 22. Gustiano, 2003: 59.

Material examined:

FRIGL 1009 (1 ex.), 396.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 31 July 2004.

FRIGL 2049, (1 ex.), 305.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 28 Nov 2005.

FRIGL 2056, (1 ex.), 432.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 29 Dec 2005.

FRIGL 2064, (1 ex.), 297.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 24 Jan 2006.

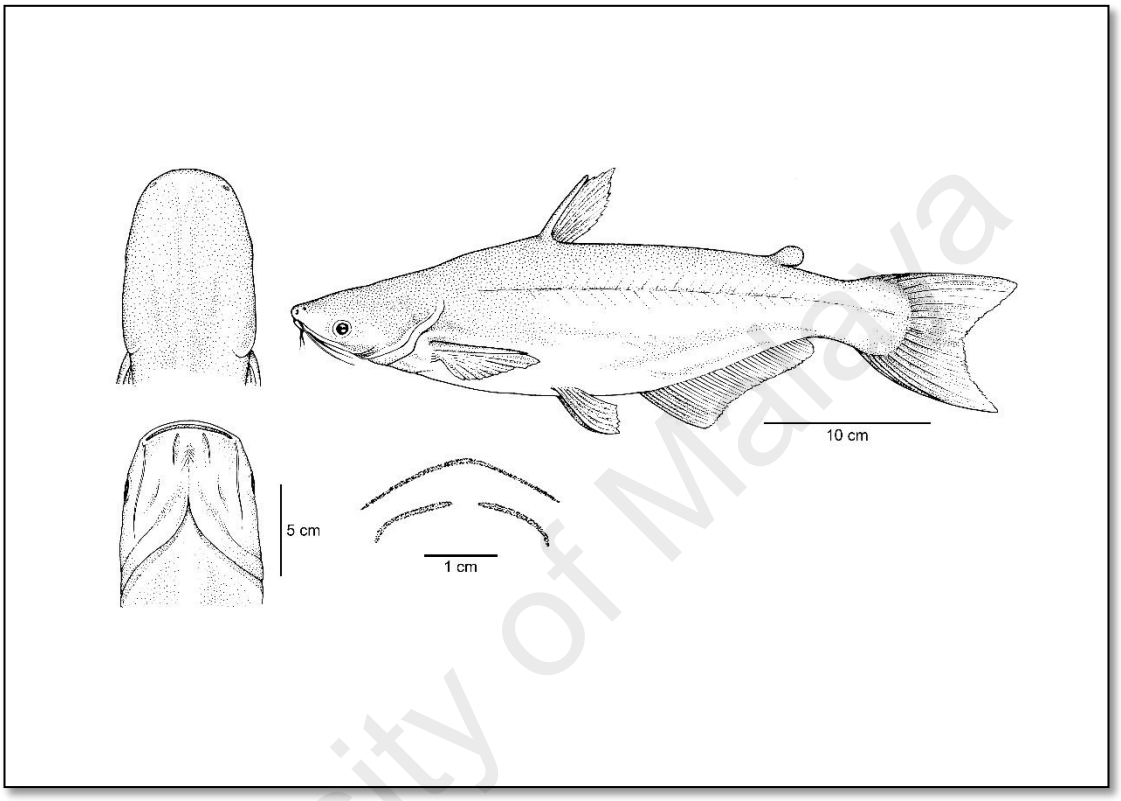


Figure 3.6: *Pangasianodon hypophthtalmus* (Sauvage, 1878). Lateral view of specimen FRIGL 2064, 297 mm SL with dorsal (top left) and ventral (below left) head features. Palatal dentition shown below figure.

Diagnosis:

The specimens were presumably thought to be escapees from the cultured cages. All specimens have 9 pelvic fin rays and this make it differs from other Pangasiid species which have only 6. The oral dentition of our specimen follows the description of Roberts & Vidthayanon (1991) with the palatine and vomerine plates of each side are more or less perfectly joined into a single curved tooth plate. The vomerine portions are widely separated at the midline as shown in Figure 3.6.

Distribution:

Endemic to Mekong River basin, but widely introduced around the world as an aquaculture species, while their juveniles are sold as ornamental specimens. Specimens also collected from the Perak River, but due to the huge size of specimen, no voucher were kept. Only DNA samples were taken and analysed in Chapter 5 and 6 (see list of complete samples, see Appendix).

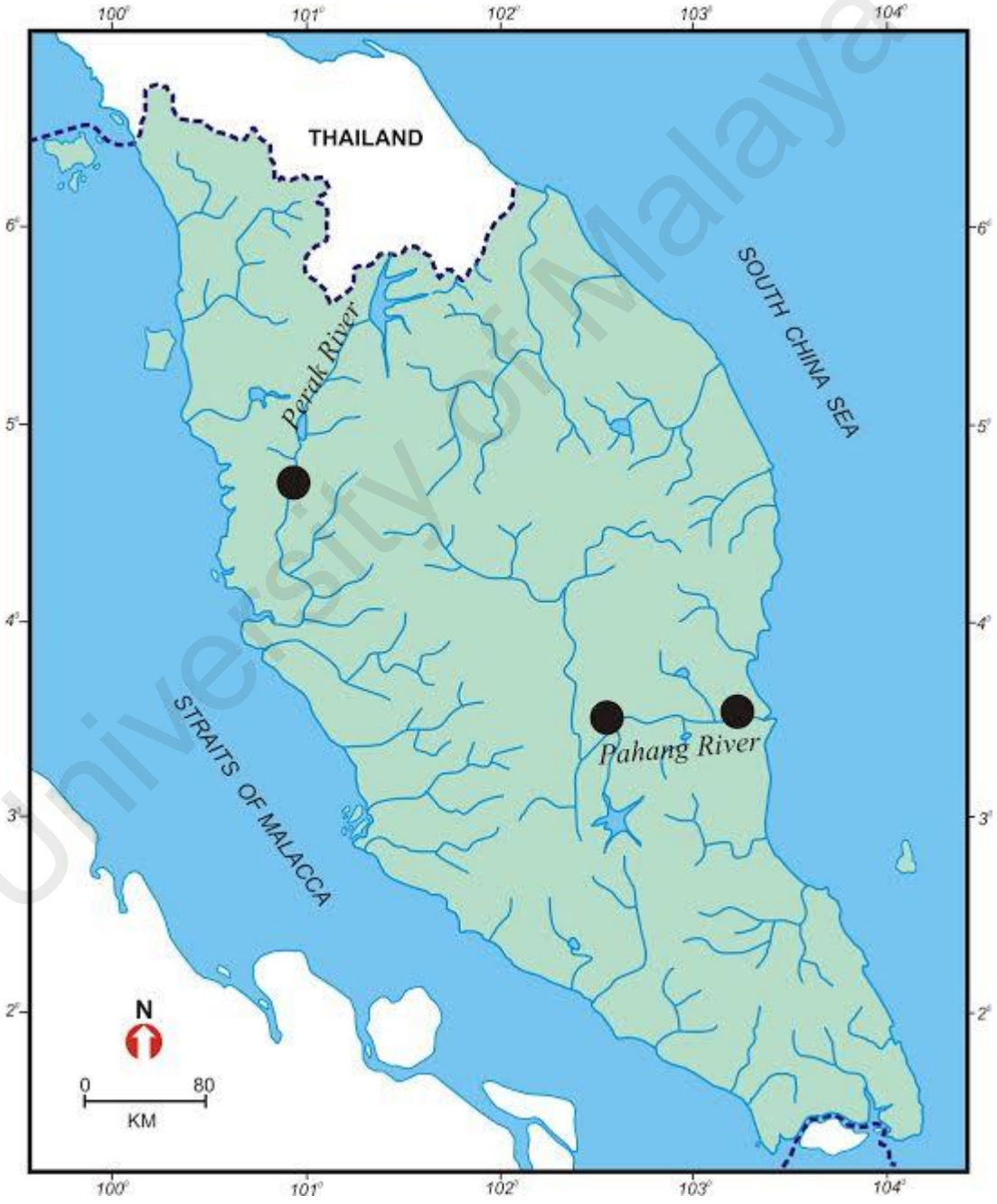


Figure 3.7: Map of Peninsular Malaysia, showing current (●) records of *Pangasianodon hypophthalmus*.

Pangasius bocourti Sauvage, 1880

(Figure 3.8)

Pangasius bocourti Sauvage, 1880: 229 (type locality Phnom Penh, Cambodia); Sauvage, 1881: 170; Kottelat, 1984: 812; Roberts & Vidthayanon 1991: 112; Vidthayanon, 1993: 47; Rainboth 1996: 154. Gustiano, 2003: 173.

Pangasius altifrons Durand, 1940: pl. 5 (type locality Tonle Sap Lake, Cambodia)

Material examined:

FRIGL 1008 (1 ex.), 460.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 31 July 2004.

FRIGL 1055 (1 ex.), 495.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 30 Nov 2004.

Description:

Our specimens have a stout and heavy body, follows the same description of Rainboth (1996). The unique characteristic that differentiates this species is that the high counts of gill rakers from the first gill arch. One specimen (FRIGL 1008) has 41 gill rakers, while the other one (FRIGL 1055) got 36 gill raker count. Another species with higher gill raker counts (more than 37) is *P. macronema*, but the different is that *P. bocourti* has a broader, more rounded head (Figure 3.8) following the description of Roberts & Vidthayanon (1991:104). Snout round, not truncate.

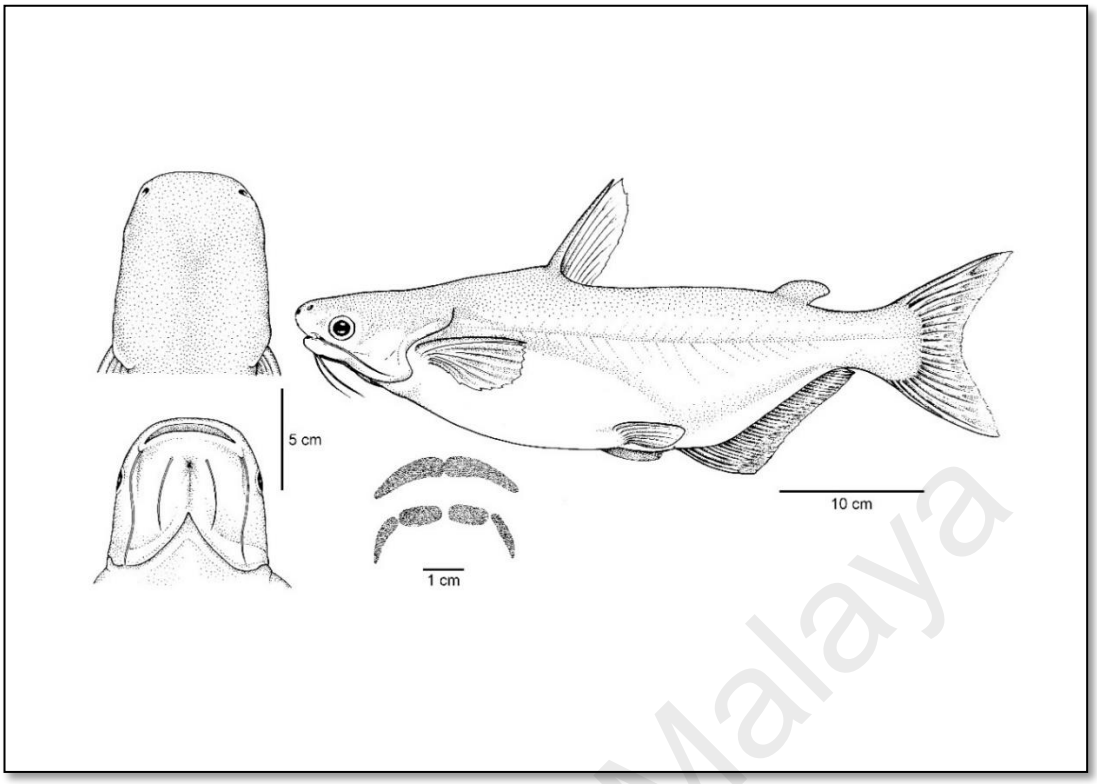


Figure 3.8: *Pangasius bocourti* Sauvage, 1880. Lateral view of specimen FRIGL 1055, 495 mm SL with dorsal (top left) and ventral (below left) head features, with palatal dentition shown below figure.

The morphologically similar species, *P. djambal* an important aquaculture species in Indonesia, has fewer gill raker counts (24-35). This species has two chambers of swimbladder. The dorsal fin is without filamentous extensions.

Distribution:

This is the first record of *P. bocourti* from Peninsular Malaysia, both specimens were from the lower reach of Pahang River (Figure 3.9). Further surveys and comparative specimens are needed to confirm this finding.

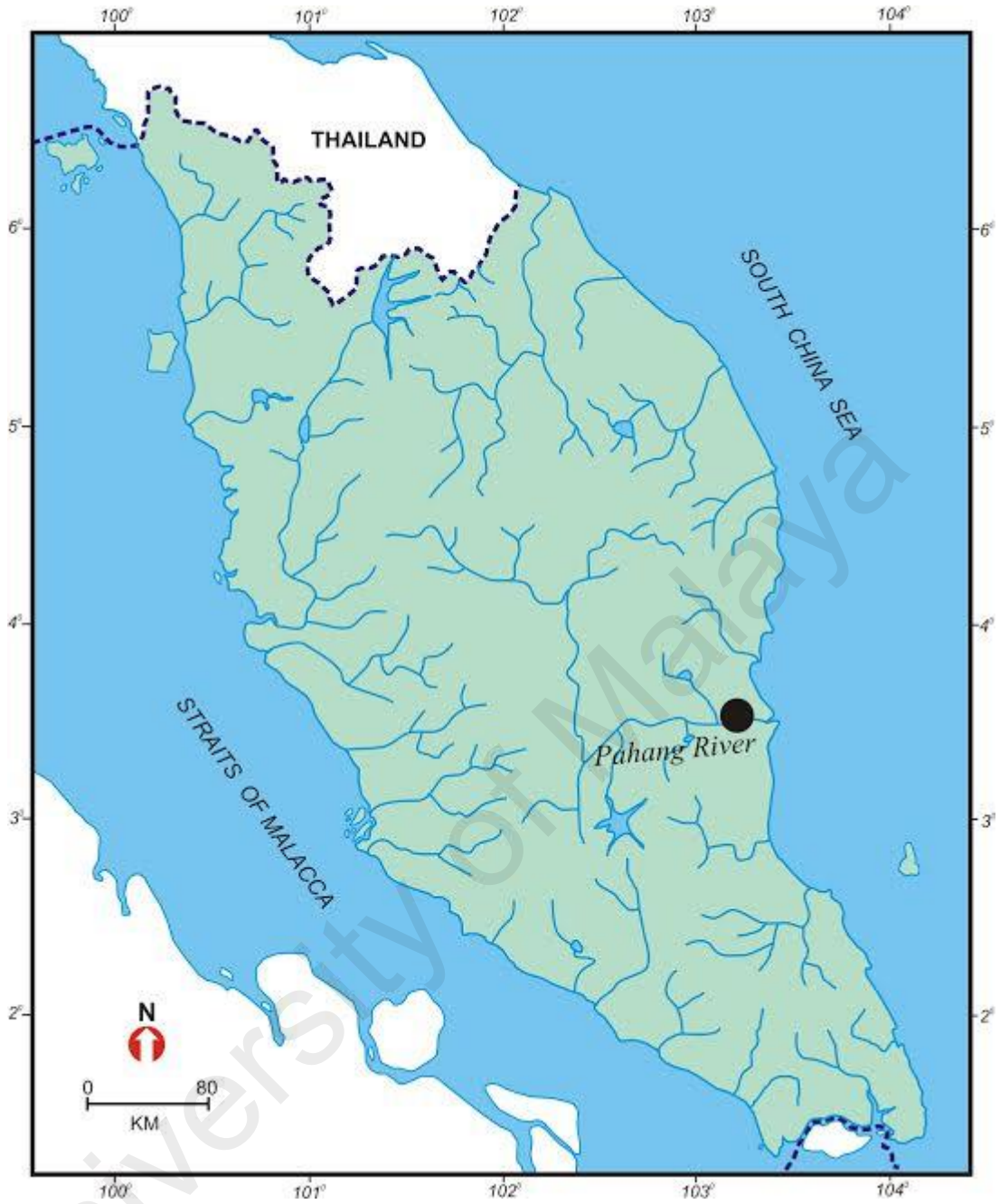


Figure 3.9: Map of Peninsular Malaysia, showing current (●) record of *Pangasius bocourti*.

***Pangasius conchophilus* (Roberts & Vidthayanon, 1991)**

(Figure 3.10)

Pangasius conchophilus Roberts & Vidthayanon, 1991: 114, Fig 5 (type locality, Mekong River, at Thabo, Nongkhai Province, Thailand). Vidthayanon, 1993: 41; Rainboth 1996: 154. Gustiano, 2003: 190.

Pangasius nasutus non Bleeker, 1962: Smith, 1945: 362

Material examined:

FRIGL 1023 (1 ex.), 313.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 25 August 2004.

LP1-2, (2 ex.), 261-479.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. M.Zafri Hassan, 22 July 2008

HB 151208, 171208, (2 ex.) 360-460 mm SL. Peninsular Malaysia: Pahang River Basin; Kampung Chenor; Maran, coll. B. Haslawati, 15-17 Dec 2008.

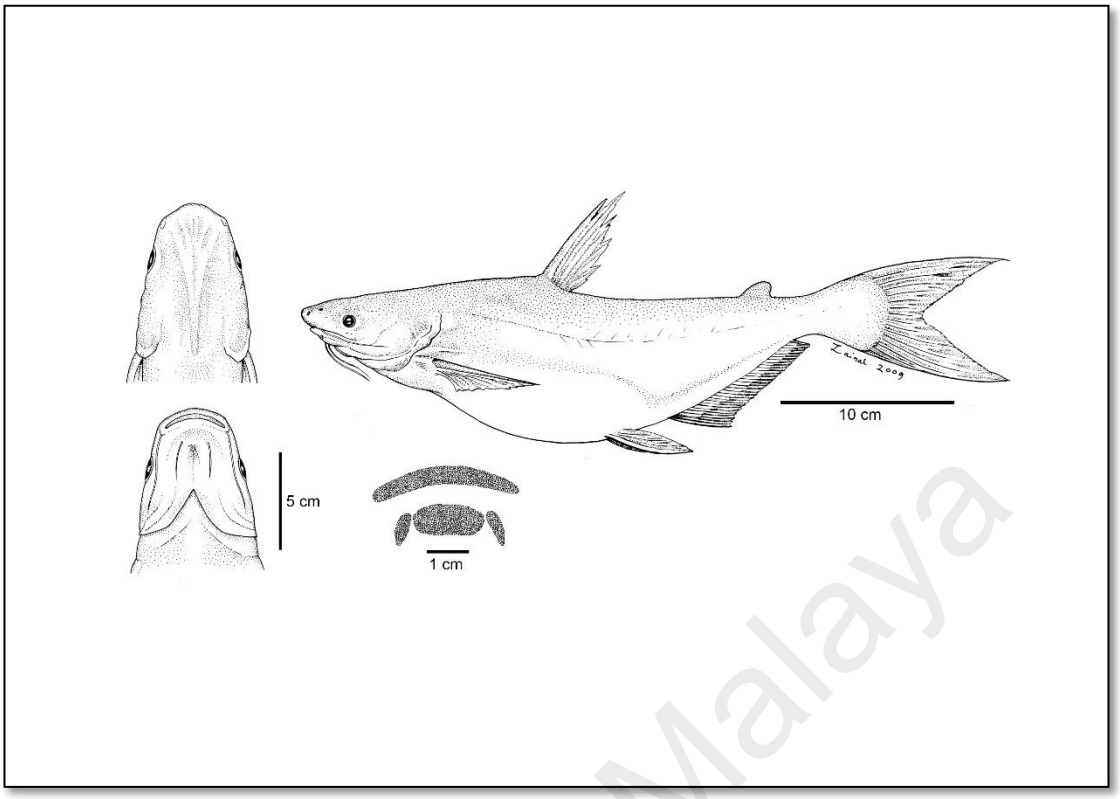


Figure 3.10: *Pangasius conchophilus* (Roberts & Vidthayanon, 1991). Lateral view of a specimen FRIGL 1023, 313 mm SL. Dorsal (top left) and ventral view of the head (below left) with palatal dentition.

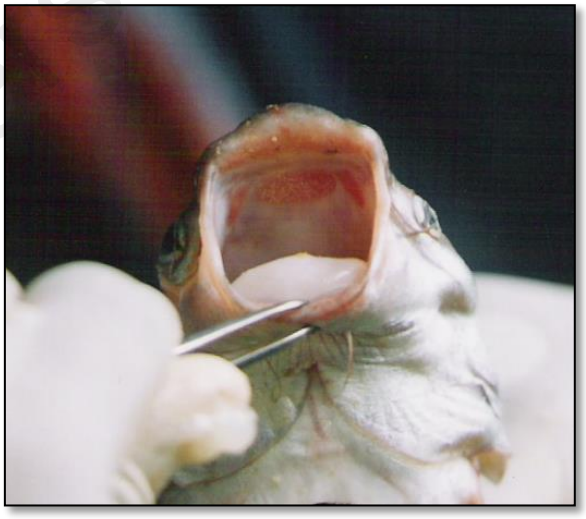


Figure 3.11: A closer look on the dentition of *P. conchophilus*

Description:

One specimen was obtained from a cage culturist within the vicinity of the study area, in Paloh Hinai, Pekan, Pahang. It is called locally as Patin Buah Kemboja since the appearance more or less resembled those of the local Patin Buah (*P. nasutus*) but cultured by the Cambodian immigrants. The fry were originally imported from Cambodia through Thailand and widely cultured in the cages along the Pahang River especially by the Cambodians.

Pangasius conchophilus differs from all other *Pangasius* known from Thailand and Indo-China except *P. bocourti* and *P. polyuranodon* by having large median vomerine toothplates (Figure 3.10 and 3.11). Another characteristic of having pointed snout in this species would lead to misidentification of this species to *Pangasius nasutus.* However, by direct comparison, *P. nasutus* has a more strongly projecting and rounded snout with smaller eyes (specimen with 295 mm SL has an eye diameter of 21.7 mm - 13.6 times in SL compared to the *P. conchophilus* (38 times SL)).

According to Roberts & Vidthayanon (1991), this species is a large, fast growing species and perhaps the most important Pangasiid in Chao Phraya and Thai Mekong. Therefore, it is no doubt that the fry of this species being imported from Cambodia for cage culture when considering this aquaculture potential.

Distribution and ecology:

This migratory species is found in Mekong River Basins. Feed mainly on molluscs and crustaceans (Roberts and Vidthayanon, 1991). In the Mekong Basin, they can migrate into the Middle Mekong, along the Thai-Lao border (Rainboth, 1996).

In this study, the samples were collected from Pahang River, from the lower reaches and then further upstream, Lubok Paku and Chenor (Figure 3.12).

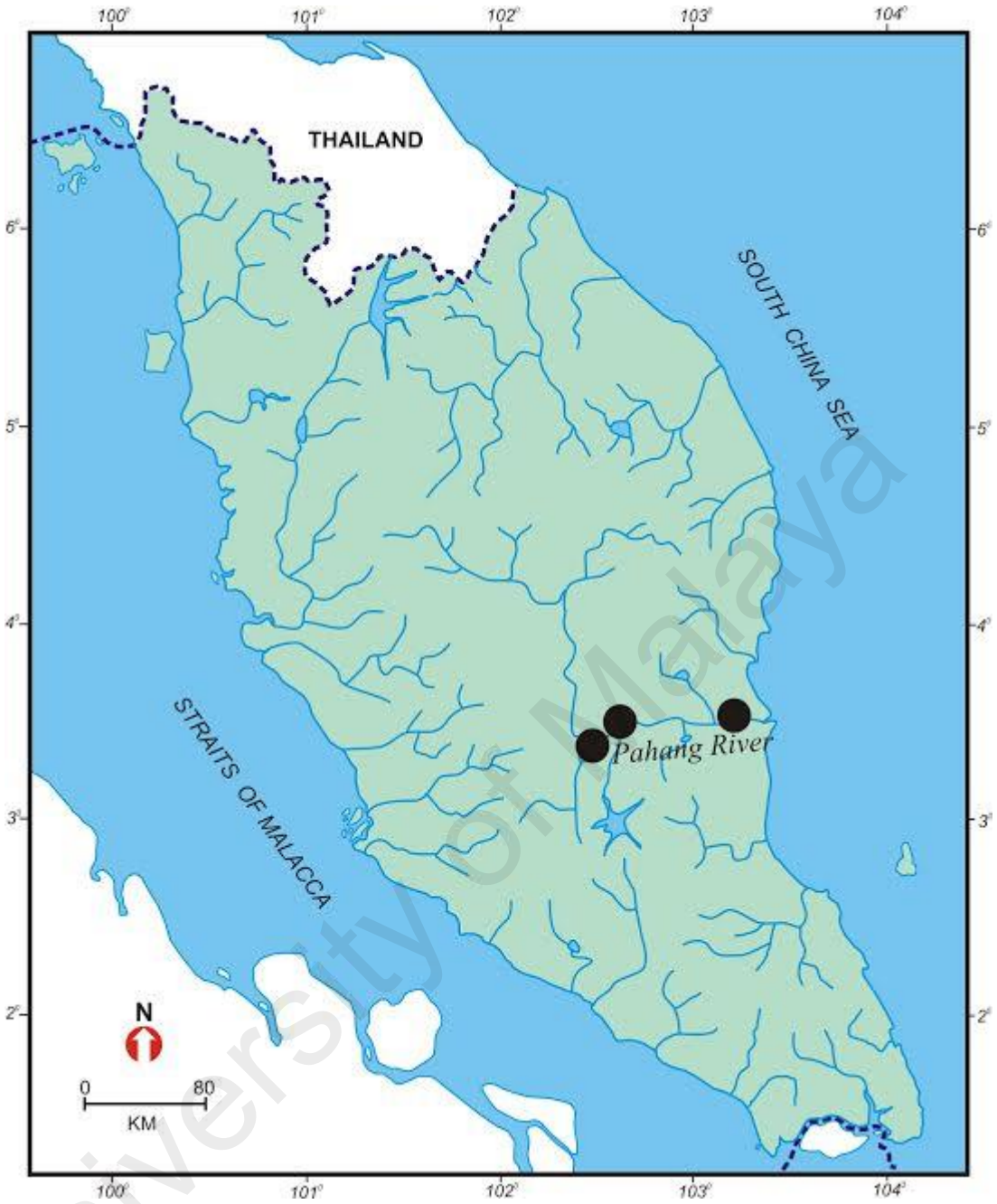


Figure 3.12: Map of Peninsular Malaysia, showing current (●) records of *Pangasius conchophilus*.

***Pangasius djambal* Bleeker, 1846**

(Figure 3.13)

Pangasius djambal Bleeker, 1846: 290 [type locality Java, Batavia (presently Jakarta), Indonesia]; Gunther; 1864: 62; Roberts & Vidthayanon, 1991: 116. Gustiano, 2003: 177. *Pangasius bedado* Roberts, 1999: 109 (type locality Musi River, Sumatera, Indonesia)

Material examined:

FRIGL 1053 (1 ex.), 500.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 27 Oct 2004.

FRIGL 1054 (1 ex.), 405.00 mm SL, Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 30 Nov 2004.

MZB 2958, holotype of *P. bedado* Roberts, 375 mm SL, Musi River, Sumatra, Indonesia, coll. T. Roberts, July 1999. (comparative specimen).

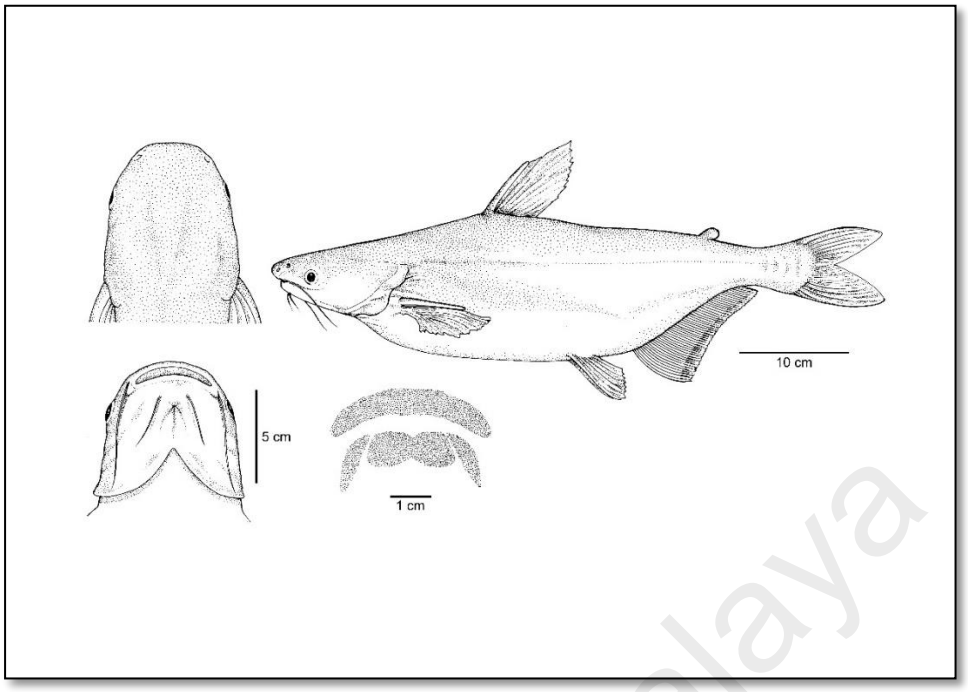


Figure 3.13: *Pangasius djambal* Bleeker, 1846. Lateral view of specimen FRIGL 1054, 405 mm SL. Dorsal (top left) and ventral head view (below left) with palatal dentition.

Description:

This specimen has a rounded, truncate snout following the description of Roberts & Vidthayanon (1991:116) for *P. djambal* (Figure 3.13). It is morphologically very similar to *P. bocourti* but the counts of gill rakers from the first gill arch from these specimens (24 and 32) are lower compared to *P. bocourti*'s (36-46) (Roberts & Vidthayanon (1991:113)**,** but within range of Gustiano's specimens (27-39) (Gustiano, 2003). This is a probable new record for Peninsular Malaysia, found in Pahang River, if it could be confirmed as a *P. djambal*.

Pangasius djambal is an important aquaculture species in Indonesia, has fewer gill rakers (24-35), compared to *P. bocourti*. Anal-fin rays, 28 and 30 (27-35 in Gustiano (2003). Anterior snout width 25.02-35.51 %HL. Subterminal mouth, width less than 10% SL. The dentition with two palatal teeth and moderately large vomerine patch follows Roberts & Vidthayanon's drawing (1991:106).

Mouth wide, width more than 30% HL (38.93 and 51.24% HL). Mandibular barbel length less than head length. Palatal teeth enlarged in 3-4 patches. Longer predorsal length 37.94-38.46% SL compared to *P. bocourti* (33.38-38.23% SL in this study. Snout length inferior or equal to distance from snout to isthmus.

Distribution:

Figure 3.14: Map of Peninsular Malaysia, showing current (●) records of *Pangasius djambal*.

Remarks:

According to the local fisheries assistant, with information from the local fishermen, the fry of *P. djambal* or *P.bocourti* are normally collected from the natural habitat prior to culture in cages. It is quite suspicious whether the species occurs naturally in the river, or are introduced from either Indo-China (*P. bocourti*) or Indonesia (*P. djambal*). There are slight differences between the physical appearance, morphological and meristic counts also close within the range (gill rakers) and fin counts.

We caught a specimen of 460 mm standard length, identified as *P. bocourti* using a drift gill net. Another specimen of 735 mm standard length (Figure 3.15) was also caught near the study area. According to the morphological appearance and dentition, this specimen was presumed to be *P. bocourti* but were not be able to be brought back and preserved due to the large size and weight. The question that arises is, whether the specimens occur naturally or if they are escapees from the cages. Thus, more samples need to be collected to clarify their status.

Figure 3.15: A 735 mm *P. bocourti* specimen caught with drift gill net from Paloh Hinai, Pekan, lower reach of Pahang River.

***Pangasius nasutus* (Bleeker 1863)**

(Figure 3.16)

Pseudopangasius nasutus Bleeker, 1862: 72 (type locality Barito River, Banjarmasin, South Kalimantan, Indonesia); Gunther, 1864: 63; Weber & De Beaufort, 1913: 256; Burgess 1989: 105; Roberts 1989: 133; 73; Tan & Ng 2000: 288. Gustiano, 2003: 194.
Pangasius ponderosus Herre & Myers, 1937 [Herre & Myers, 1937: 67, pl. 6 (type locality Chandra Dam (presently Chenderoh Dam), Perak, Malaysia)]

Materials examined:

FRIGL 1010-1022, 8 ex. 142.50-337.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 24-25 Aug 2004.

FRIGL 1049-1100, 11 ex. 142.50-337.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 26 Oct-21 Dec 2004.

FRIGL 2001, 2004, 2005, 3 ex. 340.00-470.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 26-28 July 2005.

FRIGL 2022, 2031, 2065, 3 ex. 170.00-340.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 25-29 Aug 2005.

HBCR 01-05, 5 ex., 384-506 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Chenor, Maran; 31 Aug 2009 – 2 Sept 2009.

NMS 2860, 1 ex., 694.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kuala Tahan, Pahang. *Pangasius ponderosus.* Coll. C.S. Ogilvie. Mac 1949.

ZRC 8810, 305.00 mm SL. Peninsular Malaysia. No collector name, determined by Kelvin Lim. Dec 1992.

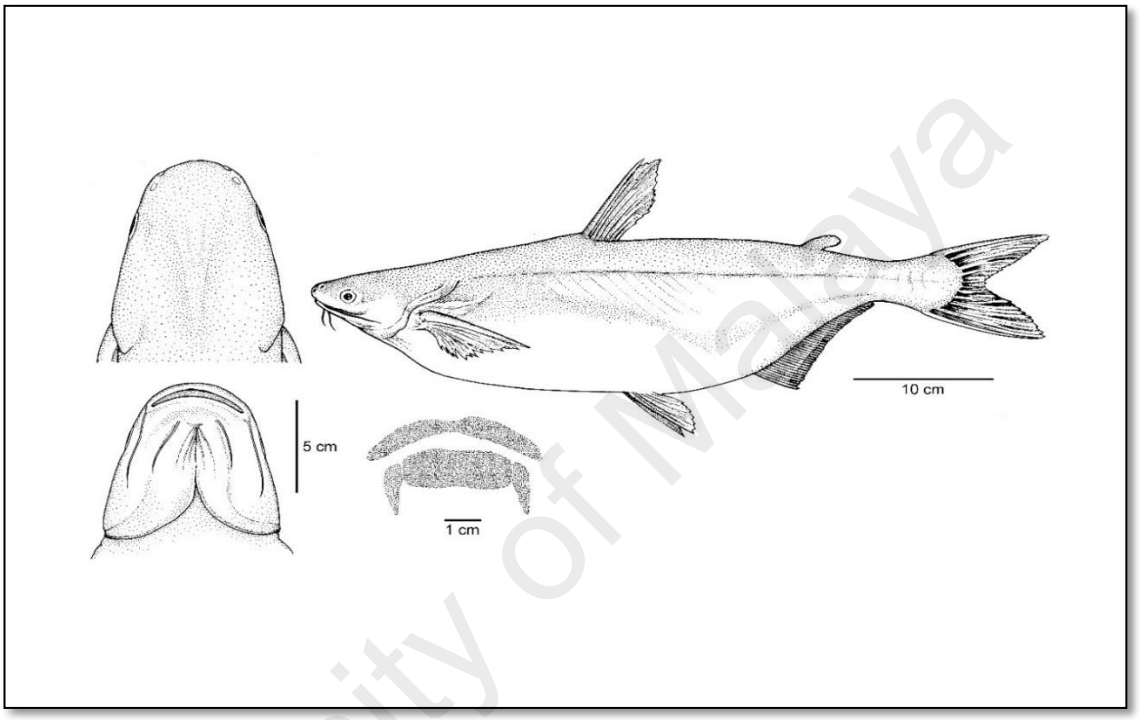


Figure 3.16: *Pangasius nasutus* (Bleeker 1862). Dorsal and ventral head view with palatal dentition.

Figure 3.17: Palatal toothbands of *P. nasutus*

Description:

Pangasius nasutus is distinguished from all other *Pangasius* species by having an inferior mouth with strongly projecting snout. The tooth band of upper jaw entirely exposed when jaws are closed. In large adults, the snout becomes rounded like most of our collections. Eye diameter is much smaller (14 times in SL) than in other specimens. Gill rakers on first gill arch are 14-19 (vs 17-21 in Roberts & Vidthayanon, 1991). Palatal teeth with large median vomerine tooth patch and smaller palatine tooth bands (Figure 3.16 and 3.17).

Distribution:

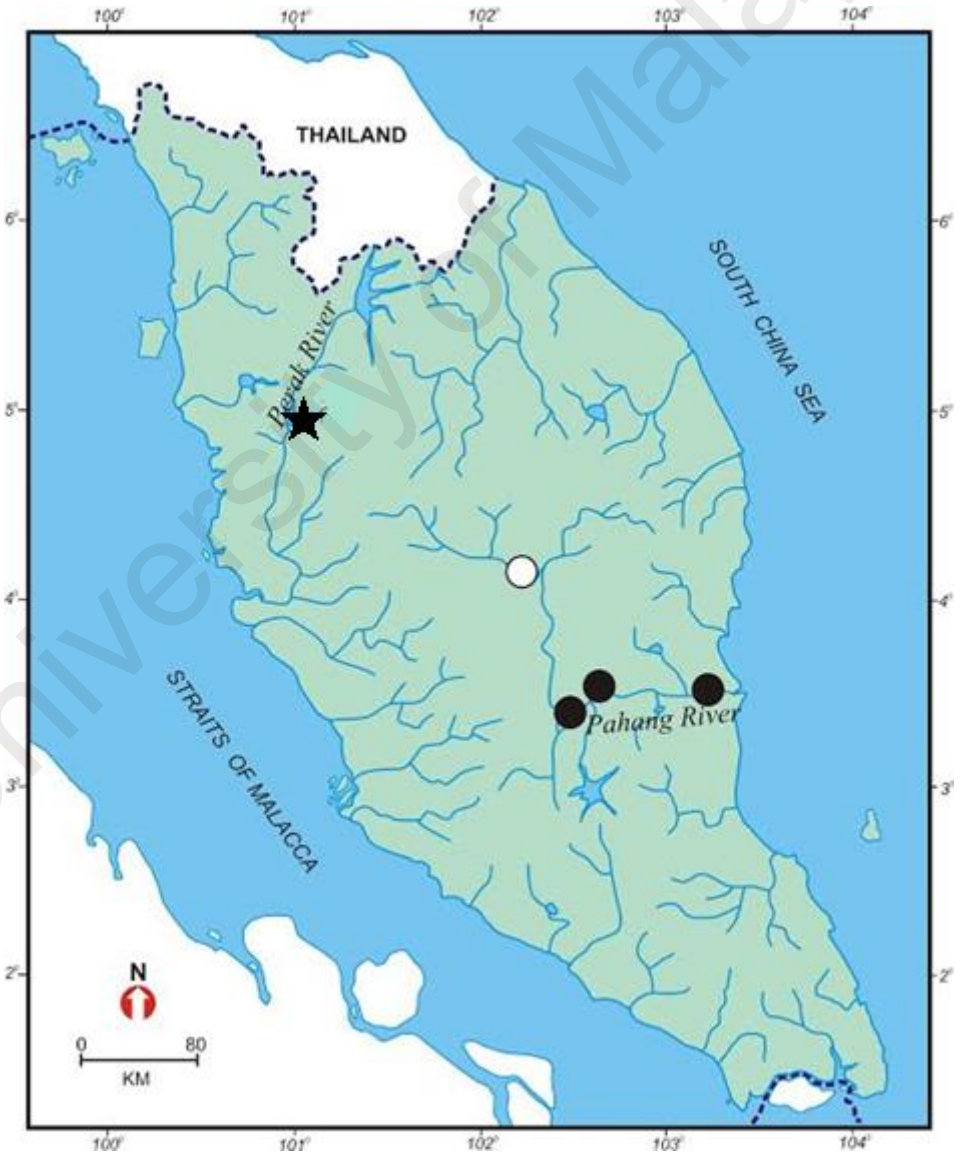


Figure 3.18: Map of Peninsular Malaysia, showing current (●) and historical record of *Pangasius nasutus* (○). Holotype locality of *Pangasius ponderosus* Herre & Myers 1937 (★)

Pangasius polyuranodon Bleeker 1852

(Figure 3.19)

Pangasius polyuranodon Bleeker, 1852: 425 (type locality Barito River, Banjarmasin, South Kalimantan, Indonesia); Weber and De Beaufort, 1913: 257; Roberts, 1989: 133; Kottelat et al., 1993: 102; Tan & Ng 2000: 288. Gustiano, 2003: 137.

Pangaisius juaro Bleeker, 1852: 589 (type locality, Palembang, South Sumatra, Indonesia)

Materials examined:

UMKL (POLY 01), 223.18 mm SL UMKL (POLY 02). 395.00mm SL. Peninsular Malaysia: Pahang River Basin, exact location unknown, dated 1992-1993.

MZB 3681, 284.5 mm SL; Indonesia: Kalimantan: Kapuas Basin; Sintang, coll. T.R. Roberts (Roberts & Vidthayanon, 1991: 136). Comparative specimen.

Description:

Roberts & Vidthayanon (1991) stated that *P. polyuranodon* is an elongate species that could achieve 800 mm in standard length (see Vidthayanon 1993), while maximum size observed by Gustiano (2003) was 602 mm SL. *Pangasius polyuranodon* can be distinguished from all congeners by the combination of a short predorsal length (25.2-29.2% SL) and a long caudal peduncle (17.2-21.8% SL) (Pouyaud et al. 2002). This species has a distinctive palatal dentition (Figure 3.19c) which consists of a large nearly squared median vomerine tooth patch with small lateral palatine toothplates, while *P. micronemus* has smaller and separate palatal tooth patches. The number of gill rakers on first gill arch is 19-30. Anal-fin rays are 33-42 (Gustiano 2003).

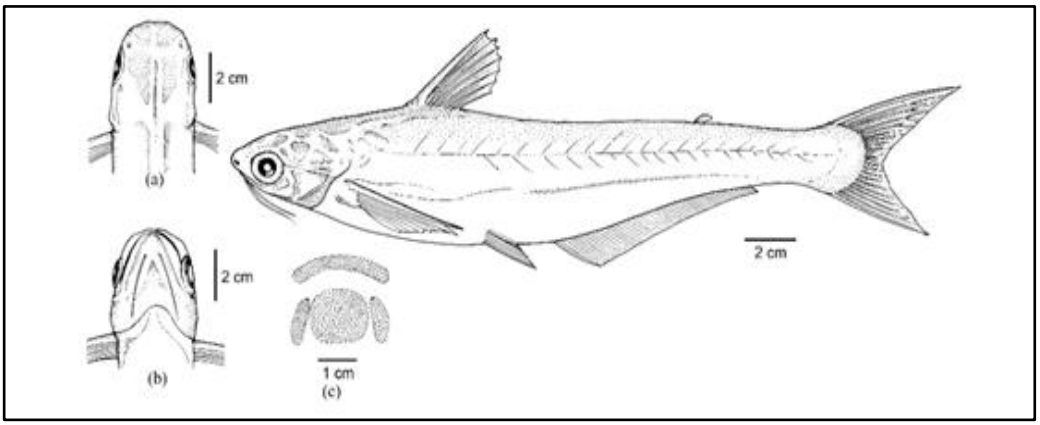


Figure 3.19: *Pangasius polyuranodon* Bleeker 1852, UMKL (POLY 02). 395 mm SL with (a) dorsal and (b) ventral head view and (c) maxillary and palatal dentition.

Distribution and ecology:-

Further details of *P. micronemus* and *P. polyuranodon* will be discussed in Chapter 4.

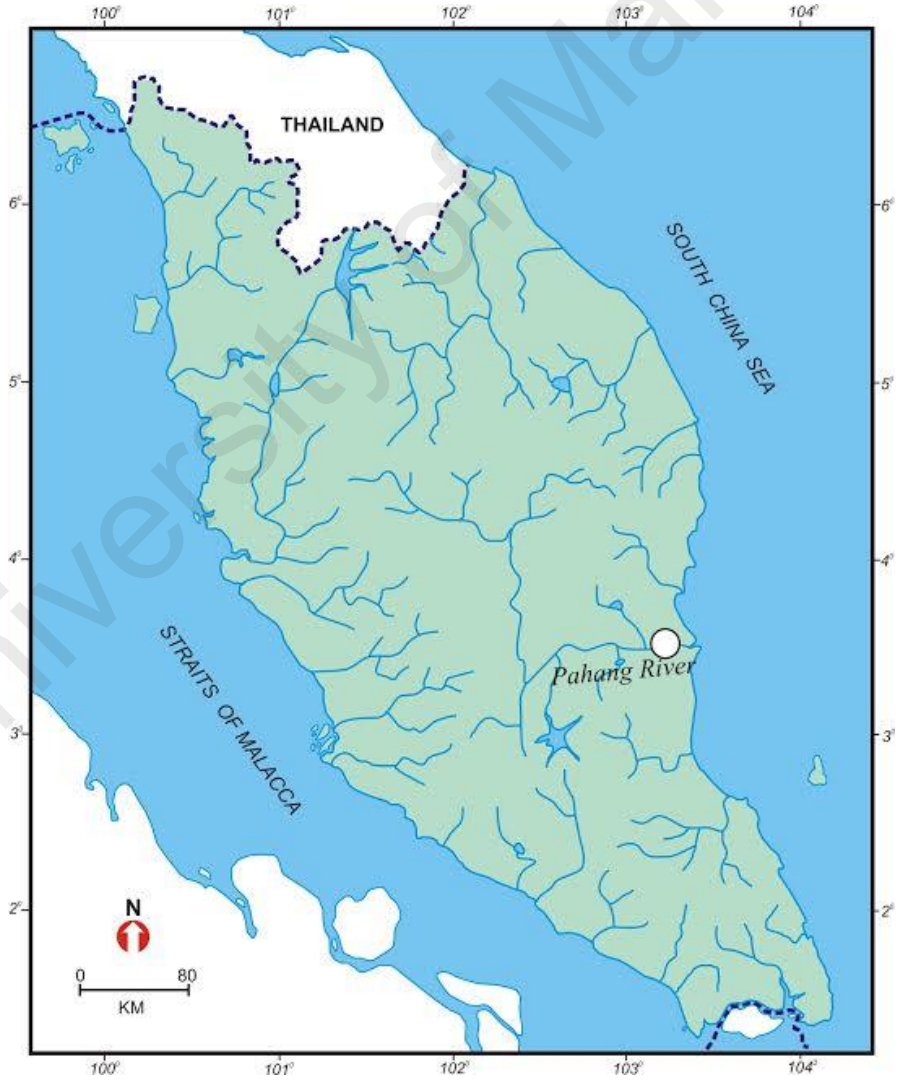


Figure 3.20: Map of Peninsular Malaysia, showing historical record for *Pangasius polyuranodon* (○)

***Pseudolais micronemus* (Bleeker, 1847)**

(Figure 3.21)

Pangasius micronemus Bleeker, 1847: 8 (type locality, Solo River, Java, Indonesia); Gunther, 1864: 63: Weber & De Beaufort, 1912: 535; Roberts, 1989: 132; Roberts & Vidthayanon, 1991: 129; Kottelat, 1993: 101; Tan & Ng, 2000: 288.

Pangasius micronema: Weber & De Beaufort, 1913: 261; Roberts, 1989: 132; Roberts & Vidthayanon, 1991: 129; Rainboth, 1996: 156.

Pangasius rios Bleeker, 1851: 205 [type locality Bandjermasing (presently Banjarmasin), South Kalimantan, Indonesia].

Pseudolais tetranema Vaillant, 1902: 52 (type locality MahakamRiver at Tepoe, East Kalimantan, Indonesia).

Pangasius dezwaani Weber & De Beaufort, 1912: 14, Figure 3 (type locality Taluk, Sumatra, Indonesia).

Pangasius hoeksi Hardenberg, 1948: 412 (type locality Kapuas River, West Kalimantan).

Pangasius tubbi Inger & Chin, 1959: 287. Fig 47 (Type localitythe confluence of the Deramakot River with Kinabatangan River, Kinabatangan District, Sabah (North Borneo), Malaysia

Pteropangasius micronemus (Bleeker, 1847) Gustiano, 2003: 74.

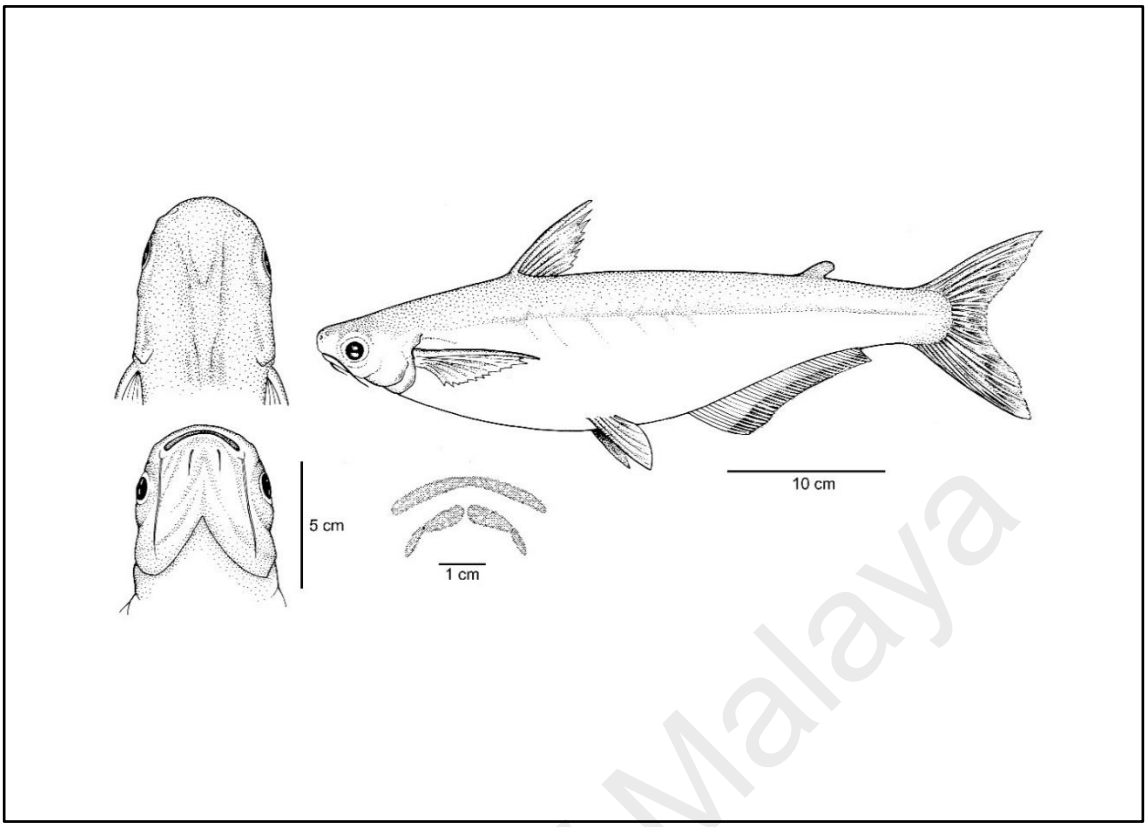


Figure 3.21: *Pseudolais micronemus* (Bleeker, 1847). Dorsal and ventral head view with palatal dentition.

Materials examined:

FRIGL 1004, 19 ex. 72.72-335.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 27 July - 21 December 2004.

FRIGL 1005, 27 ex. 14.30-364.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 28 July 2005 - 26 January 2006.

FRIGL 1006 (1), 3 ex. 334.00-393.00 mm SL, Peninsular Malaysia: Perak River Basin: Kuala Kangsar, Kg Manong. 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 2 June 2008.

FRIGL 1007. 3 ex.; 1007 (1) 366.00 mm SL. Peninsular Malaysia: Perak River Basin: Kuala Kangsar, Kg Manong; 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 31 August 2008.

FRIGL 1007 (2), 2 ex, 336.00-406.00 mm SL. Peninsular Malaysia: Perak River Basin: Kuala Kangsar, Kg Manong; 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 28 September 2008.

618, 278.82 mm SL. Peninsular Malaysia: Kelantan River Drainage Sg Galas, near Kg Lulut, MZI, 11 Sept 1984.

BIRCUM 1944, 334.00 mm SL Peninsular Malaysia: Pahang River Basin: Sg Tembeling near Jeram Pai, coll. AWB, 19 Feb 1993.

BIRCUM 1952, 343.00 mm SL: Peninsular Malaysia: Pahang River Basin; Sg Tembeling near Jeram Pai, coll. AWB, 19 Feb 1993.

BIRCUM 1972, 311.00 mm SL, Peninsular Malaysia: Pahang River Basin; no exact locality, coll. AWB. BIRCUM 1723, 250.19 mm SL. Peninsular Malaysia: Pahang River Basin; lower Pahang - P. Merah, 8 April 1993.

BIRCUM 2088, 309.00 mm SL. Peninsular Malaysia: Pahang River Basin; Lower Pahang - K. Lepar, 3-5 April 1993.

BIRCUM 2089, 322.00 mm SL. Peninsular Malaysia: Pahang River Basin, Sg. Pahang at the vicinity of Pulau Ganchong, K. Sg. Ganchong, 31 March 1993.

BIRCUM 3146(1) 247.75 mm SL., BIRCUM 3146 (2) 253.03 mm SL. Bought at fish market, presumably from Peninsular Malaysia: Pahang River Basin, 16 October 1992.

BIRCUM 2143. 261.83 mm SL. Peninsular Malaysia: Pahang River Basin; P. Lebak, 29 April - 2 May 1993. BIRCUM 2032(1) 306.00 mm SL. BIRCUM 2032(2) 249.99 mm SL. Peninsular Malaysia: Pahang River Basin; Sg Jelai at Kuala Kenong, Ulu Lepar,

Lubuk Yong, Jeti K. Lipis, BIRCUM 1843(HEAD) No exact locality, Pahang River Basin.

HB 1-6, 6 ex. 293.26-388.00 mm SL. Peninsular Malaysia: Pahang River Basin; Jerantut, Kg Kuala Pah, 11 May 2007.

BIRCUM 2849(1) 247.42 mm SL. BIRCUM 2849(2) 148.85 mm SL Peninsular Malaysia: Pahang River Basin; Sg. Pahang near Kg Perian and Jerantut Ferry, 3 Nov 1992.

ZRC 9208 274.19 mm SL. P Peninsular Malaysia: Perak River Basin; Lake Chenderoh, Malaysia. coll. Alfred E.R., April 1967.

ZRC 11660-11662. 162.15-389.00 mm SL no exact locality, Peninsular Malaysia, K. Lim, Dept. of Zoology, NUS, April 1990.

ZRC 2858 223.78 mm SL. Peninsular Malaysia: Perak River Basin; Chenderoh Lake, S.L Hora, Raffles Museum Collectors, 1938.

NMS 2859(A) 204.62 mm SL, NMS 2859(B) 172.44 mm SL, NMS 2859(C) 188.53 mm SL Peninsular Malaysia: Perak River Basin; Telok Anson**,** Fisheries Department, January 1951.

MZB 1218 (2 ex., 206.00-223.00 mm SL) Indonesia: Central Java; Bojonegoro; Solo River. Coll. Soetikno and Nurhasan, 25 June 1972.

Description:

This species is characterised by large eyes, with very thin, short barbels; maxillary barbel usually reaching but slightly posterior to eye and length of mandibular barbel less than eye diameter. Specimens collected have larger eye than any other species of *Pangasius*. However, Roberts & Vidthayanon (1991) described that there are also specimens with small eyes from Cambodia and Java which were regarded as intraspecific variation and eye size is likely to be influenced by environmental factors. Gill rakers counts are 12-21 (vs 13-15 in Roberts & Vidthayanon, 1991). Our collections of *Pseudolais micronemus* have separate palatine and vomerine bands on each side and the four tooth plates are relatively small, round and widely separated resembled the description of Roberts & Vidthayanon (1991).

Distribution and ecology:

This is the most abundant species of the family Pangasiidae in Peninsular Malaysia. *Pseudolais micronemus* can be found in large rivers, including Sg Galas, a tributary of Kelantan River, Kerian River and Perak River and along Pahang River (Figure 3.22). Further details of *P. micronemus* and *P. polyuranodon* will be discussed in Chapter 4.

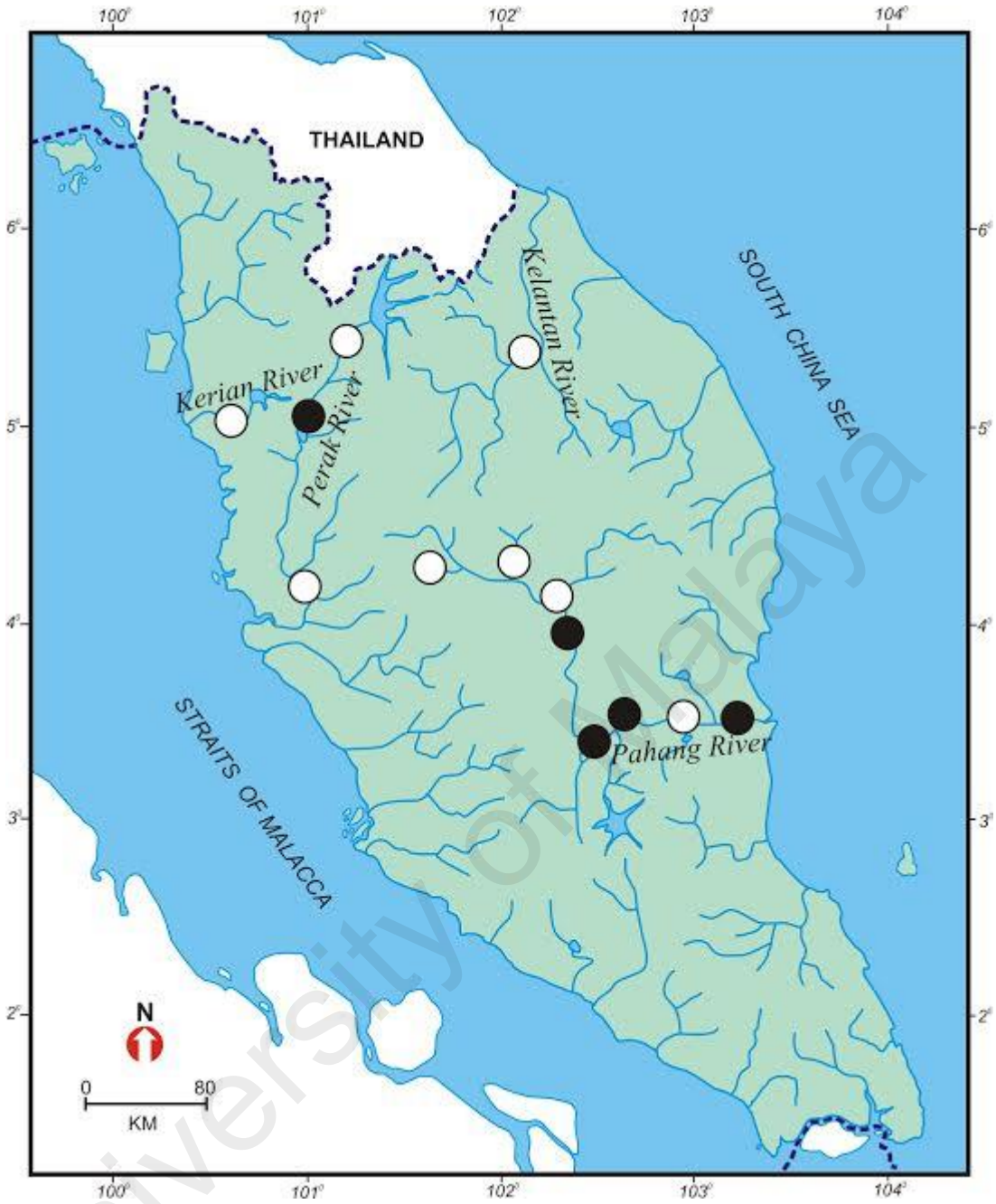


Figure 3.22: Map of Peninsular Malaysia, showing current (●) and historical record of *Pseudolais micronemus* (○)

3.4.2 Morphological Characteristics

The PCA plot from 21 out of 35 measurements for 161 specimens of the Peninsular Malaysian pangasiids are presented in Figure 3.23. PCA is the result of covariance matrix where eigenvalues which referred as characteristic roots (McGarigal et al., 2000) and the variances of the corresponding principal components represented by eigenvalues. The larger the eigenvalue, the greater the variation, hence the higher the exploratory power of that principal component. Factor-loadings (eigenvalues of the correlation matrix) as in Table 3.4, with only two factors are shown. Since eigenvalue decreases from the first component, the scree plot steep down approaching zero. The maximum number of components to be retained is indicated by the first point of straight curve, thus only two factors were selected.

The first two principal components contributed to 80.76% of the total variance (Factor 1: 70.80% and Factor 2: 9.96%) (Table 3.4). The third, fourth, fifth and following components also influenced the characterisations but have very low contributions in improving the sample separations (0.96%, 0.59% and 0.47 % respectively, with the following values also below 1%).

Principal component loadings (factor loadings) in Table 3.4, determines the correlations between the factor and morphometric variables. Factor loadings greater than 0.30 or less than -0.30 are considered significant, whereas loadings greater than 0.50 or less than -0.50 are considered very significant (Hair et al., 1987; Tabachnik & Fidell, 1989). In this analysis, all characters in factor 1 located on the negative sector (negative values) but with highly significant values except for a few characters (value 0.7 and below); Caudal Peduncle Length (CPL), Anal-fin Length, Adipose-fin Length, Adipose-fin Width, Eye Diameter (ED), and Post Ocular Length. These negative correlations, indicate an inverse relationship between the variables and the component. Those four characters which contributed to the second PCA loadings (Factor 2) with fair values (CPL

(0.30), PrePC (-0.33) and CPD (0.40) and highly significant in ED loadings (0.90) were directly correlated to the factor except for PrePC.

While Factor 1 loadings can be interpreted as characters which determines *Pangasius* species group, Factor 2 loadings can be used to differentiate *Pseudolais micronemus* and *Helicophagus waandersii*. Dominant scores of factor 2 - eye diameter (ED), caudal peduncle (length (CPL) and depth (CPD)), anal-fin length (AFL) and adipose-fin width (AdiFW) (Table 3.4) are discriminant characters for *Pseudolais*, the same characters were observed by Gustiano (2003). These characters positively contribute, discriminate between *Pangasius polyuranodon* with *H. waadersii* with adipose fin width. Eye diameter (ED) have the most contribution (0.903) compared to other characters, together with Post Ocular Length (POL) and Adipose Fin Width (AdiFW) which also contributed to the identification characters of *Helicophagus*.

Table 3.4: Summary of PCA on factor loadings, based on correlations of 21 morphometric measurements of Peninsular Malaysian pangasiids. Only the first two factors are shown.

Variables (measurements)	**Factor 1**	**Factor 2**
Head Length	-0.961	-0.183
Snout Length	-0.955	-0.007
Head Depth	-0.864	0.289
Head Width	-0.930	0.200
Predorsal Length	-0.931	-0.148
Caudal Peduncle Length	-0.787	0.303
Caudal Peduncle Depth	-0.849	0.402
Pectoral Fin Length	-0.880	0.205
Pelvic Fin Length	-0.946	-0.012
Anal-fin Height	-0.867	-0.228
Anal-fin Length	-0.743	0.467
Adipose Fin Length	-0.609	-0.359
Adipose Fin Width	-0.775	-0.315
Eye Diameter	-0.164	0.903
Mouth Width	-0.878	0.019
Lower Jaw Length	-0.819	0.054
Inter Orbital Distance	-0.948	-0.123
Distance from Snout to Ithsmus	-0.871	-0.037
Post Ocular Length	-0.781	-0.413
Body Width	-0.910	-0.010
PrePectoral	-0.833	-0.336
Eigenvalue	14.867	2.092
% Total variance	70.796	9.960

PCA were run separately for meristic variables (Table 3.5) and factor loadings of PCA factor 1 show high positive values for Pelvic-fin Rays (PvFR) and Pectoral-fin Rays (PcFR), while high negative value for Dorsal-fin Rays. Whereas, Factor 2 was dominantly contributed by Gill Raker count (GR). High loading value for Factor 3 as contributed mainly by Anal-fin Rays (AFR). Throughout this analysis, Factor 1 known to separate *Pangasianodon* from other genera by the higher number of PvFR counts, eight to nine rays instead of six. Factor 2 contribute to separation characters of *Pangasius djambal* and *P. bocourti*, with the latter has higher gill raker counts. High AFR count (more than 35) in Factor 3 further contributed to discriminate *Helicophagus waandersii* as suggested by Gustiano (2003).

Table 3.5: Summary of PCA on factor loadings, based on correlations of 4 meristic counts of Peninsular Malaysian pangasiids.

Meristic counts	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Factor 5**
Anal-fin Rays	-0.139	0.297	0.924	-0.197	-0.001
Pelvic Fin Rays	0.958	-0.191	0.172	0.108	0.074
Pectoral Fin Rays	0.591	0.516	-0.308	-0.538	-0.002
Dorsal Fin Rays	-0.953	0.201	-0.160	-0.140	0.073
Gill Rakers at the first gill arch	-0.124	-0.891	0.057	-0.432	-0.001

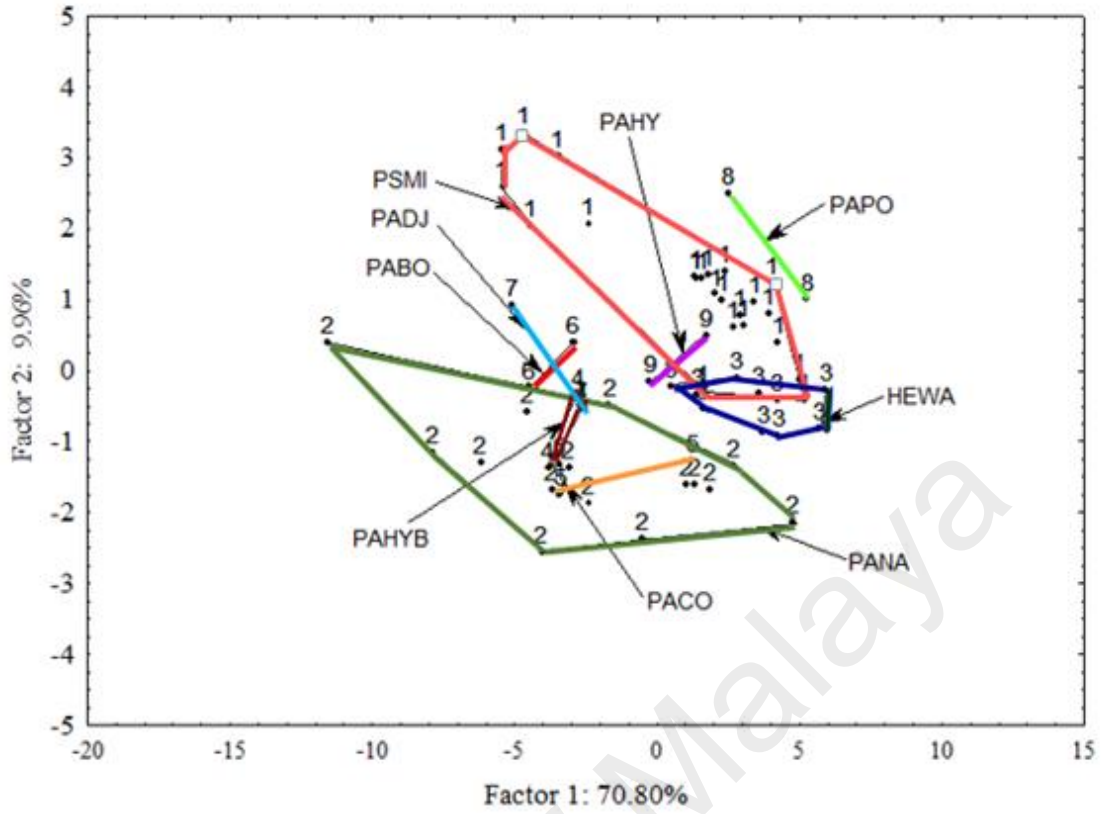


Figure 3.23. Plot of the first and second principal components of the analysis based on 21 characters of morphometric measurements on the pangasiids of Peninsular Malaysia. The sample were assigned to their respective taxa according to point numbers (1) PSMI - *Pseudolais micronemus*; (2) PANA - *Pangasius nasutus*; (3) HEWA - *Helicophagus waandersii*; (4) PAHYB - *Pangasius* hybrid; (5) PACO – *Pangasius conchophilus*; (6) PABO – *Pangasius bocourti*; (7) PADJ – *Pangasius djambal*; (8) PAPO – *Pangasius polyuranodon*.

As shown in Figure 3.23, seven species were identified - *Pseudolais micronemus*, *Pangasius nasutus*, *Helicophagus waandersii*, *Pangasius conchophilus*, *Pangasius bocourti*, *Pangasius djambal*, *Pangasius polyuranodon* with two specimens of *Pangasius* hybrid. Only three species are clearly grouped. No clear separation observed on the latter five taxa, due to sharing of some non-distinctive characters with only few specimens analysed for some species.

According to taxonomic keys as followed in the methodology section, there are three large groups corresponds to the native pangasiid species found abundantly in Peninsular Malaysia; which are *Pseudolais micronemus* (red cluster), *Helicophagus waandersii* (blue cluster) and *Pangasius nasutus* (dark green cluster). PCA however,

failed to discriminate specimens identified as *Pangasius conchophilus* and specimens identified as *Pangasius* hybrid, both are placed in the *P. nasutus* group.

Another two *Pangasius* species, *P. djambal* and *P. bocourti* which have the same characters of a rounded snout, thus located closely but not appended to the *P. nasutus* group. Another close morphological species, sharing the character of large eye size, *Pseudolais micronemus* and *Pangasius polyuranodon* are also positioned very closely but not joint as one group. However, *Pangasianodon hypophthalmus* share some characters with *P. micronemus*; presumed as thin premaxillary teeth, broad snout with wide mouth, but the latter is further distinguished by two separate palatine and two vomerine patches. *Pangasius polyuranodon* is discriminated from *P. micronemus* by large median vomerine toothplates and long predorsal length. All these characters contributed to the PCA plot factor 1.

3.5 Summary

In this chapter, seven species of Pangasiids were identified based on previous and current records. Three new species (*Pangasianodon hypophthalmus, Pangasius bocourti*, *Pangasius conchophilus* and *Pangasius djambal*) were reported for the first time from the Pahang River and Perak River (*P. hypophthalmus*), believed as escapees from the aquaculture activity in the river. Existence of *Pangasius polyuranodon*, a species which was previously unnoticed due to the close characteristics to the abundant native species, *Pseudolais micronemus,* was also confirmed.

Important characters that contribute to the identification of the species were clearly determined by the PCA, whereby, Factor 1 of the meristic PCA loadings separate *Pangasianodon* from other genera by Pelvic Fin Ray number (8-9 vs 6. High Anal-fin Rays could be used to discriminate *Helicophagus waandersii*. Combinations of PCA factors determines the complex *Pangasius* groups, while discriminant characters for

Pseudolais are Eye Diameter, Caudal Peduncle Length and Depth, Anal-fin Length as well as Adipose Fin Width.

However, there is no clear separation of some *Pangasius* species in this study, due to the small number specimens and lack of comparative materials; therefore their identification remain problematic. Referring to allopatric species previously known from the Asian Mainland; *P. conchophilus* is morphologically close to *P. nasutus* and *P. bocourti* is close to *P. djambal*. Further comparative specimens are needed to confirm the species.

Pseudolais micronemus is the most widely distributed species of pangasiid in Peninsular Malaysia, with previous records from Galas River, Kelantan also various locations along the Pahang and Perak River. Other native species, *Helicophagus waandersii, P. nasutus* and *P. polyuranodon* are confined to Pahang River only, even though the holotype of *Pangasius ponderosus*, the synonymy to *Pangasius nasutus*, was recorded previously from Chenderoh Dam of the Perak Drainage in 1937.

CHAPTER 4: THE OCCURRENCE OF *PANGASIUS POLYURANODON* BLEEKER, 1852 (TELEOSTEI: PANGASIIDAE) IN PENINSULAR MALAYSIA WITH REMARKS ON THE COMPARATIVE MORPHOLOGY WITH *PSEUDOLAIS MICRONEMUS* (BLEEKER, 1847)

(Published in *Sains Malaysiana*. 43 (11) 1707-1714)

4.1 Introduction

Catfish species of the family Pangasiidae are recognised by a laterally compressed body, having two pair of barbels (maxillary and mandibular), long anal fin, short dorsal fin and small adipose fin with free posterior margin (Gustiano, 2003; Roberts & Vidthayanon, 1991). They are widely distributed throughout Asia, from Pakistan to Indochina and the Indo-Malayan Archipelago (Roberts & Vidthayanon, 1991) and also recorded in China (Yang et al., 2007). A total of 28 valid Pangasiidae species have been reported throughout the Asian river systems (Nelson, 2006; Ferraris, 2007; Eschmeyer & Fong, 2016; Froese & Pauly, 2016), with 13 species occurring in Indo-China and 15 species found in the Indo-Malayan Archipelago (Pouyaud et al., 1999; Pouyaud & Teugels, 2000; Ng & Kottelat, 2000; Pouyaud et al., 2002; Gustiano, 2003; Kottelat, 2013).

There are four genera in the family Pangasiidae: *Helicophagus* Bleeker 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, in Cuvier & Valenciennes, 1840, and *Pseudolais*, Vaillant 1902 (see Kottelat, 2013). While the earlier molecular phylogenetic studies using partial mitochondrial Cytochrome b gene (Pouyaud et al., 2000) could not resolve the groupings, later work by Pouyaud et al. (2004) using mitochondrial 12S rRNA and recent work by Karinthanyakit & Jondeung (2012) has supported the four groupings of the genera using complete mitochondrial cytochcrome b, 12S rRNA, tRNA-Val, 16S rRNA and partial nuclear recombination-activating gene (*rag1)*. It should be noted that molecular evidence could be used to support morphological

characteristics of such species, but each must be carefully used in conjunction with the others. External features such as morphological and meristic characters should be used in the first place to confirm the species identification before using any molecular work to support the study.

Members of the Pangasiidae are very similar morphologically. Some may exhibit different morphological resemblance at different life stages (e.g. *P. bedado* Roberts, 1999 was actually a junior synonym to *P. djambal* Bleeker, 1846) (Gustiano et al., 2004), morphological variation of allopatric species that occur between the Asian Mainland and Indo-Malayan Archipelago (*Helicophagus leptorhyncus* Ng & Kottelat, 2000 vs *H. typus* Bleeker, 1858; *P. bocourti* Sauvage 1880 vs *P. djambal* Bleeker, 1846; *P. conchophilus* Robert & Vidthayanon, 1991 vs *P. nasutus* (Bleeker, 1863); *P. elongatus* Pouyaud, Gustiano & Teugels, 2002 vs *P. mahakamensis* Pouyaud, Gustiano & Teugels, 2002). These reasons, together with living in various ecological habitats might create confusion as arouse in many of the previous classification. Further taxonomical clarification and supporting evidence is clearly required for proper management and conservation of such species as some species may be previously unknown or even unnoticed.

Pangasiids are known for their great economic importance. However, many Pangasiid populations are reported to be rare, under threat of extinction or least concerned under IUCN Red List of Threatened Species (IUCN, http://www.iucn.org). Among them in the lists are *Pangasianodon gigas* (Hogan, 2011), *H. leptorhyncus* (Vidthayanon 2012a), the wild populations of *Pangasianodon hypophthalmus* (Sauvage, 1878) (Hogan & Vidthayanon 2011), *P. bocourti* (Vidthayanon 2012b), *P. conchophilus* (Vidthayanon 2012c), *P. elongatus* (Vidthayanon 2012d), *P. krempfi* Fang & Chaux, 1949 (Baird 2011), *P. larnaudii* Bocourt, 1866 (Baird 2012), *P. macronema* Bleeker, 1851 (Jenkins et al. 2009), *P. mekongensis* Gustiano, Teugels & Pouyaud, 2003 (Vidthayanon 2012e) and *P. myanmar* Roberts & Vidthayanon, 1991 (Chaudhry 2010). *P. pangasius* (Hamilton,

1822) was listed as Critically Endangered (IUCN Bangladesh) (Hossain et al. 2009) but currently its status is Least Concern (Pal 2010). Populations of *P. sanitwongsei* Smith, 1931 is seriously declining; once, it was considered endangered in Thailand (Humphrey & Bain, 1990; Roberts & Vidthayanon 1991; Hogan et al. 2009) but then reviewed as Critically Endangered (Jenkins et al. 2009). IUCN's Red List database also has regarded *Pseudolais micronemus* (Bleeker, 1947) as Data Deficient (Vidthayanon 2012f) while *P. pleurotaenia* (Sauvage, 1878) as Least Concern (Vidthayanon 2012g).

Peninsular Malaysia is the location where many Indonesian species have their northernmost distributional limit while a handful of Indian and Indochinese species have their southernmost distributional limit (Zakaria-Ismail 1994), resulting from the previous landmark change on the Sunda Shelf during the Pleistocene glacial maxima (Rainboth 1996; Voris 2000; McConnell 2011). In Peninsular Malaysia, three species of Pangasiidae have been recorded, namely *P. nasutus, P. micronemus* and *H. waandersii* (Tweedie 1936; Herre & Myers 1937; Roberts & Vidthayanon 1991; Lim & Zakaria-Ismail 1995). Fowler (1938) listed *P. polyuranodon* as having been found in Peninsular Malaysia, but its occurrence remains doubtful (Lim & Zakaria-Ismail 1995). In a recent study, all the four species of Pangasiids are listed as moderately threatened in Malaysia because of the combination factor of overharvesting and habitat degradation (Chong et al. 2010). Therefore, it is of utmost important to positively identify the species and understand its ecology to ensure proper management and conservation strategy can be taken to maintain a healthy and sustainable population, hence protecting the rich fish biodiversity and its genetic resources in the country.

The current study compares the morphological characters of *P. polyuranodon* with *P. micronemus*, and discussed its finding to the implications on the conservation of indigenous Pangasiid catfish species in Peninsular Malaysia. The findings confirmed the existence of *P. polyuranodon* in Peninsular Malaysia.

4.2 Materials and Methods

4.2.1 Morphological Analysis

While examining the pangasiid specimens housed at the Universiti Malaya Zoological Collections, the first author uncovered two specimens of *P. polyuranodon* which were first thought to be *P. micronemus.* Identification follows Roberts & Vidthayanon (1991) while morphometric measurements were taken using dial callipers and recorded to the nearest 0.1 mm, following Gustiano (2003, 2009) and Pouyaud et al. (1999). All measurements except standard length were expressed as proportional values of reference length. Measurements on the body including head length were conveyed as a percentage of the standard length (% SL). Other measurements on the head were articulated as a percentage of the head length (% HL).

4.2.2 Statistical Procedures

Univariate analysis including sample size, mean and standard deviation, minimum and maximum values were analysed using Analysis of Variance (ANOVA). Variables with significant difference were selected as distinguishable characters.

4.2.3 Specimen Examination

Specimens were compared to *P. micronemus* collections from Peninsular Malaysia (n = 82) located at the Universiti Malaya, Kuala Lumpur, Malaysia (BIRCUM and UMKL), Fisheries Research Institute (Freshwater Division), Glami Lemi, Jelebu, Malaysia (FRIGL), the Raffles Museum for Biodiversity Research, National University of Singapore (ZRC and NMS) and also my collections (HB), Mohd Zakaria-Ismail (MZI) and Asian Wetland Bureau (AWB) (now known as Wetlands International). A specimen of *P. polyuranodon* and two other specimens of *P. micronemus* from Museum Zoologicum Bogoriense (MZB) were served as comparative specimens.

The present Malaysian specimens are now housed at the Fisheries Research Institute (Freshwater Division), Glami Lemi, (FRIGL), Jelebu, Negeri Sembilan, Malaysia.

4.3 Results

4.3.1 Diagnosis

Roberts & Vidthayanon (1991) stated that *P. polyuranodon* is an elongate species that could achieve 800 mm in standard length (see Vidthayanon 1993), while maximum size observed by Gustiano (2003) was 602 mm SL. *Pangasius polyuranodon* can be distinguished from all congeners by the combination of a short predorsal length (25.2-29.2% SL) and a long caudal peduncle (17.2-21.8% SL) (Pouyaud et al. 2002). This species has a distinctive palatal dentition (Figures 4.1c and 4.2) which consists of a large nearly squared median vomerine tooth patch with small lateral palatine toothplates, while *P. micronemus* has smaller and separate palatal tooth patches (Figure 4.3c). The number of gill rakers on first gill arch is 19-30. Anal fin rays are 33-42 (Gustiano 2003).

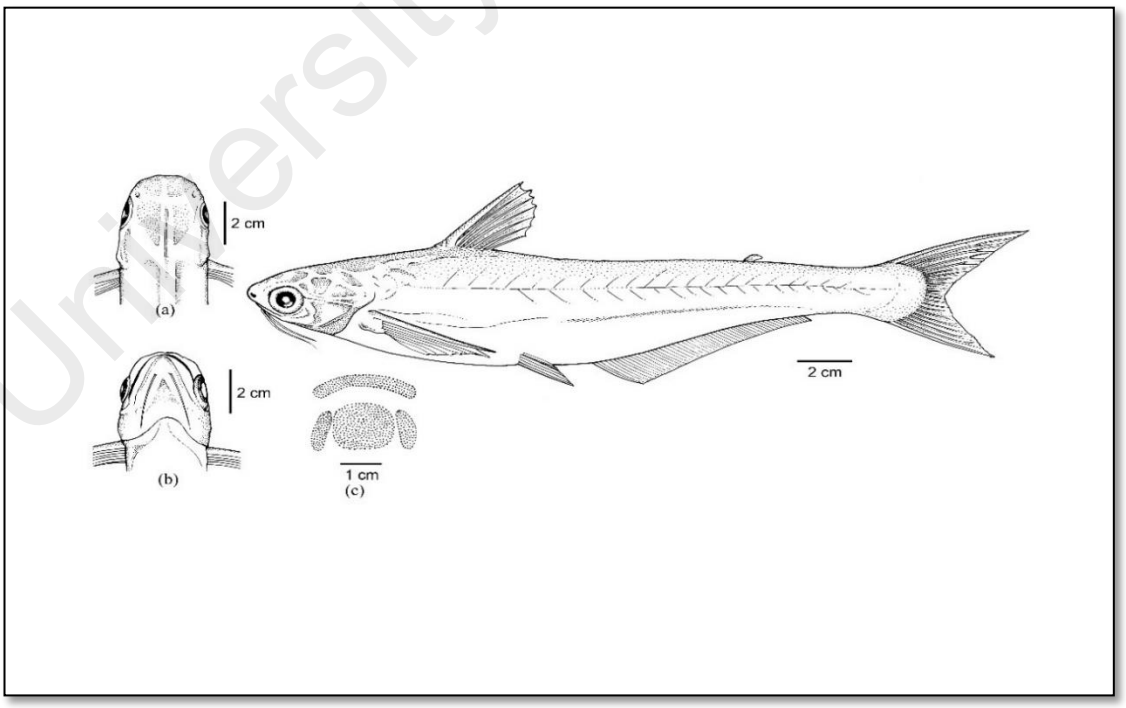


Figure 4.1: *Pangasius polyuranodon* Bleeker 1852, with (a) dorsal and (b) ventral head view and (c) maxillary and palatal dentition.

Figure 4.2: Closer look on the maxillary and palatal dentition of *P. polyuranodon*

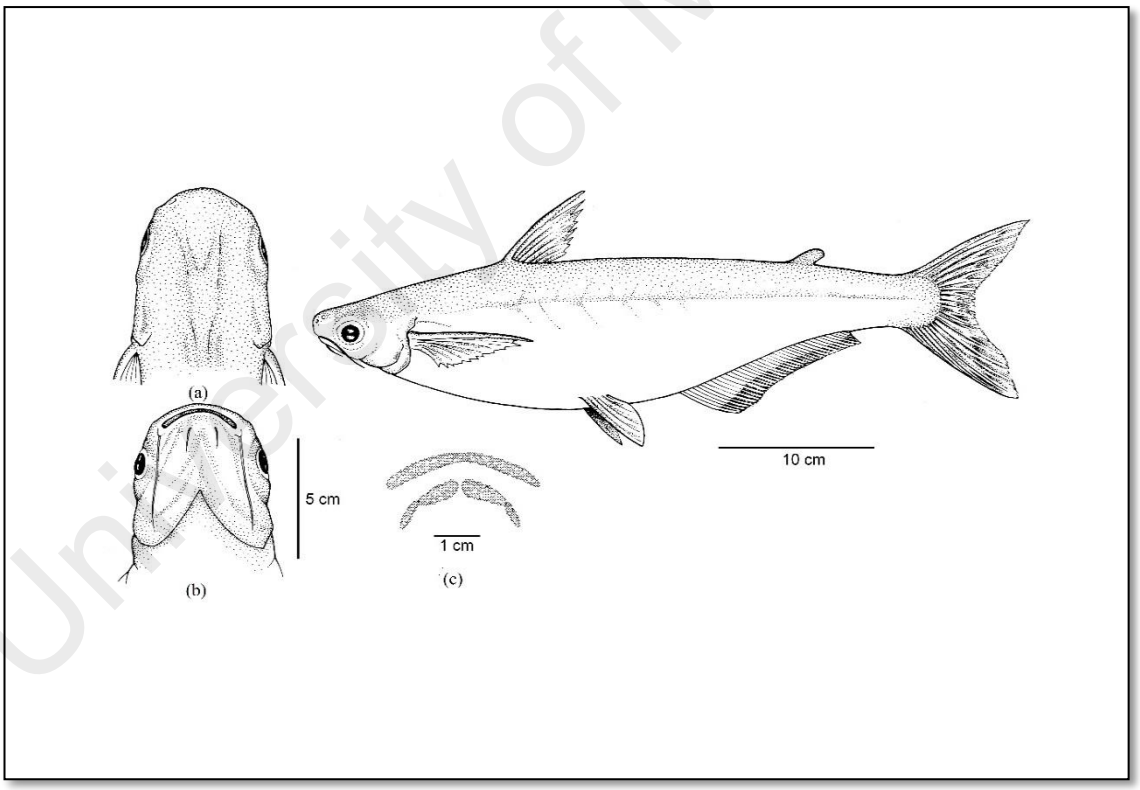


Figure 4.3: *Pseudolais micronemus* (Bleeker, 1847). (a) dorsal and (b) ventral head view, (c) maxillary and palatal dentition.

4.3.2 Description

Morphometric data of *P. polyuranodon* (Figure 4.1) specimens examined are shown in Table 4.1 in comparison with *P. micronemus*. Body elongated. Head short, flattened with snout slightly large, rounded. Mouth inferior with angulated ventral outline of lower jaw. Anterior nostrils shorter than distance of posterior nostril and situated on the anterior margin of the upper lip. Large eye diameter is a shared characteristic between both species (Table 4.1). When mouth is closed, premaxillary toothplate is hardly visible (Figure 4.1b). Wide rectangular shape of vomerine teeth, with long and slender palatine toothplates (Figures 4.1c and 4.2). Maxillary barbels reaching the posterior border of operculum while mandibular barbels shorter and hardly reaching the mid eye level. Three chambers of swimbladder extending to above anal-fin base. Short predorsal, with long caudal peduncle. Anal-fin long with high number of soft fin rays. Adipose fin minute.

From the external morphology, the two specimens recently discovered were first assumed to be *P. micronemus*, the more common and abundant indigenous pangasiid species in Peninsular Malaysia with the characteristic of large eye and elongate, slim body shape. However, closer examinations on the two specimens revealed the clear and distinctive characters of *P. polyuranodon*.

The dentition having a large and nearly squared vomerine tooth plate with discrete palatal teeth (Figure 4.2) could distinguish *P. polyuranodon* from *P. micronemus* which has four small and widely separated vomerine and palatine tooth bands (Figure 4.3c). Longer barbels (maxillary barbel length (30.6, 33.0 and 35.3% HL) and mandibulary barbel length (23.3, 20.2 and 34.9 % HL)) is another character that distinguish this species from *P. micronemus* which has minute pair of barbels with mean maxillary 28.0±7.1 and mandibulary 13.2±5.7% HL (Table 4.1).

The number of gill rakers on the first gill arch is higher in *P. polyuranodon* (28, 25 and 22; mean 25) while the number of gill rakers in *P. micronemus* found in this study is lower (12-24), similar to the findings of Gustiano (2003) with 70% majority counts of 15-18.

Higher number of anal-fin rays in the *P. polyuranodon* specimens observed (38-40) could be another discriminating characteristic. However, small number of specimens could not draw a good conclusion as compared to Gustiano (2003) that observed a range count of 33-42. Anal-fin ray counts of *P. micronemus* in Gustiano's observation were 26-40 but without frequency count distribution. In this current study, majority of *P. micronemus* specimens (67%) have anal-fin ray count of 26-30 while only small number of samples (4%) have higher count (35-40). Therefore, this could be another promising character to discriminate both the species.

Longer caudal peduncle length (19.8, 21.1 and 24.3% HL vs 18.4 ± 1.6% HL of *P. micronemus*) and shorter predorsal length 26.0, 27.3 and 28.0% HL vs 30.8 ± 4.3% HL of *P. micronemus* resemble the morphological characters described in Gustiano (2003). Detailed comparison on the morphometric and meristic characters is presented in Table 4.1.

Table 4.1: Morphometric and meristic data for *Pangasius polyuranodon* (n=3) with comparison to *Psedolais micronemus* from Peninsular Malaysia. All measurement are expressed as a percentage of standard length and percentage of head length. Data for *P. micronemus* are mean (mm) ± standard deviation, and ranges (min-max) in brackets. (*significant at $p < 0.05$)

Characters	**Comparative specimen (*P. polyuranodon*) MZB 3681**	**POLY 01**	**POLY 02**	**N**	***P. micronemus* (n=82) 72.7 – 406 mm SL**	**F ratio**	**P***
IN % STANDARD LENGTH							
Head length	17.4	19.1	17.3	82	19.2 ± 1.8 (15.0-24.1)	1.47	0.23
Head depth	9.7	10.2	9.4	82	11.5±1.4 (7.7-15.3)	4.39*	0.04
Head width	13.3	13.7	13.1	81	13.9±1.1 (10.3-17.1)	0.78	0.38
Caudal peduncle length	24.3	19.8	21.1	82	18.4±1.6 (13.9-21.7)	13.15*	0.00
Caudal peduncle depth	8.2	7.6	7.8	82	7.7±0.5 (6.1-9.1)	0.27	0.60
Pectoral spine length	16.4	-	-	49	15.1±2.3 (7.1-18.2)	30.43*	0.00
Pectoral-fin length	17.6	18.6	18.5	80	16.1±1.9 (10.5-20.2)	3.81	0.05
Dorsal spine length	16.9	15.7	14.9	47	14.9±1.7 (10.8-19.4)	1.01	0.32
Dorsal-fin length	18.9	14.9	16.1	74	16.6±1.7 (11.9-22.7)	0.01	0.92
Pelvic-fin length	11.6	9.1	10.3	82	10.7±1.0 (9.0-14.9)	0.38	0.54
Anal-fin height	8.5	9.3	11.1	81	9.5±1.5 (6.6-16.6)	0.01	0.91
Anal-fin length	31.9	32.6	31.8	82	27.8±2.9 (14.0-33.6)	6.93*	0.01
Adipose-fin height	3.7	2.7	2.7	80	2.8±1.0 (0.4-6.4)	0.06	0.81

Table 4.1: Continued

Characters	Comparative specimen (*P. polyuranodon*) MZB 3681	POLY 01	POLY 02	N	*P. micronemus* (n=82) 72.7 – 406 mm SL	F ratio	P*
IN % STANDARD LENGTH							
Adipose-fin width	1.0	1.1	0.3	80	3.2±0.9 (0.4-4.9)	1.74	0.19
Interorbital distance	11.0	10.4	10.4	82	10.3±1.1 (7.4-14.0)	0.28	0.60
Body width	13.7	14.4	15.4	82	15.5±1.4 (11.2-18.9)	1.45	0.23
Predorsal length	28.0	27.3	26.0	82	30.8±4.3 (12.2-41.2)	2.21	0.14
Prepectoral length	17.0	18.3	19.2	82	17.3±3.9 (6.0-24.5)	0.14	0.71
Prepelvic length	37.7	37.6	36.5	82	42.7±5.2 (6.4-51.7)	3.15	0.08
IN % HEAD LENGTH							
Snout length	40.5	38.3	41.2	84	41.2±3.4 (29.3-49.5)	0.33	0.56
Anterior snout width	35.5	39.1	40.6	84	35.2±2.5 (27.6-41.0)	4.57*	0.04
Posterior snout width	49.4	50.7	48.1	84	46.5±3.8 (32.3-53.6)	1.62	0.21
Eye diameter	22.3	27.7	22.4	84	25.9±3.6 (14.8-35.9)	0.67	0.41
Mouth width	45.2	59.7	47.7	84	44.4±4.2 (32.2-54.8)	6.20*	0.01
Lower jaw length	31.6	26.1	32.2	84	28.1±5.1 (7.6-41.8)	0.39	0.53

Table 4.1: Continued

Characters	**Comparative specimen (*P. polyuranodon*) MZB 3681**	**POLY 01**	**POLY 02**	**N**	***P. micronemus* (n=82) 72.7 – 406 mm SL**	**F ratio**	**P***
IN % HEAD LENGTH							
Distance from snout to isthmus	53.9	59.3	61.3	84	59.3±3.6 (49.3-67.6)	0.39	0.57
Post Ocular length	32.2	35.0	49.3	84	40.7±9.9 (22.8-61.9)	0.09	0.76
Vomerine width	24.8	23.8	24.6	45	8.0±8.6 (0.2-29.6)	10.67*	0.00
Vomerine length	28.2	37.8	19.1	45	1.7±2.0 (0.2-8.9)	258.20*	0.00
Palatine length	18.2	25.9	13.5	41	3.2±4.1 (0.5-13.7)	39.15*	0.00
Palatine width	6.1	8.7	4.8	40	1.3±1.6 (0.2-5.5)	36.69*	0.00
Dorsal spine width	13.7	7.0	5.2	83	7.9±2.6 (4.7-16.3)	0.18	0.67
Maxillary barbel length	35.3	33.0	30.6	78	28.0±7.1 (18.3-50.0)	14.75*	0.00
Mandibulary barbel length	24.9	23.3	20.2	76	13.2±5.7 (5.7-35.3)	6.21*	0.01

Table 4.1: Continued

Characters	Comparative specimen (P. polyuranodon) MZB 3681	POLY 01	POLY 02	N	*P. micronemus* (n=82) 72.7 – 406 mm SL	F ratio	P*
MERISTIC COUNTS							
Anal-fin rays	38	39	40	83	21-40	29.94*	0.00
Pelvic-fin rays	6	6	6	83	6-7	0.41	0.52
Pectoral-fin rays	13	13	13	84	10-16	0.10	0.76
Number of gill rakers on the first gill arch	22	28	25	81	12-24	33.85*	0.00

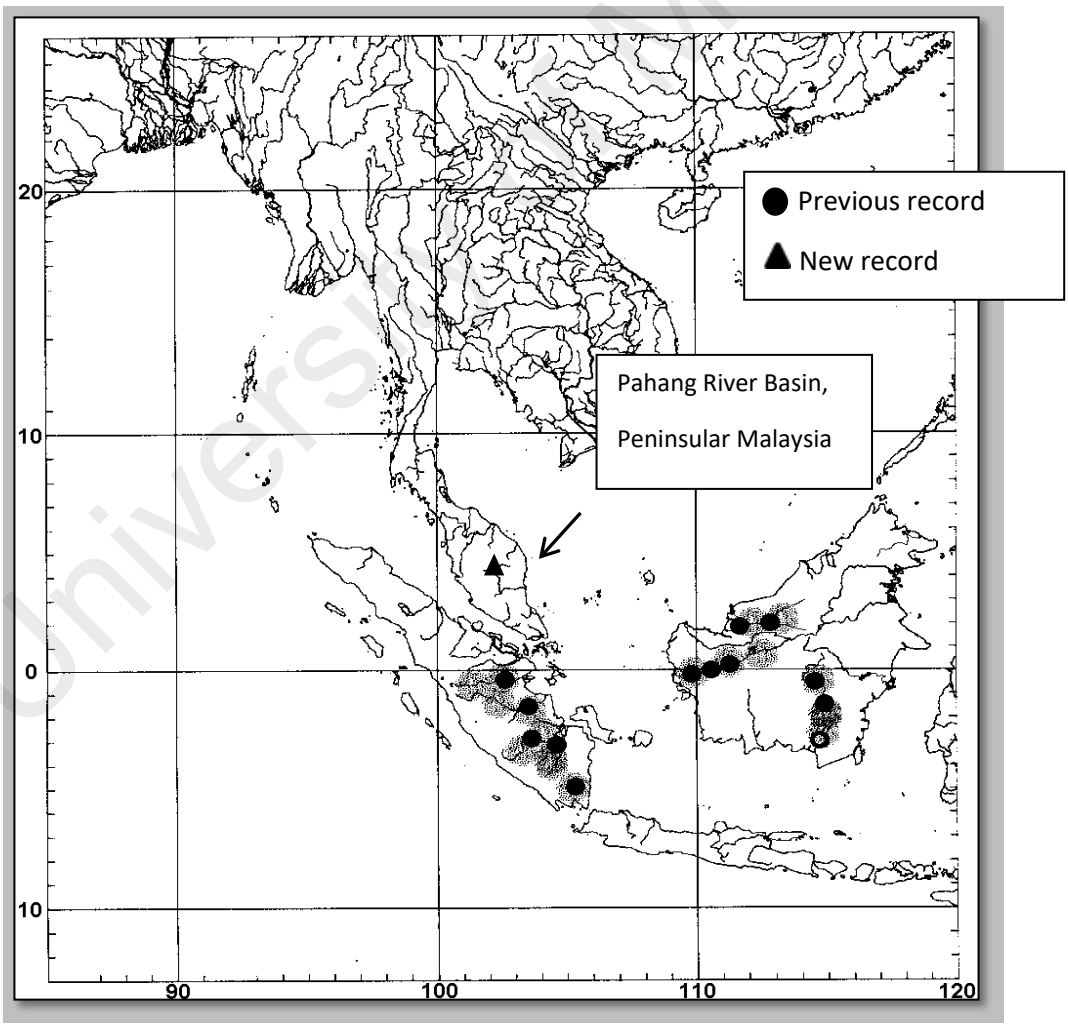


Figure 4.4: Geographic distribution of *P. polyuranodon* known to occur in Southeast Asia. Modified from Gustiano (2003).

4.4 Discussion

This study has confirmed the existence of *P. polyuranodon* in Peninsular Malaysia as previously cited by Fowler (1938) and Lim and Zakaria-Ismail (1995). The distribution of *P. polyuranodon* is now extended further north to Peninsular Malaysia (Figure 4.4), and it was previously only known from Indonesia and Borneo (Pouyaud et al. 2002; Gustiano 2003). In relationship to the lost biogeographic connection between the freshwater species occurred in continental Asia and Indo-Malayan Archipelago, this finding therefore has filled the gap on the map of aquatic faunal exchange across the high freshwater fish biodiversity of the Sunda Shelf.

Pangasius polyuranodon may have been previously misidentified as *P. micronemus* due to it close morphological appearance, with consequent confusion regarding respective distributions. Bleeker (1862) revealed the synonymy of the specimens of *Pangasius juaro* Bleeker 1852: 136 type locality Palembang, Indonesia. However, the fish which is locally called 'Ikan Juara' (in Malay language (ikan = fish)) or 'Juaro' in Indonesia is actually *P. micronemus*. During earlier revisions, both the species were kept in the same lot [RMNH 6857 (4 ex: 136-299 mm) in Leiden Museum (see Gustiano 2003: 138), before the designation of *P. polyuranodon* holotype RMNH 6855. A single *P. juaro* specimen (301 mm SL, RMNH 6857) was designated by Gustiano (2003) as the holotype based on meristic data described in the original description, not the 292 mm BMNH specimen suggested by Roberts and Vidthayanon (1991: 137).

Roberts and Vidthayanon (1991) also had difficulty in distinguishing *P. polyuranodon* from *P. micronemus*. They reported *P. polyuranodon* as widely distributed throughout Vietnam, Thailand, Malaysia and Indonesia, thus they separated the two species based on geographical variation of the number of anal-fin rays. Later, Pouyaud et al. (2002) resolved this problem by separating the *P. polyuranodon* complex into three

species with the description of two new species that occur allopatrically: *P. mahakamensis* (endemic to Mahakam River, East Kalimantan, Indonesia) and *P. elongatus* (lower reaches of Chao Phraya, Bangpakong and Vietnam Mekong) (the former was mentioned as having 32-37 anal fin rays; while the latter have 32 rays (Roberts & Vidthayanon 1991: 137). This separation resulted in *P. polyuranodon* to be occurred in the Sumatra and Borneo Islands only (Figure 4.4).

The habitats used by both the species are also different. *Pseudolais micronemus* spends most of its life in freshwater habitats (upper and middle reaches) but the migratory pattern is poorly known (Vidthayanon 2012f) while *P. polyuranodon* inhabits estuaries and lower reaches but migrates upstream during the rainy season (Pouyaud et al. 2002). In Peninsular Malaysia, *P. polyuranodon* is thought to be endangered and in fact may be nearly extinct (personal observation) since it is rarely seen or partly due to specialised habitat preferences. On the other hand, *P. micronemus* is more abundant. These findings are contrary to the assessments of Vidthayanon (2012f) who reported the uncommonality of *P. micronemus* throughout its distribution range with no recent records from Chao Phraya drainage in Thailand. The 2012 IUCN Red List status for *P. micronemus* is Data Deficient, with declining population trend in Cambodia and Vietnam. Further extensive surveys are clearly needed to establish the distribution and abundance of both *P. polyuranodon* and *P. micronemus* (which can now be done definitively using the distinguishable characters outlined in this study).

The status of the species must be identified by further specimen collection, ecological, migration patterns and spawning behaviour studies must also be conducted. If the status on risk of extinction or endangered have been confirmed, an appropriate fishery management plans must be developed for species protection. Stakeholders and public consultation could be enquired before gazetting any habitat or closing area or season. Once and while the population recovered, a long term program is required to create the

awareness on the species and habitat protection. Not only for *P. polyuranodon*, could the same management plan also be applicable to any endangered or threatened fish species.

4.5 Material Examined

Pangasius polyuranodon Bleeker 1852

UMKL (POLY 01), 223.18 mm SL UMKL (POLY 02). 395.00mm SL. Peninsular Malaysia: Pahang River Basin, exact location unknown, dated 1992-1993.

MZB 3681, 284.5 mm SL; Indonesia: Kalimantan: Kapuas Basin; Sintang, coll. T.R. Roberts (Roberts & Vidthayanon, 1991: 136). Comparative specimen.

Pseudolais micronemus (Bleeker, 1947)

FRIGL 1004, 19 ex. 72.72-335.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Tanjong Pulai, Paloh Hinai near Pekan; 3° 30’ 35”N ; 103° 08’ 32”E; coll. B. Haslawati, 27 July - 21 December 2004.

FRIGL 1005, 27 ex. 14.30-364.00 mm SL; Peninsular Malaysia: Pahang River Basin; Kampung Mentenang, Lubok Paku, Maran; 3° 28’ 59” N; 103 48’ 56”E; coll. B Haslawati, 28 July 2005 - 26 January 2006.

FRIGL 1006 (1), 3 ex. 334.00-393.00 mm SL, Peninsular Malaysia: Perak River Basin: Kuala Kangsar, Kg Manong. 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 2 June 2008 .

FRIGL 1007. 3 ex.; 1007 (1) 366.00 mm SL. Peninsular Malaysia: Perak River Basin: Kuala Kangsar, Kg Manong; 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 31 August 2008. FRIGL 1007 (2), 2 ex, 336.00-406.00 mm SL. Peninsular Malaysia: Perak River

Basin: Kuala Kangsar, Kg Manong; 04° 34’ 03”N; 100° 55’ 48”E; coll. B. Haslawati, 28 September 2008.

618, 278.82 mm SL. Peninsular Malaysia: Kelantan River Drainage Sg Galas, near Kg Lulut, MZI, 11 Sept 1984.

BIRCUM 1944, 334.00 mm SL Peninsular Malaysia: Pahang River Basin: Sg Tembeling near Jeram Pai, coll. AWB, 19 Feb 1993.

BIRCUM 1952, 343.00 mm SL: Peninsular Malaysia: Pahang River Basin; Sg Tembeling near Jeram Pai, coll. AWB, 19 Feb 1993.

BIRCUM 1972, 311.00 mm SL, Peninsular Malaysia: Pahang River Basin; no exact locality, coll. AWB. BIRCUM 1723, 250.19 mm SL. Peninsular Malaysia: Pahang River Basin; lower Pahang - P. Merah, 8 April 1993.

BIRCUM 2088, 309.00 mm SL. Peninsular Malaysia: Pahang River Basin; Lower Pahang - K. Lepar, 3-5 April 1993.

BIRCUM 2089, 322.00 mm SL. Peninsular Malaysia: Pahang River Basin, Sg. Pahang at the vicinity of Pulau Ganchong, K. Sg. Ganchong, 31 March 1993.

BIRCUM 3146(1) 247.75 mm SL., BIRCUM 3146 (2) 253.03 mm SL. Bought at fish market, presumably from Peninsular Malaysia: Pahang River Basin, 16 October 1992.

BIRCUM 2143. 261.83 mm SL. Peninsular Malaysia: Pahang River Basin; P. Lebak, 29 April - 2 May 1993. BIRCUM 2032(1) 306.00 mm SL. BIRCUM 2032(2) 249.99 mm SL. Peninsular Malaysia: Pahang River Basin; Sg Jelai at Kuala Kenong, Ulu Lepar, Lubuk Yong, Jeti K. Lipis, BIRCUM 1843(HEAD) No exact locality, Pahang River Basin.

HB 1-6, 6 ex. 293.26-388.00 mm SL. Peninsular Malaysia: Pahang River Basin; Jerantut, Kg Kuala Pah, 11 May 2007.

BIRCUM 2849(1) 247.42 mm SL. BIRCUM 2849(2) 148.85 mm SL Peninsular Malaysia: Pahang River Basin; Sg. Pahang near Kg Perian and Jerantut Ferry, 3 Nov 1992.

ZRC 9208 274.19 mm SL. P Peninsular Malaysia: Perak River Basin; Lake Chenderoh, Malaysia. coll. Alfred E.R., April 1967.

ZRC 11660-11662. 162.15-389.00 mm SL no exact locality, Peninsular Malaysia, K. Lim, Dept. of Zoology, NUS, April 1990.

ZRC 2858 223.78 mm SL. Peninsular Malaysia: Perak River Basin; Chenderoh Lake, S.L Hora, Raffles Museum Collectors, 1938.

NMS 2859(A) 204.62 mm SL, NMS 2859(B) 172.44 mm SL, NMS 2859(C) 188.53 mm SL Peninsular Malaysia: Perak River Basin; Telok Anson**,** Fisheries Department, January 1951.

MZB 1218 (2 ex., 206.00-223.00 mm SL) Indonesia: Central Java; Bojonegoro; Solo River. Coll. Soetikno and Nurhasan, 25 June 1972.

CHAPTER 5: DNA BARCODING AS A TOOL FOR DETECTING POTENTIAL INVASIVE ASIAN PANGASIID CATFISHES (TELEOSTEI: PANGASIIDAE) IN PENINSULAR MALAYSIA

(Submitted to *Biochemical Systematics and Ecology* Journal)

5.1 Introduction

Fishes of the family Pangasiidae are medium to large-sized catfishes with diverse morphologies and ecologies. Adults range from 20 to 300 cm in length, and most species are more than 50 cm. Pangasiids are generally found in freshwater areas; however, some species can also be found in brackish and marine environments (Roberts & Vidthayanon, 1991). Pangasiid catfishes are widely distributed throughout Asia, ranging of Pakistan to Indochina, the Indo-Malayan Archipelago (Roberts & Vidthayanon, 1991) and China (Yang et al., 2007). There are 28 valid species (Froese & Pauly, 2016; Nelson, 2006) within four genera: *Helicophagus* Bleeker 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, in Cuvier & Valenciennes, 1840, and *Pseudolais*, Vaillant 1902 (Ferraris, 2007; Gustiano, 2003; Kottelat, 2013).

Several taxonomic ambiguities were encountered with respect to this group because of the morphological variations between conspecifics found on the Asian Mainland and the Indo-Malayan Archipelago (Roberts & Vidthayanon, 1991), including morphologically disparate life stages (Gustiano et al., 2004), species complexes (Gustiano et al ., 2003; Pouyaud et al., 2002) and local-scale ecological variations in morphology (Haslawati et al., 2014; Gustiano et al., 2004; Roberts & Vidthayanon, 1991).

Pangasiids are valued in aquaculture and have a global demand that is expanding rapidly, especially in European countries and The Russian Federation (FAO, 2009). Major markets include European countries, other Asian countries, Mexico, Australia, the USA, the Middle-East and Russia (Nguyen, 2008). Vietnam is the major producer, with more than 1.1 million tonnes of production in 2008 (POSMA, 2009). The most

commercially farmed *Pangasiu*s species are *Pangasianodon hypophthalmus* and *Pangasius bocourti* catfishes, which are exported, mainly in filleted forms, to more than 130 countries (Phan et al., 2009). The juveniles of striped catfish (*P. hypophthalmus*) are also traded as ornamental fish (Singh & Lakra, 2012; Więcaszek et al., 2009). Despite their economic importance, however, several other pangasiid species are reported to be rare in the wild, with declining populations due to threats of extinction (Haslawati et al., 2014) . They are *P. gigas* (Hogan, 2013), *Pangasius krempfi* (Baird, 2013), *Pangasius myanmar* (Chaudhry, 2010), *Pangasius pangasius* (Hossain et al., 2009; Pal, 2010), *Pangasius sanitwongsei* (Jenkins et al., 2009) as well as *Pseudolais micronemus* (Vidthayanon, 2012f) and *Pangasius pleurotaenia* (Vidthayanon, 2012g) among few others.

In Malaysia, pangasiid catfishes are also among the most popularly consumed freshwater fish (Department of Fisheries Malaysia, 2010-2015). Only four species are recorded as being native to Peninsular Malaysia: *Helicophagus waandersii, Pangasius nasutus, Pangasius polyuranodon* and *Pseudolais micronemus* (Haslawati et al., 2014; Lim & Zakaria-Ismail, 1995; Roberts & Vidthayanon, 1991; Herre & Myers, 1937; Tweedie, 1936). *Pangasius nasutus* and *P. polyuranodon* are currently listed as Least Concern in the IUCN Red List (Vidthayanon, 2013a; 2013b). Additionally, Chong et al. (2010) listed *H. waandersii* and *P. nasutus* as Moderately Threatened (MT), which is equal to endangered (EN) in the IUCN Red List. In contrast, *P. micronemus*, which is very abundant in Malaysia, has declining population trends reported in Cambodia and Vietnam (Haslawati et al., 2014; Vidthayanon, 2012f).

The wild Malaysian native pangasiid populations can fetch high market prices: 40-60 MYR/kg for *P. nasutus* and as much as 280-300 MYR/kg for *H. waandersii.* (1 USD = MYR 4.39). High local demand has grabbed the interest of breeders and aquaculturists, with regard to importing and culturing the species, especially from Chao

Phraya and Mekong rivers. Some of the introduced species might have established themselves in the new environment and affected the native populations in some way.

Hybridisation between the wild and imported pangasiids is being practised by local breeders to cater to the high demand and preference for the local species (personal observation; Anuar, 2011). Moreover, in any aquaculture practice, there is a large possibility of having escapees and of introduced species becoming hybrids with the natural populations (Gross, 1998; McGinnity et al., 2003; Na-Nakorn et al., 2004; Singh & Lakra, 2012; Slaney et al., 1996).

Hybridisation would lead to heterosis due to over dominance and heterozygosity at many loci, with transmission of genes from one species to another. Species loss via direct genetic changes can occur in two ways; reduction of effective population size by ecological/biological and genetic effects and alteration/extinction of gene pools of the fish stocks by crossbreeding (hybridisation) and backcrossing (Nguyen & Na-Nakorn, 2004). In Thailand, populations of the native *Clarias macrocephalus* are facing a threat of extinction due to the hybridisation of female *C. macrocephalus* and male introduced *C. gariepinus*. These fertile hybrids are capable of back crossing with their parents; contribute to 90% of Thailand's *Clarias* production (Nukwan et al., 1990). Eventually, these introgressed populations might escape to a natural water body and destroy the genetic integrity of the native *Clarias* (Senanan et al., 2004; Na-Nakorn et al., 2004). Similarly, aquaculture hybrid *C. batrachus* x *C. gariepinus* also caused problems in Bangladesh (Rahman et al., 1995).

Given the taxonomic challenges discussed above, we proposed DNA barcoding be incorporated into the identification of pangasiid catfishes in Peninsular Malaysia. DNA barcoding is the use of a short standard mitochondrial DNA sequence – *cytochrome c oxidase I* (COI) gene sequence - as a genetic marker for species identification in many

fish biodiversity studies (Bhattacharjee et al., 2012; Chu et al. 2013; Hebert et al., 2003; Ratnasingham, & de Waard, 2003; Hubert et al., 2008; Serrao at al., 2014; Ward et al., 2005). The COI gene is short enough to be sequenced quickly and cheaply yet long enough to characterise variations among species.

Here, we investigate the utility of COI-based DNA barcoding as a tool for the rapid and accurate identification of invasive pangasiid catfishes by evaluating its ability to distinguish between the native and potentially invasive species. Implications for conservation will be discussed for fisheries management and native pangasiid conservation.

5.2 Materials and Methods

5.2.1 Sample Collection

Pangasiids were collected from two rivers in Peninsular Malaysia in which the species are known to be abundant: the Pahang River and Perak River (Fig 5.1). There were more site collections for Pahang River (four sites) compared to only one site for Perak River because the Pahang River is well known for its native pangasiid population. Local fishing gear such as gill nets, drift gill nets, long-lines, hook and lines and other traditional methods were used for specimen collection. Details on the site description and fishing methods were discussed in Chapter 3 – Materials and Methods section.

Specimens were identified on site based on their external morphology (following Gustiano, 2003; Roberts & Vidthayanon, 1991; Vidthayanon, 1993). Doubtful specimens were further examined in the laboratory. Voucher specimens were fixed in 10% formalin for at least seven days; they were rinsed with tap water and soaked for another seven days

and, then, transferred to 70% ethanol. They were then deposited in the Fisheries Research Institute (Freshwater Division) FRI Glami Lemi, Negeri Sembilan, Malaysia.

5.2.2 Molecular Analysis

Muscle tissues were taken from the caudal region on the right side of the fish. Photographs were taken on the left side of the specimens. Forceps and scissors were carefully rinsed with distilled water to avoid cross-contamination between the tissue samples. Tissue samples were preserved in 90% ethanol and stored at 4°C until further analysis.

5.2.2.1 DNA extraction, PCR amplification and sequencing

DNA was extracted using Promega Wizard Genomic Animal Tissue Extraction Kit (Mouse Tail Procedure) (Promega, Madison, WI, USA) following the manufacturer's guidelines. Cytochrome c Oxidase I (COI) was amplified using primers from Ward et al. (2005) with slight modifications. Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl that contained 1.5 µl DNA template, 2.5 µl 10x PCR buffer, 3.3 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 1.5 µl of each primer and 5U Taq DNA polymerase. Thermocycling conditions were conducted with an initial denaturation at 94° C for 2 min, followed by 35 cycles of denaturation at 94°C at 30 s, annealing for 40 s at 58° C, extension at 72° C for 1 min and a final extension for 10 min at 72° C. Three µl of successful amplified PCR product was checked by electrophoresis with 1% agarose that contained ethidium bromide staining, and the results were visualised under UV illumination. Both strands of PCR fragments were sequenced to obtain a consensus sequence.

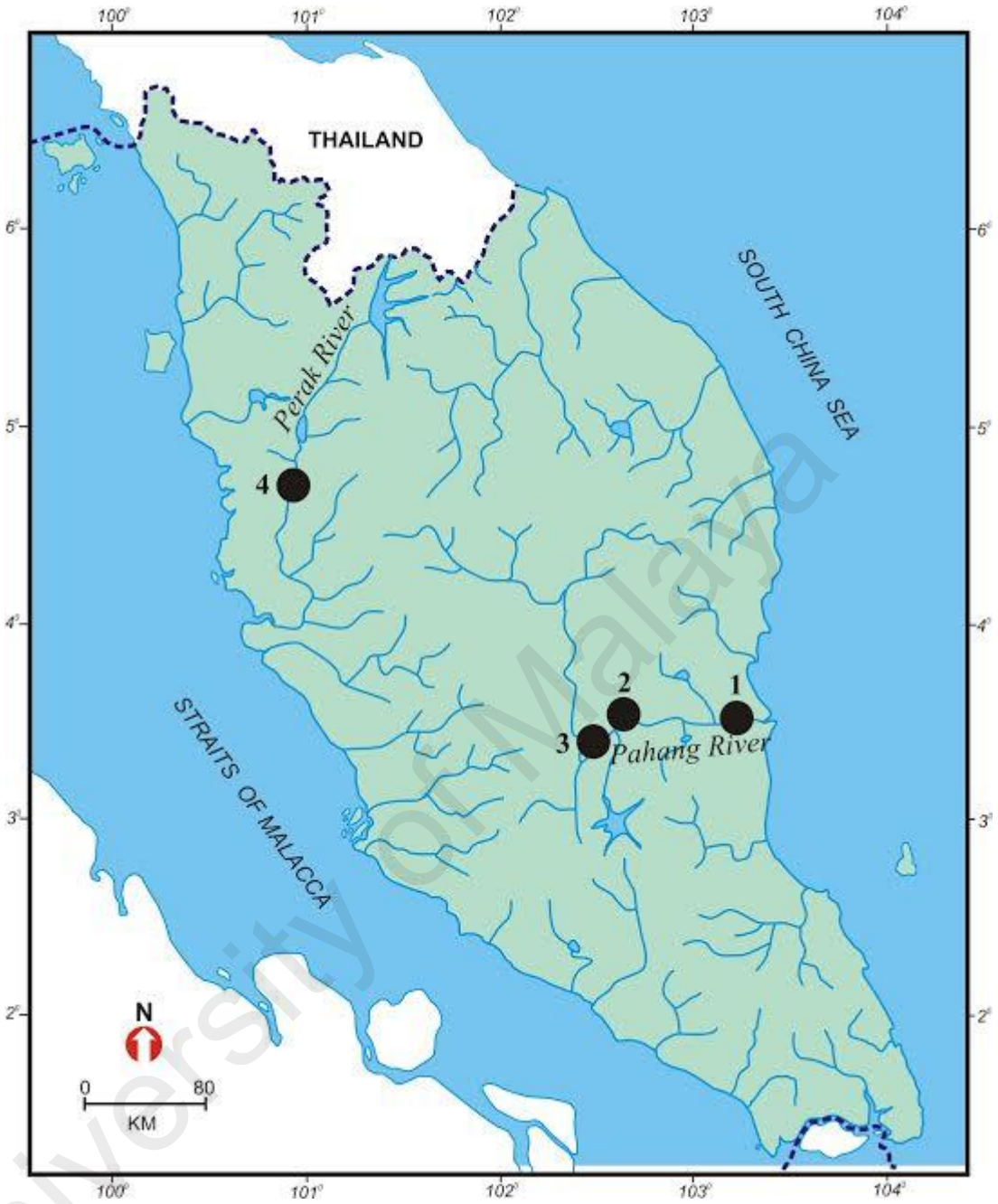


Figure 5.1: Map of Peninsular Malaysia showing showing major rivers and its tributaries. Collection sites of the pangasiid species from two main river basins, the Pahang River and the Perak River are presented as (●). Numbers refer to the following locations: (1) Paloh Hinai, Pekan, Pahang; (2) Lubok Paku, Maran, Pahang; (3) Chenor, Maran, Pahang (Pahang River); and (4) Manong, Kuala Kangsar, Perak (Perak River).

PCR products were purified with the Promega PCR Purification Kit following the manufacturer's protocol. Purified samples were then sent for the sequencing service at First Base Laboratories, Sri Kembangan, Selangor, Malaysia. To control the sequence accuracy and resolve any ambiguous bases, the same primer pairs in both directions were used for cycle sequencing using the ABI PRISM Dye-Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresis (on an Applied Biosystems Automated Sequencer).

5.2.2.2 Sequence analysis

Sequences were viewed and edited manually using Chromas version 1.45 (MacCarthy, 1996), whereas contiguous sequence (contig) assembly and multiple sequence alignment was performed using ClustalW (Thompson et al., 1997). All of the sequences generated were deposited in the NCBI GenBank, together with the specimen voucher numbers, with nomenclature suggested by Chakrabarty et al. (2013) (Table 5.1). Other published Pangasiid COI sequences from the GenBank database were also included in the analysis.

The final alignment was screened for the stop codons and insertion-deletion mutations using the same software, to ensure that there were no amplifications of non-target fragments (Song et al., 2013). In addition, the determinations of base compositional frequencies and nucleotide substitutions between pairwise comparisons were performed using MEGA 6.0 (Tamura et al., 2013).

A phylogenetic analysis was performed to illustrate the divergence and relationships among the taxa using the same software. Other Siluriformes sequences, *Hemibagrus macropterus* (family Bagridae), *Clarias batrachus* and *Clarias macrocephalus* (family Clariidae), were chosen as outgroups to root the tree. The mean pairwise genetic distance matrix was calculated using the Kimura two parameter (K2P) model with the pairwise deletion of gaps (Felsenstein, 1985). A neighbour-joining tree

was constructed and the tree robustness was assessed by bootstrapping analysis with 1000 replicates.

5.2.2.3 Automatic Barcoding Gap Discovery (ABGD)

The online version of the Automated Barcoding Gap Discovery (ABGD) web tool (http://wwwabi.snv.jusieu.fr/public/abgd/abgdweb.html) (Puillandre, Lambert, Brouillet, & Achaz, 2012) was used to determine the barcode gap occurrence, and it partitioned the sequences into putative groups or species by first implementing the default parameters (Khare et al., 2014; Khedkar et al., 2014) (Pmin = 0.001, Pmax = 0.1, Steps = 10, X (relative gap width) = 1.5, Nb bins = 20). Kimura 2-parameter (K2P) distances were used to correct the transition rate bias in the substitutions. The default for the minimum relative gap width was set to values between 0 and 1.2 (Puillandre et al., 2012).

Table 5.1: Species list, sampling location/rivers, country, specimen voucher, GenBank accession number of specimens used for barcoding analysis.

Species	Sampling location, river	Country	Specimen voucher	Accession number[@@]	Reference
Helicophagus waandersii	Lubok Paku, Pahang River	Malaysia	Hewa03LP	KP036415**	This study
H. waandersii	Lubok Paku, Pahang River	Malaysia	Hewa07LP	KP036416**	This study
H. waandersii	Paloh Hinai, Pahang River	Malaysia	Hewa24PH	KP036417**	This study
P. hypophthalmus	na	na	na	NC021752	Zhao et al. (2013)
P .hypophthalmus	Kuala Kangsar, Perak River	Malaysia	Pahy0901KK	KP036425**	This study
P. hypophthalmus	Paloh Hinai, Pahang River	Malaysia	Pahy60PH	KP036426*	This study
P. hypophthalmus	Laguna de Bay, Laguna, Calabarzon	Philippines	Phyp1- Phyp4-LdB	HQ682713-16	Aquino et al. (2011)
P. hypophthalmus	Nakhon Ratchasima Province	Thailand	AUPH1 - AUPH8	JF292393 - JF292399	Wong et al. (2011)
P. hypophthalmus	Nakhon Ratchasima Province	Thailand	AUPH10 - AUPH20	JF292400 - JF292410	Wong et al. (2011)
P. hypophthalmus	na	na	PANGHYPO-J01-009	EU752151	Yancy et al. (2008)
P. hypophthalmus	An Giang	Vietnam	BW-1778	EF 609427	Ward & Holmes (2007)
P. bocourti	Paloh Hinai, Pahang River	Malaysia	Pabo55PH	KP036428*	This study
P. bocourti	na	na	PANGBOCO-J01-003	EU752149	Ward & Holmes (2007)
P. bocourti	An Giang	Viet Nam	BW-1791	EF 609425	Ward & Holmes (2007)
P. bocourti	Yasothon Province	Thailand	AUPB2	JF292411	Wong et al. (2011)
P. bocourti	Yasothon Province	Thailand	AUPB4 - AUPB7	JF292412 - JF292415	Wong et al. (2011)
P. bocourti	Yasothon Province	Thailand	AUPB9 - AUPB22	JF292416 - JF 292429	Wong et al. (2011)
Pangasius conchophilus	Paloh Hinai, Pahang River	Malaysia	Paco23PH	KP036413*	This study
P. conchophilus	Chenor, Pahang River	Malaysia	Paco03CR	KP036414**	This study
P. conchophilus	na	Vietnam	BW-1796	EF609426	Ward & Holmes (2007)
Pangasius djambal	Paloh Hinai, Pahang River	Malaysia	Padj53PH	KP036427*	This study

Table 5.1: Continued

Species	Sampling location, river	Country	Specimen voucher	Accession number	Reference
Pangasius nasutus	Paloh Hinai, Pahang River	Malaysia	Pana57PH	KP036410*	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	Pana61PH	KP036411*	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	Pana93PH	KP036412*	This study
P. nasutus	Pahang River	Malaysia	SLM-PN(PH)-01- 04	JF781172 - JF781175	Song et al. (2013)
Pseudolais micronemus	Kuala Kangsar, Perak River	Malaysia	Pami0801KK	KP036418**	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	Pami50PH	KP036419*	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	Pami52PH	KP036421*	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	Pami59PH	KP036422*	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	Pami51PH	KP036420*	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	Pami85PH	KP036423*	This study
P. micronemus	Kuala Kangsar, Perak River	Malaysia	Pami0902KK	KP036424**	This study
P. micronemus	Pahang River	Malaysia	SLM-PM(PH)-01 - SLM-PM(PH)-05	HM156360 - HM156364	Song et al. (2013)
Hemibagrus macropterus	Hechuan, Chongqing	China	na	NC019592	Zeng, et al. (2012)
Clarias macrocephalus	Faculty of Fisheries, Kasetsart University	Thailand	AUCM19	JF292337	Wong et al. (2011)
C. batrachus	Nakhon Ratchasima Province	Thailand	AUCB1	JF292297	Wong et al. (2011)
C. batrachus	Laguna de Bay	Philippines	Cbat3-LdB	HQ682679	Aquino et al. (2011)

@@GenSeq nomenclature from Chakrabarty et al. (2013)
*genseq-4: collection-vouchered non-types
**genseq-5: non-types that have photo vouchers but lack a specimen voucher

5.3 Results

5.3.1 DNA sequence variation

The analyses involved 75 nucleotide sequences of Pangasiids with four outgroup catfishes, which produced a final trimmed alignment of 620 base pairs. The alignment contains 492 (79.35%) conserved sites and 128 (20.65%) variable sites, of which 120 sites (19.35%) are parsimony informative. No indels or stop codons were observed, which suggests that there were no pseudogenes. The average base composition was 30.4 % (T), 26.0% (C), 26.2% (A) and 17.2% (G). The estimated transition/transversion (si/sv) ratio, R, ranged from 0.10 to 18.25.

The mean corrected pairwise distances (K2P) among the eight species of Pangasiids are shown in Table 5.2. Interspecific divergence between the species ranged from 3% to 19.7%, which is sufficiently sensitive to delineate species. A barcode gap is detected where the minimum interspecific divergence (3.0 %) is lower than the maximum intraspecific divergence (5.10 %).

The lowest interspecific divergence value (3 %) was found between *P. nasutus* and *P. conchophilus,* which indicates that there is a close genetic distance. However, the intraspecific value of 0.051 for *P. conchophilus* is higher than the interspecific pairwise value (0.030) between *P. conchophilus* and *P. nasutus,* which suggests a possible sign of species-complex or evidence of hybridisation following unclear species delineation for *P. conchophilus.*

Pangasius djambal and *P. bocourti* were the second lowest in divergence value (7.1%). Within *Pangasius*, the genus that has the largest number of species in the family, the highest divergence value (11.1%) was observed between *P. bocourti* and *P. conchophilus*.

As expected, the highest pairwise divergence value (19.7% divergence) was found to be between the species of two genera, *H. waandersii* (*Helicophagus*) and *P. hypophthalmus* (*Pangasianodon*). Apart from *Pangasius*, other genera in the Pangasiidae family (*Pangasianodon*, *Pseudolais* and *Helicophagus*) were represented by only a single species each and do not have any comparisons of COI sequences either from Genbank or from this work. With respect to the interspecific divergence, the lowest value was observed in *H. waandersii* (1.0% divergence), while the highest (5.1% divergence) was observed in *P. conchophilus* (Table 5.2).

5.3.2 Neighbour-joining (NJ) analysis

Relationships between Pangasiid COI sequences were reconstructed using K2P model and showed in NJ tree (Figure 5.2). The branches are monophyletic according to their respective genera (*Pangasius, Helicophagus, Pseudolais* and *Pangasianodon*) relative to the outgroup. Using Siluriformes as outgroups, an 86% bootstrap value separated the Pangasiidae family into the five clades. Five major clades (A-E) were identified based on high bootstrap values (> 90%) of distinctive lineages. Clades A (*P. bocourti* and *P. djambal*) and B (*P. conchophilus* and *P. nasutus*) represent the genus *Pangasius*, while Clades C, D and E identify the monotypic genera of *Helicophagus* (*H. waandersii*), *Pseudolais* (*P. micronemus*) and *Pangasianodon* (*P. hypophthalmus*), respectively. Although the five clades were well defined, their branching order was not resolved (polytomy) due to the low bootstrap values (< 70%), which suggests simultaneous radiation.

Clade E (*Pangasianodon*) is well distinguished from the other four clades (Clades A, B, C and D) with a high confidence level (99%). A medium bootstrap value (51%) separated the genus *Pangasius* (Clades A and B) from Clade C (*Helicophagus*) and Clade D (*Pseudolais).* The two clades within *Pangasius* were divided by a 64% bootstrap value

and further differentiated into their respective clades by a strong bootstrap confidence (99%).

Within the clades, our sequences show some differences in the evolutionary distance from other sequences obtained from Genbank. *Pangasius bocourti* KP036428 is very close to *P. djambal* KP036427 but has a small divergence from other *P. bocourti* sequences, with a 37% bootstrap value. Other *P. bocourti* are clustered together with an 86% bootstrap confidence.

A similar result was also observed in Clade B, where our sequences of *P. conchophilus* (KP036413 and KP036414) matched with EF609426 from Vietnam (Ward & Holmes, 2007). In this clade, *P. nasutus* and *P. conchophilus* should have been separated into two groups with a bootstrap value of 99%. Clearly, *P. nasutus* sequences from this study (KP036410-12) showed a consistent grouping, together with one sequence (JF781172) from Song et al. (2013). However, the other three sequences of *P. nasutus* from their study (JF781173-75) were clustered into the *P. conchophilus* group. One of the possible explanations was most likely due to misidentification during sampling, by a wrongly identified sample or samples taken from hybrid specimens.

5.3.3 Automatic Barcoding Gap Discovery (ABGD)

The ABGD analysis indicates that by using the default standard settings, a barcode gap is detected between the intraspecific and interspecific distances. A barcode gap is observed whenever the divergence among the organisms from the same species (intraspecific) is smaller than the divergence between different species (interspecific) (Puillandre et al., 2012). In this analysis, the sequences were partitioned in an initial approach into a very stable five species, as shown in Figure 5.3b. In the recursive approach, another two additional species can be recognised. This finding is congruent with the primary species concept; the threshold value (P = 0.021544), which defines the species boundary of Pangasiids COI sequences analysed in this study, followed the

observations on the Indian Mahseers (Khare et al., 2014) and the Narmada River fishes (Khedkar et al., 2014).

The value above, which partitioned the sequences into five groups, has also produced the same groupings as in the phylogenetic tree. The values below threshold are treated as false positives because real species would split into two or more partitions. No barcode gap is shown in the values above the threshold value, which are thus treated as false negatives. Conversely, the ABGD method, which is based on pairwise distances, clearly defined the groupings of sequences to be congruent with the pairwise intraspecific divergence in Table 5.2, which could not be differentiated into distinctive clusters according to species using the phylogenetic concept.

Table 5.2. Mean corrected (K2P) genetic distance (pairwise intraspecific, *p* divergence) and interspecific divergence value, *d*, of Pangasiid cytochrome c oxidase I (COI) sequences from this study. The percentage of divergence is shown in parentheses.

No	Species	1	2	3	4	5	6	*d*
1	*Pangasius nasutus*	-						0.011
2	*Pangasius conchophilus*	0.030 (3.0)	-					0.051
3	*Pangasius bocourti*	0.120 (12.0)	0.111 (11.1)	-				0.012
4	*Pangasius djambal*	0.065 (6.5)	0.083 (8.3)	0.071 (7.1)	-			n/c
5	*Pangasianodon hypophthalmus*	0.157 (15.7)	0.144 (14.4)	0.117 (11.7)	0.163 (16.3)	-		0.014
6	*Pseudolais micronemus*	0.077 (7.7)	0.097 (9.7)	0.133 (13.3)	0.074 (7.4)	0.166 (16.6)	-	0.008
7	*Helicophagus waandersii*	0.113 (11.3)	0.129 (12.9)	0.168 (16.8)	0.097 (9.7)	0.197 (19.7)	0.090 (9.0)	0.001

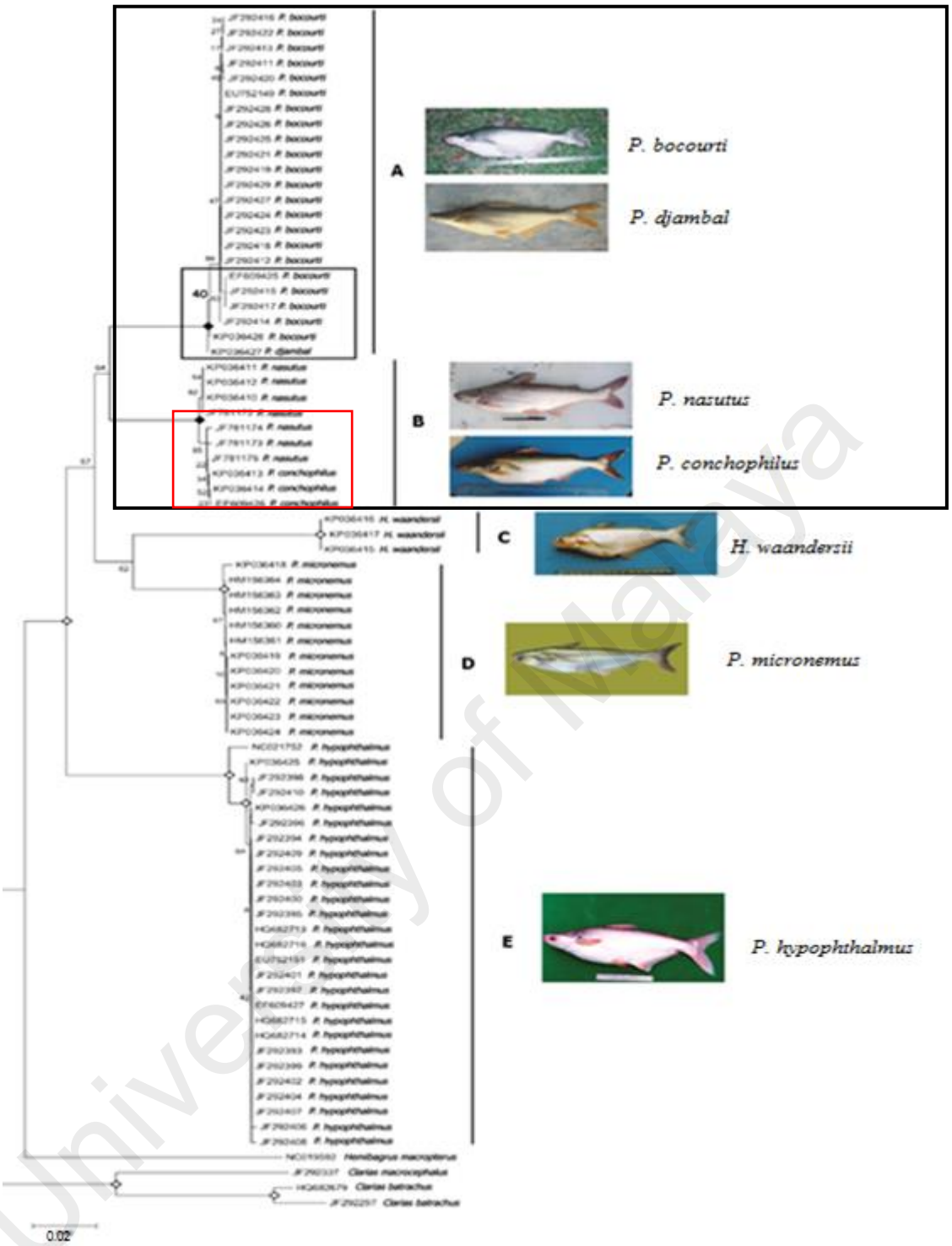
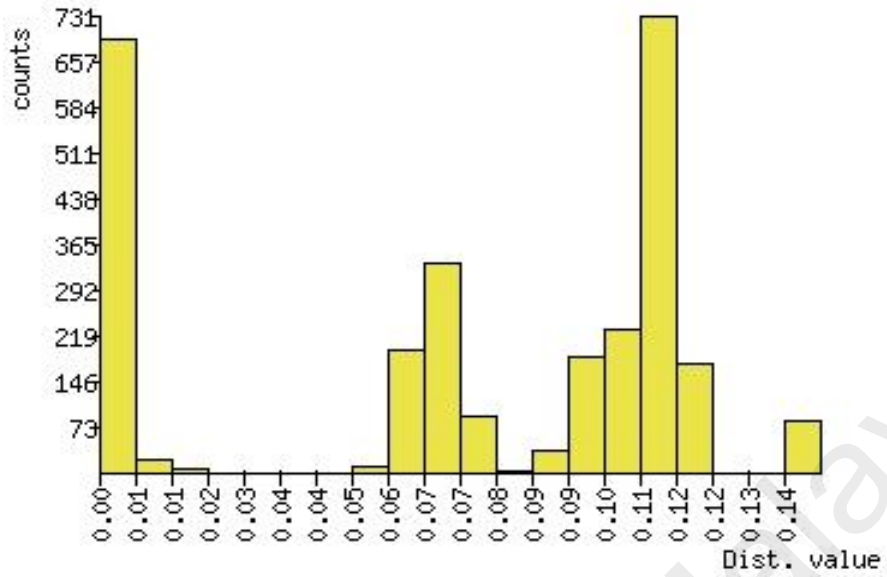


Figure 5.2. Neighbour-joining tree of Kimura-2-Parameter distances for Peninsular Malaysian pangasiids combined with the publicly available pangasiid COI sequences from GenBank and outgroups from the Siluriformes family. Bootstrap values are given near branches. Both black and white dots indicate support values higher than 95%. Outer box and full black dots near branch (◆) indicate problematic groups. The scale below indicates the branch length.

(a)



(b)

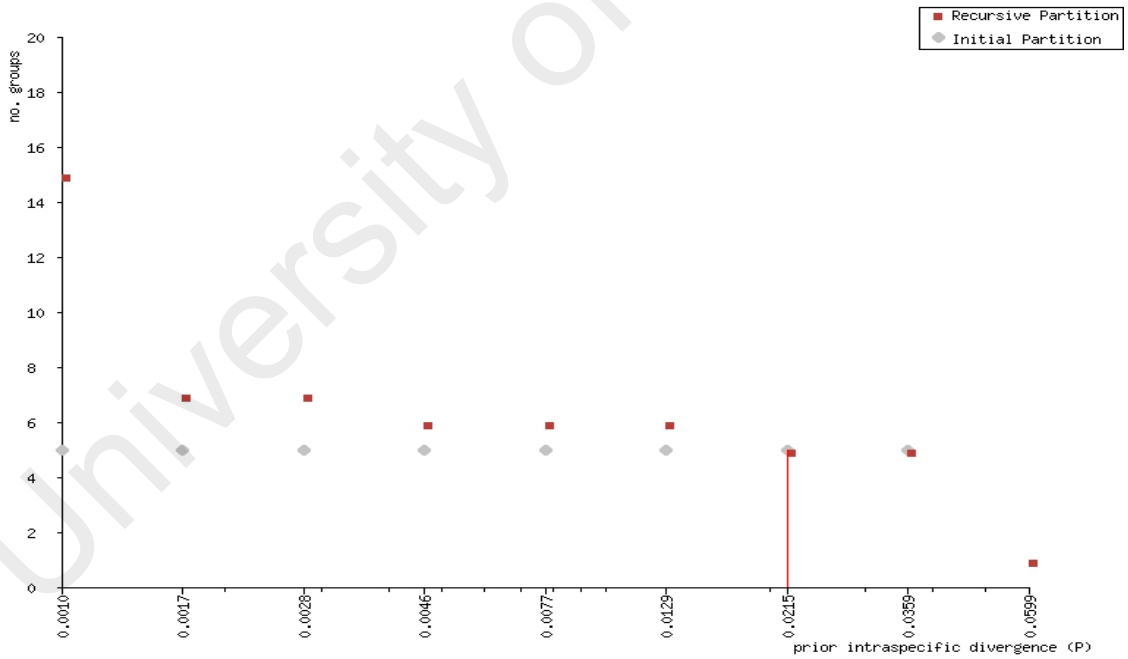


Figure 5.3: Barcode gap analysis of pangasiid species generated by Automatic Barcode Discovery Gap Discovery. Distributions of K2P distances and between each pair of specimens for the COI gene (a) histogram of the distance and (b) number of species obtained for each prior intraspecific divergence.

5.4 Discussion

5.4.1 Species relationship and taxonomic accounts

The reconstructed neighbour-joining tree demonstrates the relationships between the native and introduced pangasiids species. The clades were monophyletic according to their genera except for the genus *Pangasius*. In this analysis, *Pangasianodon* is clearly separated as the basal lineage, which is similar to the observations of Karinthanyakit & Jondeung (2012). The difference in the swimbladder chamber (a single chamber vs two to four in other Pangasiids) and pelvic fin rays (eight to nine pelvic fin rays vs six in other genera) might have contributed to this character (Karinthanyakit & Jondeung, 2012). Sharing six pelvic fin rays, the genus *Helicophagus* is characterised as having a combination of a slender anterior snout (< 16.5% HL) and a predorsal length of 34.5-40.5% SL. While *Pseudolais* can be differentiated from other genera by large eye diameter, minute maxillary barbel and minute adipose fin. *Helicophagus* and *Pseudolais*, are defined as sister groups. The final genus, *Pangasius,* can be differentiated by these characters; relatively long maxillary barbel, robust dorsal and pectoral fins as well as robust adipose fin (Gustiano, 2003).

Relationships between species in the genus *Pangasius* using COI remain unresolved. Previous studies have also failed to resolve their genetic relationships using different genetic markers (Karinthanyakit & Jondeung, 2012; Na-Nakorn et al., 2006; Pouyaud et al., 2004; Pouyaud et al., 2000) even though several genes have been tested in defining their intraspecies relationships. In the present study, the COI analysis shows the clustering of *P. bocourti* and *P. djambal* in a common group, although both have a 7.1% genetic distance. In fact, they are two allopatric species that have a very close morphological appearance (Roberts & Vidthayanon, 1991; Vidthayanon, 1993). Two taxonomic keys of pangasiids were created to distinguish *P. bocourti* (described from the

Asian mainland) from *P. djambal* (a species from the Indo-Malayan Archipelago) (Roberts & Vidthayanon, 1991). *Pangasius bocourti* is widely cultured in the Mekong Delta, mostly in southern Vietnam (Cacot et al., 2003). The taxonomy of this species is still problematic, and the species currently described as *P. bocourti* could be an undescribed species (Poulsen et al., 2004). However, from the sequences analysed in this study, there is no evidence for this confusion.

The morphologically closest species to *P. bocourti*, *P. djambal*, has the same features of palatal dentition but with a much wider median vomerine toothplate and a larger palatine juxtaposed to it. In addition, the head shapes are indistinguishable. The two species differed from each other only by the higher number of gill raker counts in *P. bocourti* (36-46 vs 24-35 (Roberts & Vidthayanon, 1991), 35-47 vs 27-39 (Gustiano et al., 2004). Roberts & Vidthayanon (1991) observed some well-marked colour patterns on the fins, but these were not observed in this study. Some of the distinguishing characters might be well developed only upon maturation (such as the gill raker counts and dentition characters); therefore, it is challenging to differentiate the fish at a younger age (we refer to the description of *P. bedado* in Roberts, 1999, as a junior synonym of *P. djambal* (Gustiano et al., 2004)). Therefore, it is somewhat difficult to distinguish between these two species only by using the partial COI gene; other characters that are expressed only by proteins must be incorporated if the DNA method is to be used.

The NJ tree also revealed other allopatric species, *P. nasutus* and *P. conchophilus*, to be in the same clade, discriminated by a genetic divergence of 3%, which is a threshold value for species delimitation (Meier et al., 2005). These two species differ morphologically by only two main distinguishing features; the more pointed snout (in large adults of *P. nasutus*) and larger eye diameter (only observed in specimens of more than 300 mm of *P. conchophilus*).

In ABGD analysis, a barcode gap is detected between the intraspecific and interspecific distances. A barcode gap is the difference between the maximum intraspecific and minimum interspecific distances, which is well defined in this analysis (Puillandre et al., 2012). Five stable species were observed and partitioned initially, and by using a recursive approach, another two additional species could be recognised. However, this approach requires confirmation by integration with other methods; initial morphological identification, additional genetic loci and specimens (Khedkar et al., 2014; Puillandre et al., 2012). The ABGD method is solely based on pairwise genetic differences; it does not rely on the genealogical tree and the properties of the internal nodes. As a result, it works well on speciation radiations, bifurcating events or both speciation mixed.

However, for recently diverged speciation, a barcoding gap might not be present (Meyer & Paulay, 2005), and it is not possible to use genetic data inference (Puillandre et al., 2012). This hindrance could be attributed to the unclear separation in the *Pangasius* genus, which diverge more recently (in the late Pleistocene period – 3.99 Million years before present, MBp) compared to *Helicophagus* and *Pseudolais* (4.26 MBp) and *Pangasianodon* (late Miocene, 6.75 MBp) (Karinthanyakit & Jondeung, 2012). *P. nasutus* and *P. conchophilus* diverged as recently as 1.74 MBp in the mid-Pleistocene period. Although there has been no report on the divergence of *P. bocourti* and *P. djambal* when calibrated using the *Pseudotropius* basis (Karinthanyakit & Jondeung, 2012), a similar recent divergence was predicted to occur in the late Pleistocene (0.3-1.0 MBp) (Pouyaud et al., 2013), at nearly the same time as another allopatric species in the Pangasiid family (*H. leptorhynchus* vs *H. typus* – 0.29 MBp) (Karinthanyakit & Jondeung, 2012).

5.4.2 Pangasiid species diversity and impacts of aquaculture practices

This study utilises the COI-based barcoding gene as a genetic tool to evaluate the Pangasiid catfish species found in Peninsular Malaysia and assesses the impact of human activities, especially the aquaculture practices for the abundance and survival of native species.

From the results, three species known as native (*H. waandersii*, *P. nasutus* and *P. micronemus*) are potentially threatened by the current aquaculture practices, with introductions of new pangasiid species from Indo-China and Thailand, namely *P. conchophilus*, *P. hypophthalmus* and *P. bocourti*. DNA barcoding revealed their close identity through phylogenetic relationships and further genetic analyses (ABGD method and K2P genetic distance). These genetic analyses have facilitated the identification of morphologically close pangasiid species thus evaluate their impacts on conservation strategies.

Many factors can affect the survival of the natives, among them is the genetic impacts from the introduced species. As shown in Figure 5.2, *P. conchophilus*, shows a close genetic relationship to the native *P. nasutus,* due to the position in the same group (Clade B). Close genetic distance (3% divergence) indicates that they are two different species but very closely related. These two morphologically similar species were once known as one species until Roberts & Vidthayanon (1991) revealed *P. conchophilus* as a new, distinct species. Due to the close morphological characters, in the field, they can be easily misidentified, like the samples of *P. nasutus* JF781173-75 from Song et al. (2013), which clustered with *P. conchophilus*. One of the possible explanations was most likely due to misidentification during sampling, by a wrongly identified sample or samples taken from hybrid specimens. In this case, further confirmation must include other marker such as nuclear gene, since COI as a maternally inherited marker could not detect hybrids.

Pangasius conchophilus was introduced in the 1990s by immigrants from Cambodia. From personal observation, the authors were informed that the reason for the introduction was mainly to fulfill the high demand for the native *P. nasutus.* The close morphological appearance could create confusion to the non-expert, which would make it marketable. They are cultured by the Cambodian immigrants near Pekan, Pahang (near location 1- Figure 5.1), in the lower reaches of the basin. Ironically, one specimen in this analysis (KP036414) was collected from Chenor, Pahang, which is located in the middle stretch of the river (location 3 – Figure 5.1). Local fishermen reported that they often found both species together in their nets, which meant that they are now occupying the same habitat and living sympatrically in harmony. Their close genetic and morphological relationship might produce natural crossbreeds or hybrids in the near future.

The Pahang River is home to many freshwater fish species; among those that are popularly known are the *Jullien*'s River Carp, *Probarbus jullienni*, and the Pangasiids; many of them share a preference for a special feeding item, the moluscivourous bivalve, *Corbicula* sp. Competition for habitat (space) and food are one of the factors that affect the biodiversity (Gozlan et al., 2010; De Silva et al., 2006). This special mollusc preference behaviour, which was not observed in other *Pangasius* spp., derived the name *P. conchophilus*, (etymology; concho = mollusc, philus/philic = like) (Roberts & Vidthayanon, 1991) is likely to harm or compete with the local fish, not only the pangasiids.

Pangasius bocourti is a commonly cultured pangasiid species. However, there is no record on the introduction of this species into Malaysia. It is believed that aquaculturists imported the fry from Thailand in bulk and misidentified the species as *P. nasutus* (a species that has a high local demand). It is a common practice to culture pangasiids without knowing what species they are. The close morphological appearance to *P. djambal* has caused the locals to believe that this species as a native species even

though both were not recorded previously (Herre & Myers, 1937; Lim & Zakaria-Ismail, 1995; Roberts & Vidthayanon, 1991; Tweedie, 1936).

Pangasianodon hypophthalmus, a popular worldwide culture species, was introduced in the 1980s by the Department of Fisheries, Malaysia. Ever since successful induced breeding (Saidin et al., 1988), the culture of this species has gained popularity among the farmers because of its ideal culture characteristics. The species has a fast growth rate and can breed artificially with low culture maintenance (in floating cages) (Saidin et al., 1988). It can be cultured using many different types of culture practices, including pond cultures, and more specifically, is popular in floating cages along the Pahang River basin.

In many Asian countries, where emphasise is more towards aquaculture development (De Silva et al., 2006), to increase production, thus the impacts of introduced species on biodiversity is rarely evaluated (De Silva et al., 2009). There are many examples of the effect of introduced species on biodiversity conservation: through predation, genetic interactions (hybridisation, introgression and other indirect genetic effects), habitat use and modification and transmission of a novel disease (Gozlan et al., 2010). Chong et al. (2010) considered that the establishment of *P. hypophthalmus* to Malaysia is not as invasive as Tilapia (*Oreochromis* spp.). However, there are reports in many other countries that show that such establishments in the wild have bad deleterious ecological impacts (Database on Introduction of Aquatic Species (DIAS) http://www.fao.org/fishery/dias/en; Fish Base www.fishbase.org; Singh & Lakra, 2012; Więcaszek et al., 2009).

Singh & Lakra (2012) discussed many possible impacts due to the introduction of *P. hypophthalmus* into India. Escapees from nearby cultured ponds and hatchery sites were detected in the open waters of West Bengal, Andhra Pradesh (Kolleru Lake), Kerala

and Uttar Pradesh. These escapees could possibly hybridise in the wild and, thus, could be a concern in the future (De Silva et al., 2006; 2009; Singh & Lakra, 2012).

Likewise, Więcaszek et al. (2009) found that hybrid refugees of *Pangasius* are frequently common in Poland waters and that it is also difficult to obtain pure species in aquarium trade. These *Pangasius* hybrids were also associated with novel Asian monogenoid parasites, which is a very alarming sign in European inland waters.

Fish health and management are also considered to be an issue that affects *Pangasius* introduction. There are reports on the risk of disease and parasites associated with catfish culture. A novel disease, bacillary necrosis of *Pangasius* caused by *Edwardsiella ictaluri* (a bacteria native to North America, from ictalurid catfish), was identified in farmed *P. hypophthalmus* cultures in the Mekong River (Crumlish et al., 2002; Ferguson et al., 2001) Another disease that is related to the Pangasiid culture is Aeromonad Septicaemia, which is caused by *Aeromonas hydrophila* (Crumlish et al., 2002; Subagja et al., 1999). Occurrences of pangasiid disease were reported in Bangladesh (Faruk, 2008) and New Zealand (Reed, 2008). Various ecto- and endo-parasites have also been found (Székely et al., 2008), including *Ichthyophthirius, Myxobolus* spp., and *Trichodina* sp., among other parasites. Very recently, Siti-Zahrah et al. (2014) reported the occurrence of a viral disease in farmed cages in Malaysia caused by the Channel Catfish Virus (CCV). They reported that this disease has caused economic loss to farmers, due to having a high mortality rate (30%-40%) within two years of the study (2010-2012). It is possible that this virus could also spread to the native species, considering that the commercial pangasiids are now well established in the river.

It is suggested that in the future, the introduction of exotic species (either for aquaculture, recreational fishery or aquarium trade) should follow the Code of Conduct for Responsible Fisheries, which was developed by FAO, and that these guidelines must

be further extended to the fisheries workers and general public. Control by people's actions is more effective than control through imposing laws (Welcomme & Vidthayanon, 2003). Under these circumstances, Department of Standard Malaysia (2007) has coordinated a development of multi-departmental consensus document called 'Malaysian Standard - Good Aquaculture Practice (GAqP) – Aquaculture Farm – General Guidelines MS 1998:2007', which is on appropriate biosecurity measures, disease control and trans-boundary fish. In this document, farmers are advised to conserve genetic diversity and maintain the aquatic ecosystem by minimising the effects of introducing alien species and genetically modified organism (GMO) fish in their culture practices (Department of Standard Malaysia, 2007).

Congruently, Gozlan et al. (2010) recommended several methods of characterising and managing the impacts, such as the usage of a fish invasiveness scoring kit (FISK) to evaluate the potential invasiveness. For those confirmed invasive populations, remediation or mitigation action could be implemented.

CHAPTER 6: PHYLOGENETIC RELATIONSHIPS OF PANGASIIDS (TELEOSTEI: PANGASIIDAE) IN PENINSULAR MALAYSIA BASED ON MITOCHONDRIAL DNA 16S RRNA AND CYTOCHROME OXIDASE I PARTIAL GENE SEQUENCES

6.1 Introduction

The catfishes of the family Pangasiidae are important Asian catfish widely distributed throughout Asia, from Pakistan to Indochina and the Indo-Malayan Archipelago (Roberts & Vidthayanon 1991). There are 28 valid species (Nelson 2006; Eschmeyer & Fong, 2016; Froese & Pauly, 2016), under four genera (Gustiano, 2003; Ferraris, 2007; Kottelat, 2013) namely *Helicophagus* Bleeker 1858; *Pangasianodon* Chevey, 1930; *Pangasius* Valenciennes, in Cuvier & Valenciennes, 1840, and *Pseudolais*, Vaillant 1902. Thirteen species occurring in Indo-China, fifteen species found in the Indo-Malayan Archipelago (Pouyaud et al. 1999; Pouyaud & Teugels 2000; Ng & Kottelat 2000; Pouyaud et al. 2002; Gustiano 2003, Kottelat 2013) while four species could be found in the Malay Peninsula (Gustiano 2003).

However, these groupings are still unstable at the genus level as discussed in Chapter 2 (Section 2.3). Roberts & Vidthayanon (1991) recommended two valid genera; *Pangasius* (19 species) and *Helicophagus* (2 species) which was further adjoined with two other genera (*Pangasianodon* and *Pteropangasius*) with further support from osteological characteristics (Vidthayanon & Roongthongbaisuree, 1993); Vidthayanon, 1993). Further, molecular systematic studies tried to support these classification and inferred phylogenetic relationships of this catfish by using allozyme and partial cytochrome b gene sequence of mitochondrial DNA (mtDNA) (Pouyaud et al., 2000), partial 12S rDNA mtDNA (Pouyaud et al., 2004), partial 16S rRNA mtDNA gene (Na-

Nakorn et al., 2006) and multigene combination (mtDNA and nuclear gene) (Karinthanyakit & Jondeung, 2012).

The genus *Helicophagus* and *Pangasianodon* were validated with allozyme and cytochrome b data (Pouyaud et al. (2000) but the status of other genera; *Pteropangasius* (*Pseudolais*) and *Pangasius* were not clarified. *Pteropangasius pleurotaenia* and '*micronema*' (*P. micronemus*) were suggested to be distinct groups and poor phylogenetic resolution within the Pangasiidae do not allow the possibility of finding subgenera. The unresolved phylogeny was also observed when using 16S rRNA (Na-Nakorn et al., 2006). The interspecies relationships were not well supported particularly in the genus *Pangasius*, whereby *Pteropangasius* is not recognised as a separate genus.

In another study using partial 12S rDNA, Pouyaud et al. (2004) revealed a clear groupings of the four genera by rooting to *Pteropangasius*, even with low confidence support. With exception to *P. lithostoma* (which joined the *Helicophagus* group) this gene recognised the generic separation and endorsed the closely related species, *P. djambal* vs *P. bocourti* and *P. nasutus* vs *P. conchophilus* with allopatric distributions between continental Asia and the Sunda region (Chapter 2, Figure 2.7).

Recently, the work of Karinthanyakit & Jondeung (2012) have further supported the four groupings of genera using complete mitochondrial cytochcrome b, 12S rRNA, tRNA-Val, 16S rRNA and partial nuclear gene of recombination-activating gene (*rag1*).

In order to develop any conservation strategies for species that have potential threats of extinction, levels of genetic variation should be examined further. Highly distinctive biota shows larger contribution to diversity, therefore should be given higher conservation priority (Vrijenhoek, 1998; Bowen, 1999). Gustiano (2003) suggested conservation priorites based on species, region and ecological niches. Taxonomically, high conservation priority should focus on species with distinct phylogenetic positions

such as *Pangasianodon gigas*, *Pteropangasius pleurotaenia*, *Pangasius pangasius* and *Helicophagus typus*. Regional area of species endemism (Kalimantan) and species that occupy ecologically different niches should also be given priority concern.

Na-Nakorn et al. (2006) highlighted the need to study the intraspecific genetic variations and interspecific relationships of pangasiids; however, in their study, only low levels of intraspecific variations were observed. These previous studies have added systematic and phylogenetic information for this catfish group, however, there is no attempt to include specimens or samples from Peninsular Malaysia which is in the middle region of freshwater faunal distribution in South East Asian region as previously discussed in Chapter 2, section 2.3.

This chapter presents an analysis on two partial mitochondrial DNA genes (non-protein coding gene partial mtDNA 16S rRNA (16S) and protein-coding Cytochrome c Oxidase I (COI)) to determine the phylogenetic relationships and genetic variation of the pangasiid catfishes in Peninsular Malaysia. 16S gene sequences was chosen for easier comparison on the pangasiid species since many sequences were available from previous studies, while COI as barcoding gene was proven to have better resolution on larger variation of fish populations (Bhattacharjee et al., 2012; Hebert et al., 2003; Ratnasingham, & de Waard, 2003; Hubert et al., 2008; Ward et al., 2005).

6.2 Materials and Methods

6.2.1 Sample collection

Pangasiids were collected from two rivers in Peninsular Malaysia in which the species are known to be abundant: the Pahang River and Perak River (Chapter 5, Fig 5.1). There were more site collections for Pahang River (four sites) compared to only one site

for Perak River because the Pahang River is well known for its native pangasiid population. Local fishing gear such as gill nets, drift gill nets, long-lines, hook and lines and other traditional methods were used for specimen collection. Details were outlined in Chapter 3 (Section 3.2.1 – Field technique).

Specimens were identified on site based on their external morphology (following Gustiano, 2003; Roberts & Vidthayanon, 1991; Vidthayanon, 1993). Doubtful specimens were further examined in the laboratory. Specimens identified as *Pangasius* 'hybrid' were also analysed. Voucher specimens were fixed in 10% formalin for at least seven days; they were rinsed with tap water and soaked for another seven days and, then, transferred to 70% ethanol. They were then deposited in the Fisheries Research Institute (Freshwater Division) FRI Glami Lemi, Negeri Sembilan, Malaysia.

6.2.2 Molecular analysis

Muscle tissues were taken from the caudal region on the right side of the fish. Photographs were taken on the left side of the specimens. Tissue samples were preserved in 90% ethanol and stored at 4°C until further analysis.

6.2.2.1 DNA extraction, PCR amplification and sequencing

Tissue samples were cut and preserved in 90% ethanol and stored at 4°C until required. DNA was extracted using Promega Wizard Genomic Animal Tissue Extraction Kit (Mouse Tail Procedure) (Promega, Madison, WI, USA) following the manufacturer's manual. Two mitochondrial gene region, a non-protein coding partial region of 16 S rRNA gene and protein coding Cytochrome C Oxidase I (COI) was amplified using primers 16 Sar (5´-CGC CTG TTT AAC AAA AACAT-3´) and 16 Sbr (5´-CCG GTC TGA ACT CAG ATC ATG T-3´) (Palumbi et al., 1991) and Ward et al. (2005) respectively .

Polymerase Chain Reaction (PCR) was performed in a total volume of 30 µl for 16S rRNA containing 50-200 ng genomic DNA (2µl) template DNA, 6 µl of 10x PCR buffer, 25 mM $MgCl_2$, 0.2mM dNTPs, 0.5 µM of each primer (forward and reverse) and 1 unit of Taq Polymerase (Promega, Madison, WI, USA). Reactions were carried out on an Eppendorf Mastercycler under these following conditions: initial denaturation at 94° C for 3 min followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min and extension at 72° C, while final extension at 72° C for 5 min.

Partial mtDNA Cytochrome c Oxidase I gene was amplified using primers of Ward et al. (2005). Primer optimisation was done to find the best primer pair. Majority of the samples were successfully amplified using primer FISH F1 (forward) and FISH R2 (reverse). However, other low productivity sample products were amplified with a combination of other primer pairs.

For CO1, a total volume of 25 µl PCR mixture containing 50-200 ng genomic DNA in 1.5 µl, 2.5 µl 10xPCR buffer, 3.3 µl 25mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 1.5 µl of each primer and 5 units of Taq DNA polymerase. Each PCR reaction is included with a negative control with no DNA template in the PCR mix. PCR condition for CO1 is: initial denaturation at 94° C for 2 min, followed by 35 cycles of denaturation at 94°C at 30 s, annealing for 40 s at 58° C and extension at 72° C for 1 min. Final extension ran for 10 min at 72° C.

For both of the gene amplifications, three µl of successful amplified PCR product was checked by electrophoresis of 1% agarose containing ethidium bromide staining and visualised under UV illumination. Both strands of PCR fragments were sequenced to get consensus sequence.

PCR products were purified with Promega PCR Purification Kit following the manufacturer's protocol. Purified samples were then sent for sequencing service at First Base Laboratories, Sri Kembangan, Selangor, Malaysia.

In order to control the sequence accuracy, and resolve any ambiguous bases, same primer pair of both directions were used for cycle sequencing using the ABI PRISM Dye-Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresis on an Applied Biosystems Automated Sequencer.

6.2.2.2 Sequence analysis

Sequences were viewed and edited manually using Chromas version 1.45 (MacCarthy, 1996), whereas contiguous sequence (contig) assembly and multiple sequence alignment was performed using ClustalW (Thompson et al., 1997). All of the sequences generated were deposited in the NCBI GenBank (Table 6.1). Complete specimen voucher numbers, with nomenclature suggested by Chakrabarty et al. (2013) are shown in Appendix. Other published 16S rRNA and COI sequences from the GenBank database were also included in the analysis to get a better overview on the pangasiid distribution in the phylogenetic analyses. They are species known to exist in both region (Indo-China/Thailand and Indo-Malayan Archipelago) but excluding the Southern Asian species.

Both mtDNA were homologous in length and could be easily aligned by eye. The number of polymorphic sites and nucleotide diversity (*Pi*), nucleotide composition and number of transition and transversion between species were determined by DnaSp version 5.0 (Rozas et al., 2009). Determinations of base compositional frequencies and nucleotide substitutions between pairwise comparisons were performed using MEGA 6.0 (Tamura et al., 2013).

For protein coding region (COI), the final alignment was screened for the stop codons and insertion-deletion mutations using the same software, to ensure that there were no amplifications of non-target fragments (Song et al., 2013) as outlined in Chapter 5, section 5.2.2. A Siluriformes sequence, *Hemibagrus macropterus* from family Bagridae, was chosen as outgroup to root the tree. The mean pairwise genetic distance

matrix was calculated using the Kimura two parameter (K2P) model with the pairwise deletion of gaps (Felsenstein, 1985), implemented in MEGA 6.0 Software (Tamura et al., 2013).

6.2.2.3 Phylogenetic inference

Analysis was done separately for each of the two genes and then combined. MEGA 6.0 was used for Neighbour-Joining Analysis (NJ) and Maximum Parsimony (MP). The NJ analysis used Kimura-2-Parameter method, while MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar, 2000). Modeltest version 3.7 (Posada & Crandall, 1998) was used to get the best evolution model for each gene region for running the ML and Bayesian Inference (BI) phylogenetic analyses. The optimal model used for all the analysis which was determined by hierarchical likelihood ratio tests (hLRT) using Modeltest 3.7 (Posada & Crandall, 1998). The model selected on the Hierarchical Likelihood Ratio Tests (hLRTs) for 16S and COI were TrN+I+G and TrN+G, respectively. Concatenated data set were performed on reduced set of specimens which used only specimens from this study, since no other previous sequences that combined the two genes. The model selected for this analysis was TrN+I+G.

The software PAUP* version beta 10 (Swofford, 2001) was used to perform the Maximum Likelihood (ML) tree. ML was run by unweighted heuristic search option via random stepwise addition of taxa, with 1000 bootstrap replicates. The 50% majority rules consensus trees were computed using tree-bisection-reconnection (TBR) option as branch-swapping algoritm and confidence limits were assessed using bootstrap procedure (Felsenstein, 1985).

Bayesian phylogeny analysis was done using MrBayes version 3.0 (Huelsenbeck & Ronquist, 2001), implementing Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analyses, with trees sampled every 1000 generation. Four Markov chains were run simultaneously starting from a random tree for one million generations,

sampling from the chain every 100th tree. The first 25% of samples were discarded as burn in. Results were summarised as consensus trees with posterior probabilities for clades.

Table 6.1: List of species that were used in this study with information on collection sites and river, country, GenBank accession numbers of mitochondrial DNA 16S rRNA and Cytochrome Oxidase sub unit I (COI).

Species	Collection site, River	Country	GenBank accession numbers		Reference
			16S	COI	
Helicophagus waandersii	Lubok Paku, Pahang River	Malaysia	KR349214	-	This study
H. waandersii	Lubok Paku, Pahang River	Malaysia	-	KP036415	This study
H. waandersii	Lubok Paku, Pahang River	Malaysia	KR349215	KP036416	This study
H. waandersii	Lubok Paku, Pahang River	Malaysia	KR349216	-	This study
H. waandersii	Paloh Hinai, Pahang River	Malaysia	KR349217	KP036417	This study
H. waandersii	Mekong River	Thailand	DQ334322	-	Na-Nakorn et al. (2006)
H. waandersii	Mekong River	Thailand	DQ334323	-	Na-Nakorn et al. (2006)
H. typus	Jambi, Batang Hari	Indonesia	HM355764	-	Karinthanyakit & Jondeung (2012)
H. leptorhynchus	Ubon Ratchathani, Mekong	Thailand	HM355763	-	Karinthanyakit & Jondeung (2012)
Pangasianodon hypophthalmus	Kuala Kangsar, Perak River	Malaysia	KR349218	KP036425	This study
P. hypophthalmus	Paloh Hinai, Pahang River	Malaysia	KR349219	KP036426	This study
P. hypophthalmus	Lubok Paku, Pahang River	Malaysia	KR349220	-	This study
P. hypophthalmus	Nakhon Ratchasima Province	Thailand	-	JF292409	Wong et al (2011)
P. hypophthalmus	Mekong River	Thailand	DQ334282	-	Na-Nakorn et al. (2006)
Pangasius bocourti	Pahang River, Pahang River	Malaysia	KR349221	KP036428	This study
P. bocourti	na	na	-	EF609425	Ward & Holmes (2007)
P. bocourti	Yasothon Province	Thailand	-	JF292429	Wong et al. (2011)
P. bocourti	Mekong River	Thailand	DQ 334299	-	Na-Nakorn et al. (2006)
P. bocourti	Ubon Ratchathani, Mekong	Thailand	HM355769	-	Karinthanyakit & Jondeung (2012)

Table 6.1: (continued)

Species	Collection site, River	Country	GenBank accession numbers		Reference
			16S	COI	
Pangasius conchophilus	Paloh Hinai, Pahang River	Malaysia	KR349222	KP036413	This study
P. conchophilus	Chenor, Pahang River	Malaysia	KR349223	KP036414	This study
P. conchophilus	Mekong River	Thailand	DQ334302	-	Na-Nakorn et al. (2006)
P. conchophilus	na	na	-	EF609426	Ward & Holmes (2007)
Pangasius djambal	Paloh Hinai, Pahang River	Malaysia	KR349224	-	This study
P. djambal	Paloh Hinai, Pahang River	Malaysia	KR349225	KP036427	This study
Pangasius macronema	Mekong	Thailand	DQ334314	-	Na-Nakorn et al. (2006)
P. macronema	Mekong	Thailand	HM355776	-	Karinthanyakit & Jondeung (2012)
Pangasius nasutus	Paloh Hinai, Pahang River	Malaysia	KR349226	-	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	KR349227	KP036411	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	KR349228	KP036412	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	KR349229	-	This study
P. nasutus	Paloh Hinai, Pahang River	Malaysia	KR349230	-	This study
P. nasutus	Kapuas, River	Indonesia	HM355778	-	Karinthanyakit & Jondeung (2012)
P. nasutus	Pahang River	Malaysia	-	JF781172 - JF781175	Song et al. (2013)
Pangasius polyuranodon	Chao Phraya	Thailand	HM355779	-	Karinthanyakit & Jondeung (2012)
Pseudolais micronemus	Paloh Hinai, Pahang River	Malaysia	-	KP036419	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	KR349231	KP036420	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	KR349232	KP036421	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	KR349233	KP036422	This study
P. micronemus	Paloh Hinai, Pahang River	Malaysia	KR349234	KP036423	This study

Table 6.1: (continued)

Species	Collection site, River	Country	GenBank accession numbers 16S	COI	Reference
P. micronemus	Kuala Kangsar, Perak River	Malaysia	KR349235	KP036424	This study
P. micronemus	Kuala Kangsar, Perak River	Malaysia	KR349236	-	This study
P. micronemus	Chenor, Pahang River	Malaysia	KR349237	-	This study
P. micronemus	Pahang River	Malaysia	-	HM156360 - HM156364	Song et al. (2013)
P. pleurotaenia	Mekong	Thailand	DQ334329	-	Na-Nakorn et al. (2006)
P. pleurotaenia	Chao Phraya	Thailand	HM355765	-	Karinthanyakit & Jondeung (2012)
Pangasius hybrid	Paloh Hinai	Malaysia	KR349238	-	This study
Pangasius hybrid	Paloh Hinai	Malaysia	KR349239	-	This study
Hemibagrus macropterus	Hechuan, Chongqing	China	NC019592	NC019592	Zeng et al. (2012)

6.3 Results

6.3.1 Sequence Characteristics

For the 16S rRNA gene, a total of 550 nucleotide basepairs from thirty-nine sequences of pangasiids were analysed with one outgroup, *Hemibagrus macropterus* (Table 6.1). The alignment contains 480 (87.27%) conserved sites and 70 (12.72%) variable sites, of which 48 sites (8.73%) are parsimony informative. The average base composition was 22.04 % (T), 24.46% (C), 30.75% (A) and 22.80% (G). The estimated transition/transversion (si/sv) ratio, R, is 5.00.

For COI, we analysed thirty-three sequences, with a total of 582 nucleotide basepairs, with *Hemibagrus macropterus* as the outgroup. The final alignment is of 441 (75.77%) conserved sites, 141 (24.23%) variable sites, with 112 parsimony informative sites (19.24%). The average base composition was 30.27 % (T), 26.61% (C), 26.38% (A) and 16.74% (G). The estimated transition/transversion (si/sv) ratio, R, for 3rd codon is 6.00 (Table 6.2).

6.3.1.1 Model of evolution

For 16S rRNA gene base frequencies for A=0.3200, C=0.2455, G=0.2105 and T=0.2241 were recorded. The substitution model incorporated the following rate matrix [A-C]=1.0000, [A-G]=3.3020, [A-T]=1.0000, [C-G]=1.0000, [C-T]=9.1256, [G-T]=1.0000 and proportion of invariable sites *(Pinvar) I*= 0.6963. The shape parameter of the discrete gamma distribution was G=0.5283. The model of evolution selected for 16S wasTrN+G, with –lnL of 1355.5927.

For COI, base frequencies of A=0.2794, C=0.2679, G=0.1487 and T=0.3040 were obtained and the substitution model rate matrix was [A-C]=1.0000, [A-G]=10.1210, [A-T]=1.0000, [C-G]=1.0000, [C-T]=18.1256, [G-T]=1.0000. Proportion of invariable site,

I=0 while the Gamma shape parameter distribution for COI was 0.1121. The model selected for evolution for COI was TrN+G. The –lnL value for this model was 1733.2992.

For combined sequences, base frequencies found were A=0.2966, C=0.2601, G=0.1810, T=0.2623. The substitution model rate matrix were [A-C]=1.0000, [A-G]=5.1298, [A-T]=1.0000, [C-G]=1.0000, [C-T]=19.8406, [G-T]=1.0000. Proportion of invariable site, *I* of these combined gene=0.7518 while the Gamma shape parameter distribution was 2.0294. The model selected for evolution was TrN+I+G and the –lnL value was 2917.5679.

Table 6.2: Summary of 16s rRNA, Cytochrome c Oxidase I (COI) and combined sequence characteristics, mean base compositions, models of evolution selected and model parameters implemented by the Hierarchical Likelihood Ratio Tests (hLRTs) in Modeltest v3.7 for Maximum Likelihood (ML) and Bayesian Inference(BI); (*Pinvar*, *I*), and α, gamma shape parameter).

Sequence characteristics	**Data set**		
	16S rRNA	**COI**	**Combined data**
Number of pangasiid sequences analysed	39	33	16
Fragment length (bp)	550	582	1132
Number of variable sites (%)	70 (12.72%)	141(24.23%)	191(17.00%)
Transition/transversion, R (si/sv) ratio	5.00	6.00	2.97-9.00
Mean base composition (%)			
A	30.75	26.38	28.53
C	24.46	26.61	25.57
G	22.8	16.74	19.63
T	22.04	30.27	26.26
Model of evolution parameters			
Best-fit model	TrN+I+G	TrN+G	TrN+I+G
Pinvar (I)	0.6963	0	0.7518
α	0.5283	0.1121	2.0294

Table 6.3: Mean corrected (K2P) genetic distance, percentage of divergence is shown as pairwise intraspecific (*p* divergence) and interspecific divergence value (*d*) of Pangasiid 16S rRNA sequences from this study. Standard Error (S.E) in parentheses.

No	Species no*	1	2	3	4	5	6	7	8	9	10	*d*
1	*Pangasius nasutus*	-	[0.001]	[0.007]	[0.007]	[0.007]	[0.007]	[0.006]	[0.007]	[0.007]	[0.007]	0.2 (0.001)
2	*Pangasius conchophilus*	0.2	-	[0.008]	[0.007]	[0.007]	[0.007]	[0.007]	[0.007]	[0.007]	[0.007]	0.1 (0.001)
3	*Pangasius bocourti*	3.0	3.0	-	[0.009]	[0.009]	[0.007]	[0.007]	[0.001]	[0.007]	[0.009]	0.0 (0.000)
4	*Pangasius djambal*	3.1	3.1	4.0	-	[0.000]	[0.000]	[0.008]	[0.008]	[0.008]	[0.000]	0.0 (0.000)
5	*Pangasianodon hypophthalmus*	3.1	3.1	4.0	0.0	-	[0.008]	[0.008]	[0.008]	[0.008]	[0.008]	0.0 (0.000)
6	*Pseudolais micronemus*	2.9	3.1	2.9	3.6	3.6	-	[0.007]	[0.008]	[0.008]	[0.008]	0.2 (0.001)
7	*Helicophagus waandersii*	2.9	3.0	3.4	3.8	3.8	3.1	-	[0.001]	[0.001]	[0.008]	1.2 (0.003)
8	*H. typus*	2.8	2.9	3.8	3.5	3.8	3.5	0.6	-	[0.000]	[0.008]	n/c
9	*H. leptorhyncus*	2.8	2.9	3.8	3.8	3.8	3.5	0.6	3.8	-	[0.008]	n/c
10	*Pangasius* hybrid	3.1	3.1	4.0	0.0	3.5	3.6	3.8	3.8	3.8	-	0.0 (0.000)

Table 6.4. Percent pairwise mean corrected (K2P) genetic distance (intraspecific divergence (*p*)) and interspecific divergence value (*d*) of pangasiid COI sequences from this study. Standard Error (S.E) in parentheses.

No.	Species	1	2	3	4	5	6	7	Interspecific value, *d*
1	*Helicophagus waandersii*		[0.016]	[0.018]	[0.014]	[0.016]	[0.014]	[0.014]	0.1 [0.001]
2	*Pangasianodon hypophthalmus*	15.8		[0.017]	[0.011]	[0.014]	[0.011]	[0.012]	0.1[0.001]
3	*Pangasius bocourti*	12.0	11.3		[0.017]	[0.019]	[0.011]	[0.017]	0.2 [0.002]
4	*Pangasius conchophilus*	12.6	11.0	6.8		[0.011]	[0.015]	[0.014]	0
5	*Pangasius djambal*	11.7	11.1	0.2	6.7		[0.011]	[0.012]	n/c
6	*Pangasius nasutus*	12.6	11.1	6.7	0.2	6.5		[0.012]	0.3 [0.002]
7	*Pseudolais micronemus*	9.9	12.2	8.1	8.1	7.9	8.0		0.1 [0.000]

6.3.1.2 Divergence between pangasiid species

Mean corrected (K2P) genetic distance showed low interspecific divergence value, *d*, of 16S rRNA pangasiids between 0-1.2% with the highest value in *H. waandersii*. While intraspecific divergence differs from as low as 0 to 4.0 % (Table 6.2).

Pangasius nasutus varied from its morphologically similar species, *P. conchophilus*, by only 0.2 % of divergence. They could not be differenciated in phylogenetic analysis and clumped together as one clade as shown in the phylogram (Fig 6.1-NJ/ML/BA). Genetic distance for majority of other groups are within range 0 to 4.0%, with the highest distance observed between *P. djambal* vs *P. bocourti* and *Pangasianodon hypophthalmus* vs *P. bocourti*. It is surprising that even though *P. djambal* and *P. bocourti* is morphologically similar but they have the highest divergence value (4.0%), while the lowest divergence observed between *P. hypophthalmus* and *P. djambal* (no divergence, 0%).

Helicophagus waandersii has genetic divergence of 0.6 % to both congeners (*H. typus* and *H. leptorhyncus*) means that they are genetically very close to each other but still have some differences in morphology.

Low percentage of interspecific divergence value, *d*, found in the pangasiids COI sequences (0-0.3 %). The highest value (0.3%) was found in *P. nasutus* sequences, while the lowest observed in *P. conchophilus* (0 %). For intraspecific percentage of divergence, the highest value (15.8%) was observed between *P. hypophthalmus* vs *H. waandersii*, while the lowest (0.2%) were found in *P. nasutus* vs *P. conchophilus* and *P. bocourti* vs *P. djambal* (Table 6.4).

Pangasius nasutus varied from its morphologically similar species, *P. conchophilus*, by only 0.2 % of divergence. They could not be differenciated in phylogenetic analysis and clumped together as one clade as shown in the Bayesian phylogram (Fig 6.2). The same distance (0.2%) was also observed in the morphologically

similar allopatric pair of *P. djambal* and *P. bocourti*, which also grouped together in the Bayesian tree.

Genetic distance for majority of other groups are within range 6.5 % (*P. djambal* vs *P. nasutus*) to the highest distance observed between *H. waandersii* vs *Pangasianodon hypophthalmus* (15.8%).

6.3.2 Phylogenetic analysis

6.3.2.1 Mitochondrial DNA 16S ribosomal RNA (16S rRNA) partial gene

Bayesian tree of Peninsular Malaysian pangasiids based on ribosomal mitochondrial DNA 16S rooted to *Hemibagrus macropterus* produces five clades (Figure 6.1) namely *Pseudolais, Pangasianodon, Helicophagus, Pangasius conchophilus-nasutus* and *Pangasius bocourti*. Two additional species from GenBank, *Pangasius macronema* and *Pangasius polyuranodon* were included as comparison and they are sister group to *Pseudolais*. *Pangasius djambal* and *Pangasius* hybrid were included in the *Pangasianodon* group. All the three *Helicophagus* species were clumped as one group.

Although separated by low bootstrap support at the outer clade, groupings at inner nodes were backed by strong bootstrap values (more than 95%) to well defining clades.

From earlier observation in Chapter 4, *P. micronemus* and *P. polyuranodon*, were found to be similar morphologically, therefore analysis were done for molecular comparison to complement their close morphological characters with their closely related species (*P. pleurotaenia, P. polyuranodon* and *P. macronema*). As shown in Figure 6.1, *P. polyuranodon* is genetically closer to *P. macronema* than to *P. micronemus*. However, these two *Pangasius* species are sister o the genus *Pseudolais* compared to another relative species in the genus *Pangasius*.

Two closely related species, *P. nasutus* and *P. conchophilus* grouped together, the rest of the sequences seems to be evolutionary derived from the *P. nasutus* from Kapuas Indonesia, with the consensus value of 1.00.

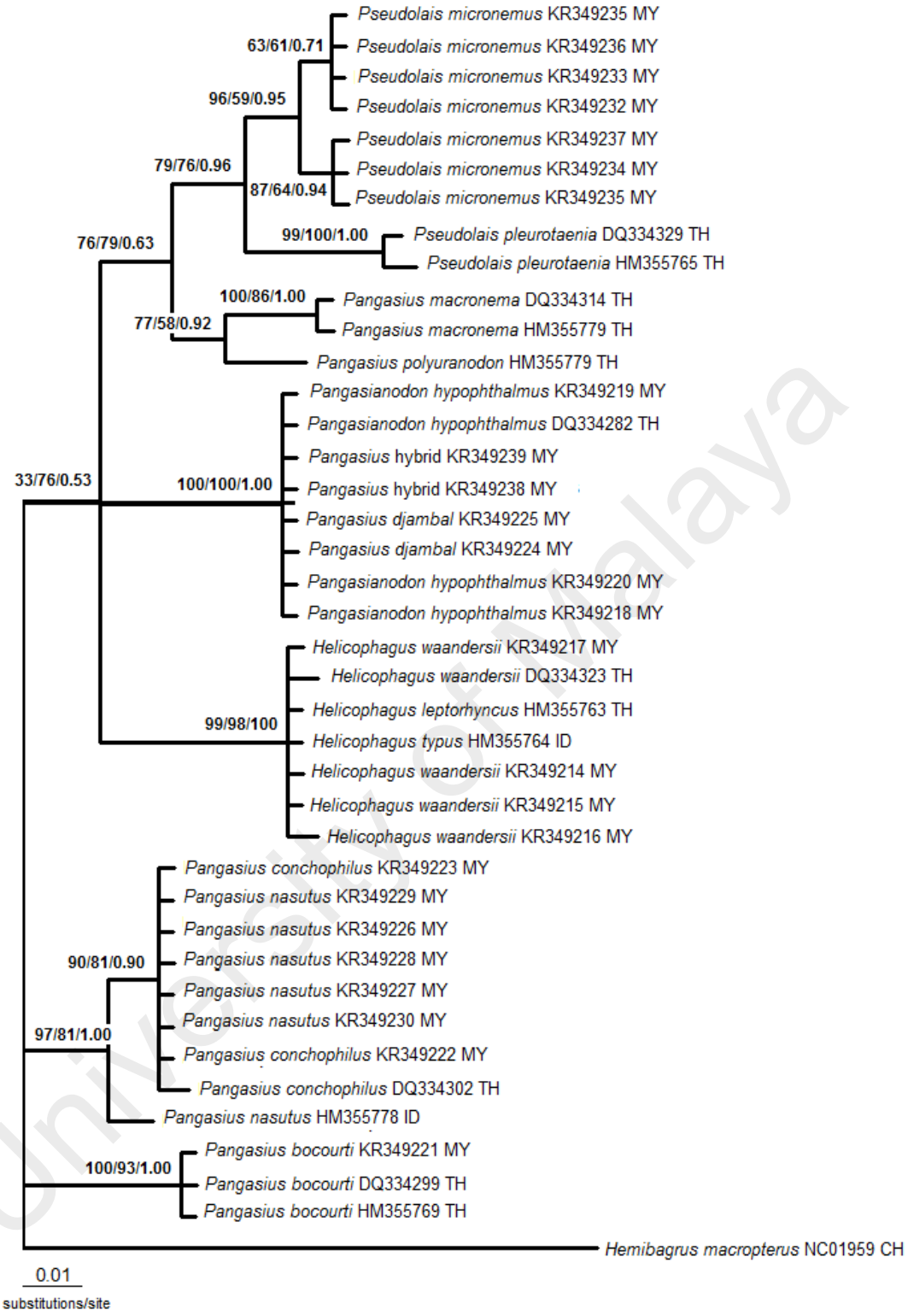


Figure 6.1: Bayesian phylogram inferred from mtDNA 16S rRNA partial gene of Peninsular Malaysian pangasiid catfish sequences, with selected sequences from previous studies. The numbers at each node represent bootstrap proportion for NJ/ML and Bayesian posterior probabilities for each tree (NJ/ML/BI), respectively.

6.3.2.2 Mitochondrial DNA Cytochrome Oxidase 1 (COI) partial gene

The relationships between pangasiid COI sequences were reconstructed using K2P model and shown in the NJ tree (Chapter 5 - Figure 5.2). The detailed analyses were done in the previous chapter (Chapter 5) with more sequences and outgroups species. However, for comparison with 16s rRNA gene sequences, a phylogeny was reconstructed using fewer GenBank sequences (thirteen sequences only) (Table 6.1) and further analysed using MP, ML and Bayesian analyses. Bayesian phylogram as showed in Figure 6.2 shows monophyletic branches which are assigned according to their respective genera (*Pangasius, Helicophagus, Pseudolais* and *Pangasianodon*).

Using *Hemibagrus macropterus* (Family Bagridae) with outgroup, five major clades (A-E) were identified, the same result as in Figure 5.2 (Chapter 5). In this chapter, Clades A and B representing the genus *Pangasius* (A- *P. bocourti* and *P. djambal*) and B - *P. conchophilus* and *P. nasutus.* Clades C - *Helicophagus* (*H. waandersii*), D - *Pseudolais* (*P. micronemus*) and E - *Pangasianodon* (*P. hypophthalmus*). Although the five clades were well defined, their branching order was not resolved due to the low bootstrap values (< 70%), which suggests simultaneous radiation.

Clade E (*Pangasianodon*) is well distinguished from the other four clades (Clades A, B, C and D) with a high confidence level (99%). A medium bootstrap value (51%) separated the genus *Pangasius* (Clades A and B) from Clade C (*Helicophagus*) and Clade D (*Pseudolais).* The two clades within *Pangasius* were divided by a 64% bootstrap value and further differentiated into their respective clades by a strong bootstrap confidence (99%).

Within the clades, our sequences show some differences in the evolutionary distance from other sequences obtained from Genbank. *Pangasius bocourti* KP036428 is very close to *P. djambal* KP036427 but has a small divergence from other *P. bocourti*

sequences, with a 37% bootstrap value. Other *P. bocourti* are clustered together with an 86% bootstrap confidence.

A similar result was also observed in Clade B, where our sequences of *P. conchophilus* (KP036413 and KP036414) matched with EF609426 from Vietnam (Ward & Holmes, 2007). In this clade, *P. nasutus* and *P. conchophilus* should have been separated into two groups with a bootstrap value of 99%. Clearly, *P. nasutus* sequences from this study (KP036410-12) showed a consistent grouping, together with one sequence (JF781172) from Song et al. (2013). However, the other three sequences of *P. nasutus* from their study (JF781173-75) were clustered into the *P. conchophilus* group. One of the possible explanations was most likely due to misidentification during sampling, by a wrongly identified sample or samples taken from hybrid specimens.

Figure 6.2: Bayesian phylogram inferred from COI partial gene of Peninsular Malaysian pangasiid catfishe sequences, with selected sequences from previous studies. The numbers at each node represent bootstrap proportion for NJ/ML and Bayesian posterior probabilities for each tree (NJ/ML/BI), respectively

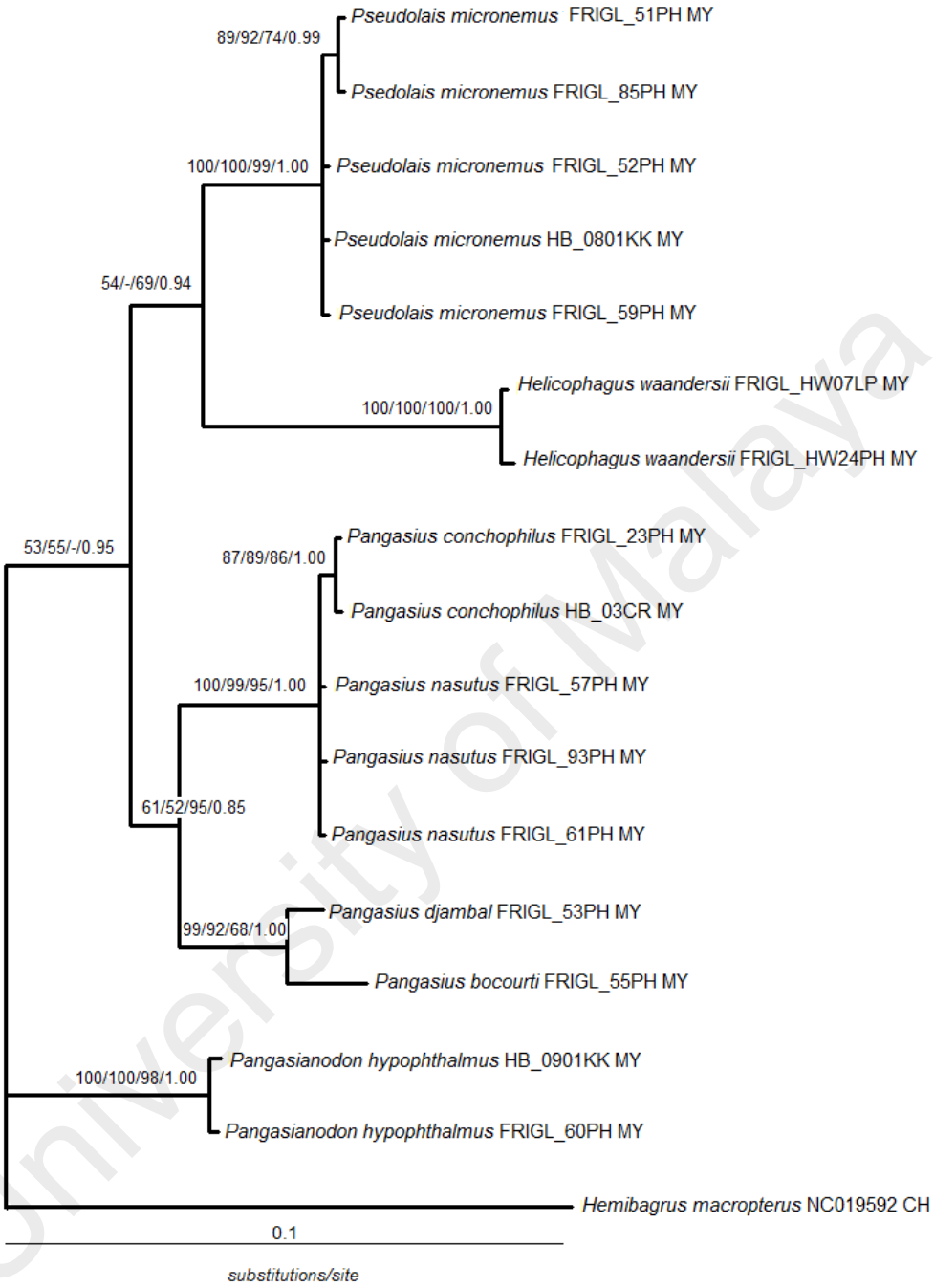


Figure 6.3: Bayesian phylogram inferred from concatenated 16S rRNA and COI mitochondrial DNA sequences of the pangasiids from Peninsular Malaysia. Numbers at each node indicates boostrap values for NJ/MP/ML and Bayesian Inference, respectively.

6.3.3 Genetic variation/ DNA polymorphism

6.3.3.1 Mitochondrial DNA 16S ribosomal RNA (16S rRNA) partial gene

A total of 40 sequences (including outgroup) produced 15 haplotypes with high haplotype diversity, *h*, of 0.9077 and nucleotide diversity *Pi* (%) 0.03016. All the Peninsular Malaysian *P. nasutus* specimens have the same haplotype number (Hap1), which are genetically different from the species originated from Kapuas, Indonesia which is designated as Hap9. Three nucleotide variations occur at sites 268 (transversion - A to G), 352 and 418 (transition - T to C). Their close relative, *P. conchophilus* that also found in Peninsular Malaysia also included in the same haplogroup (Hap1).

Helicophagus waandersii was represented by three haplotypes (Hap2, Hap3 and Hap10). Three Peninsular Malaysian species (Hewa02-KR349214), Hewa07 (KR349215) and Hewa24 (KR349217)) are in the same haplogroup (Hap2) with another two congeners, *H. leptorhynchus* from Thailand and *H. typus* from Jambi, Indonesia. Whereas, one Peninsular Malaysian sequence has a different polymorphic variation, designed as Hap3 (Hewa08LP) and another *H. waandersii* from Mekong Thailand (DQ33432) (Hap2), only differentiated by one nucleotide change (transversion) from A to T at site 200.

For *P. micronemus*, two haplotypes observed from seven sequences (Hap3 and Hap7), having less polymorphism compared to *H. waandersii* samples (five sequences with three haplotype variations) as listed in Table 6.5.

Three individuals of Peninsular Malaysian *P. hypophthalmus*, combined with two samples identified as *P. djambal* and two samples suspected to be *Pangasius* hybrids designed as Hap6, same haplotype as a sample from Mekong, Thailand (DQ334299).

Similar to *P. hypophthalmus*, which harbour only one haplotype, *P. bocourti* sequences also generated a single haplotype (Hap4) with the only *P. bocourti* from Pahang River has the same haplotype variation with both sequences from Thailand.

6.3.3.2 Mitochondrial DNA Cytochrome Oxidase 1 (COI) partial gene

From 75 sequences, seven species of pangasiid produced eleven haplotypes with haplotype diversity of 0.828 and nucleotide diversity 0.0713. *Helicophagus waandersii* produced one haplotype (Hap 6), while *P. micronemus* produced two haplotypes (Hap 7 and 8). For *P. micronemus*, the sample KP036418 (Hap 7) was from Perak River, while the other eleven samples designated as Hap 8 came from Pahang River basin except for KP036424 (another sample from Perak River).

Pangasianodon hypophthalmus, with the most individuals, has the highest haplotype numbers (Hap 9, 10, 11). Among these three haplotypes, only one haplotype (Hap 9) was represented by a majority of the sequences including sample KP036426 from Pahang River. Hap 10 (KP036425) came from the Perak River, while Hap 11 was represented by a single specimen (NC021752) from Thailand.

Interestingly, Hap 3 and Hap 5 were shared by two conspecific group of species. Hap 3 is shared by two species from Peninsular Malaysia (this study – *P. bocourti* KP036428 and *P. djambal* KP036427). Meanwhile, Hap 5 is shared by both *P. conchophilus* (three samples) and *P. nasutus* (three samples). Hap 4 represented by only *P. nasutus* from Pahang River.

The sequences were assigned according to their haplogroups to identify the variable sites that assigned them into their haplotypes. For the three haplotypes of the group *P. bocourti* - *P. djambal* (Hap 1, 2 and 3), only two parsimony informative sites at 180 and 513 nucleotide locations found. For another *Pangasius* group (*P. nasutus*-*P. conchophilus*) (Hap 4 and 5) parsimony informative sites are located at sites 306 and 579.

A haplotype phylogram was constructed to show relationships of the five genera within pangasiid family (Figure 6.4). Relative to the outgroup, a monophyletic tree with medium to strong bootstrap values (66 – 100 %) and able to distinguish four genera (*Pangasianodon* (Hap 9, 10 and 11); *Pseudolais* (Hap 7 and 8); *Helicophagus* (Hap 6); and *Pangasius* (Hap 1, 2, 3, 4,and 5).

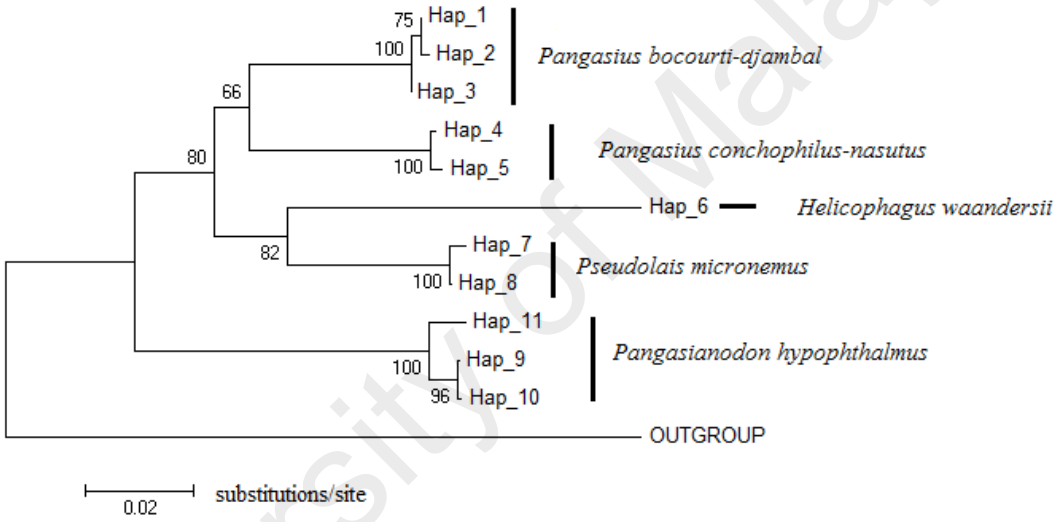


Figure 6.4: NJ tree phylogram inferred from mtDNA COI showing haplotype variation within Pangasiid family

Table 6.5. Partial mtDNA COI haplotype variation, haplotype diversity and nucleotide diversity of pangasiid species analysed.

No	Species	n	Nhap	Hap_no	Haplotype diversity, *h*	Nucleotide diversity, *Pi* (%)
1	*Helicophagus waandersii*	3	1	6	0.667	0.0012
2	*Pangasianodon hypophthalmus*	27	3	9, 10, 11	0.279	0.0014
3	*Pangasius bocourti**	22	3	1, 2, **3**	0.379	0.0007
4	*Pangasius conchophilus***	3	1	5	-	-
5	*Pangasius djambal**	1	1	3	-	-
6	*Pangasius nasutus***	7	2	4, **5**	0.733	0.0026
7	*Pseudolais micronemus*	12	2	7, 8	0.167	0.0006
	Total	**75**	**11**	**-**	**0.828**[#]	**0.0713**[#]

n = number of samples (sequences); Nhap = number of haplotypes; Hap_no= Haplotype number/designation
*share haplotype 3, **share haplotype 5; the total value x[#] were obtained from pooled analysis, not the sum of individual *h* and *Pi*

6.4 Discussion

The results of phylogenetic reconstructions presented here shows that even though the relationships is not fully resolved as showed by low bootstrap supports, but enough to provide supporting evidence on the five genera of pangasiids. Strong boostrap supports (95% -100%) on each of the clades defined the monophyletic clades of *Helicophagus, Pangasianodon, Pangasius* and *Pseudolais.*

The basal group was interchangeable (in both analyses – 16S and COI) between *P. micronemus* and *Pangasianodon hypophthalmus,* which is not an issue since the bootstrap value do not strongly support this.

Pseudolais micronemus observed to be the sister group to *P. macronema-P. polyuranodon* (Figure 6.1–16S rRNA). In Figure 6.2, COI tree produces the same topology with the analysis of Karinthanyakit & Jondeung (2012), whereby the *Pseudolais* group is related as the sister group to *Helicophagus.*

Even though 16S rRNA can differentiate the genera by genetic distance, but the relationships is not well defined, therefore COI tree which has similar topology to the work by Karinthanyakit & Jondeung (2012), is more reliable in terms of phylogenetic relationships.

For the *Pangasius* group, *P. nasutus* could not be separated from *P. conchophilus* in all the analyses, even though genetic distance clearly delineated their difference, (Table 6. 2 – 0.2% in 16S rRNA, Table 5.2 – 3.0% in COI). Thus, in this study, genetic characters provided by these two genes were not able to differenciate the species status which were achieved successfully using multiple genes in Karinthanyakit & Jondeung (2012).

As reviewed in Chapter 5, intraspecies relationships between *P. djambal* and *P. bocourti* for COI gene which clustered in the same group of *Pangasius*, with 7.1% genetic distance, the value observed in 16S rRNA is also high (4.0% distance). However in 16 S

rRNA tree, *P. djambal* is grouped with *P. hypophthalmus* but not *P. bocourti*. *Pangasius bocourti* is grouped with other GenBank *P. bocourti* sequences confirming its species status. As observed in COI tree, both *P. djambal* and *P. bocourti* is in the same grouping with other *P. bocourti* sequences from GenBank, but little divergence observed in the Peninsular Malaysian specimen, which shows closer relationship of *P. bocourti* to *P. djambal* compared to other *P. bocourti* in the group.

Throughout analyses in this chapter (phylogenetic tree and genetic distance), it is clear that both mitochondrial genes (16S rRNA and COI) can be used for taxonomic purposes. While COI seems to show better performance in species delineation (discussed in detail in Chapter 5), 16S rRNA is still a good marker for species identification.

Conservation strategies using phylogenetic inference

Morphologically similar species which are very closely related, like *P. nasutus* and *P. conchophilus* and *P. djambal* and *P. bocourti* are the subject of conservation interest. Their location in the phylogenetic tree means that they are genetically close, which is a potential to conservation issue. They can be hybridised for aquaculture purposes, and such programs could lead to negative environmental impacts (Chapter 5 – Discusion). As far as conservation is concerned, information from phylogenetic analyses is also useful, when pointing to highly distinct taxa (Gustiano, 2003). Nevertheless, all the native species analysed in this study are under consideration for conservation effort. *P. nasutus* has potential threat of hybridisation with introduced *P. hypophthalmus* and *P. conchophilus*. As such, artificial hybridisation is also practised between *P. nasutus* (male) and *P. hypophthalmus* (female) to produce hybrids. These offsprings (locally called 'Patin Mas') have the *P. nasutus* appearance but are easier to breed and cultured like the *P. hypophthalmus* parent (personal observation).

On the other hand, *Pseudolais micronemus* which was reported to be rare in the Mekong Delta (Gustiano, 2003), the real population status is yet to be studied. Its morphologically similar species *P. polyuranodon*, though only analysed using GenBank sequence, with a close position to *P. macronema*, not *P. micronemus.* They might be linked by ecological relationship, referring to the appearance of the big eye size, which is related to their omnivorous food preferences, hence their wide spread distributions.

The same remark also applied to another widely abundant species, *Helicophagus waandersii.* Ecological disturbance by competition to specialised molluscivorous food preference from the introduced pangasiid species, might threaten its wild survival. With reference to its feeding habit, it is hard to breed this species in captivity, although some efforts have been made by the Malaysian Department of Fisheries in the 1990s (personal observation).

CHAPTER 7: CONCLUSION

This study examined the diversity of Pangasiid catfishes in Peninsular Malaysia, based on past and present collections. A combination of morphological and molecular methods were employed, with further focus on the implementation of the barcoding gene (Cytochrome Oxidase sub unit I) as species identification tool for some *Pangasius* species complexes.

Findings from this study evaluates the diversity and distribution of pangasiid in Peninsular Malaysia using morphological and meristic characters following the studies conducted by Roberts & Vidthayanon (1991), Vidhayanon (1993) and Gustiano (2003). Some details on the species that occur in Peninsular Malaysia are presented. Establishment of identification keys of eight species are supported by Principal Component Analysis, with meristic factor loadings that distinguished *Pangasianodon* (higher number of Pelvic-Fin Rays), while Anal-Fin Rays could be used to distinguish *Helicophagus waandersii*. Discriminant characters for *Pseudolais* identification are the combinations of big eye diameter, longer caudal peduncle, anal-fin length and adipose fin width. However, in this study, no clear separation was found for morphologically close species, *P. conchophilus* and *P. nasutus* as well as *P. bocourti* and *P. djambal*.

The record on the occurrence of *P. polyuranodon* which were officially reported, remarks the taxonomic status of *Pangasius polyuranodon* in Peninsular Malaysia and the importance of correct taxonomical identification in the conservation program of indigenous fish species. Potential threat to the survival of the native Pangasiid species could be detected, thus appropriate plans could be initiated. *Pangasius polyuranodon* has a wide distribution in this region, which was recently divided into three remarkably different species according to their location. In Peninsular Malaysia, this species was previously reported but there is no conclusive proof for its presence. Through comparative

evaluation with a morphologically similar species, *Pseudolais micronemus*, together with reference to available regional collections from the Raffles Museum for Biodiversity Research, Singapore and the Museum Zoologicum Bogoriense, Bogor, Indonesia, an identification key was established in order to clarify the status and presence of *P. polyuranodon* in Peninsular Malaysia.

The usefulness of the partial sequence of mitochondrial DNA (mtDNA) Cytochrome Oxidase sub unit I (COI) gene in detecting potential invasive Asian Pangasiid catfishes was evaluated and possible threats and implications to the native species was raised. However, using this gene, the recently diverged *Pangasius* (late Pleistocene period – 3.99 Million years before present, MYA) could not be separated clearly using barcoding gap or phylogenetic tree. Barcoding gaps were successfully detected using Automated Barcode Gap Discovery (ABGD) between intraspecific and interspecific distances. DNA barcoding can be used as a demonstrative tool to identify closely related species thus justifying its application in the biodiversity conservation of these fishes.

It is clear that barcoding gene can be utilised as an identification tool to evaluate the pangasiid catfish species found in Peninsular Malaysia and able to detect the genetic variation between a taxonomically problematic groups. Potentially invasive species that could threaten the survival of native species may also been determined. The results are beneficial to develop guidelines on the sustainable fisheries and aquaculture practices.

This study also demonstrated that both mitochondrial genes (16S rRNA and COI) can be used for taxonomic purposes to support morphological characters. While COI seems to show better performance in species delineation, but 16S rRNA is still a good marker for species identification. Unfortunately, morphologically similar species which is very closely related like *P. nasutus* and *P. conchophilus* and *P. djambal* and *P. bocourti*

are still the subject of conservation interest. Their location in the phylogenetic tree means that they are genetically close, which is potential for conservation issues. They can be hybridised for aquaculture purposes, and such program could lead to other negative environmental impact.

Further work with more extensive collections should focus on understanding the status of the genetic diversity of the established pangasiid population by assessing the potential impact on the native population, either ecological or sociological. Public awareness should be concentrated on the reduction of a potential negative impact of introduced species by practising sustainable aquaculture/fishery. A management decision for the protection and conservation of native species can now be facilitated partly through the findings in this study.

It is suggested that in the future, the introduction of exotic species (either for aquaculture, recreational fishery or aquarium trade) should follow the Code of Conduct for Responsible Fisheries, which was developed by FAO, and that these guidelines must be further extended to fisheries workers and general public. Control by public actions is more effective than control through imposed laws (Welcomme & Vidthayanon, 2003). Under these circumstances, Department of Standards Malaysia (2007) has coordinated the development of multi-departmental consensus document called 'Malaysian Standard - Good Aquaculture Practice (GAqP) – Aquaculture Farm – General Guidelines MS 1998:2007', which focusses on appropriate biosecurity measures, disease control and trans-boundary fish. In this document, farmers are advised to conserve genetic diversity and maintain the aquatic ecosystem by minimising the effects of introducing alien species and genetically modified organism (GMO) fish in their culture practices (Department of Standards Malaysia, 2007).

Towards sustainable fisheries management, assessment of biodiversity is considered crucial before outlining any strategy. Precise knowledge on species

identification (taxonomy), biology and ecology is a strong drive to successful conservation planning. In order to keep balance between the economic importance of aquaculture and wild stock protection, a systematic approach should be considered. Knowledge of the genetic status of species that have an ambiguous taxonomy is crucial and there is a state of urgency for better control in the introduction of exotic species.

REFERENCES

Adams, D. C., Rohlf, F.J. & Slice, D.E. (2004). Geometric Morphometrics: ten years of progress following the 'revolution'. *Italian Journal of Zoology, 71*, 5-16.

Adamson, E. A. S. (2010). *Influence of historical landscapes, drainage evolution and ecological traits on patterns of genetic diversity in Southeast Asian freshwater snakehead fishes*. (Unpublished Doctoral Thesis). Queensland University of Technology, Brisbane, Australia.

Adamson, E. A S., Hurwood, D. A., & Mather, P. B. (2010). A reappraisal of the evolution of Asian snakehead fishes (Pisces, Channidae) using molecular data from multiple genes and fossil calibration. *Molecular Phylogenetics and Evolution*, *56*(2), 707-717.

Albertson, R. C., & Kocher, T. D. (2001). Assessing morphological differences in an adaptive trait: a landmark-based morphometric approach. *Journal of Experimental. Zoology, 289*, 385-403.

An, H.S., Jee, Y.-J., Min, K.-S., Kim, B.-L., & Han, S.J. (2005). Phylogenetic analysis of six species of pacific abalone (Haliotidae) based on DNA sequences of 16s rRNA and cytochrome c oxidase subunit I mitochondrial genes. *Marine Biotechnology,*. *7*(4), 373-380.

Anderson, S., Banlier, A. T., Barrell, B.G., de Bruijn. M. H., Coulson, A. R., Drouin, J., ….. Young, I.G. (1981). Sequence and organization of the human mitochondrial genome. *Nature, 290*, 457-465.

Ansfield, M., Reaney, S. D., & Jackman, R. (2000). Production of a sensitive immunoassay for detection of ruminant and porcine proteins, heated to >130°C at 2.7 bar, in compound animal feedstuffs. *Food and Agricultural Immunology, 12*, 273– 284.

Anuar, H., Ambak, M.A., & Abdul-Samad, A.P. (2011). Crossbreeding of *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Pangasius nasutus* (Bleeker, 1863) and their larval development. *Journal of Sustainability Science and Management*, *6*(1), 28-35.

Aquino, L. M. G., Tango, J. M., Canoy, R. J. C., Fontanilla, I. K. C., Basiao, Z. U., Ong, P. S. & Quilang, J. P. (2011). DNA barcoding of fishes of Laguna de Bay, Philippines. *Mitochondrial DNA*, *22*(4), 143-53.

Armbruster, J. W. (2011). Global Catfish Biodiversity. *American Fisheries Society Symposium*, 77, 15-38.

Armbruster, J. W. (2012). Standardized measurements, landmarks, and meristic counts for cypriniform fishes. *Zootaxa, 16*, 8-16.

Asensio, L. (2007) PCR-based methods for fish and fishery products authentication. *Trends in Food Science & Technology, 18*, 558–566.

Avise, J. C. (1994). *Molecular Markers, Natural History and Evolution*. New York: Chapman & Hall.

Baird, I. (2011). *Pangasius krempfi*. The IUCN Red List of Threatened Species 2011: e.T181328A7668262.http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T181328A7668262.en. Downloaded on **14 November 2016**.

Baird, I. (2012). *Pangasius larnaudii*. The IUCN Red List of Threatened Species 2012: e.T180760A1659795.http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T180760A1659795.en. Downloaded on **14 November 2016**.

Barr, N.B., Copeland, R.S., De Meyer, M., Masiga, D., Kibogo, H.G., Billah, M.K., …, McPheron, B.A. (2006). Molecular diagnostics of economically important fruit fly species (Diptera: Tephritidae) in Africa using PCR and RFLP analyses. *Bulletin of Entomological Research, 96*, 505–521.

Bataille, M., Crainic, K., Leterreux, M., Durigon, M., & de Mazancourt, P. (1999). Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. *Forensic Science International, 99*, 165–70.

Berg, L. S. (1947). *Classification of fishes both recent and fossil*. Ann Arbor, Mich: J.W. Edwards.

Bermingham, E., & Lessios, H.A. (1993). Rate variation of protein and mtDNA evolution as revealed by sea urchins separated by the Isthmus of Panama. *Proceedings of the National Academy of Sciences, USA. 90*, 2734–2738.

Brown, W.M. (1985). The mitochondrial genome of animals: Molecular evolutionary genetics. In: MacIntyre R.J. (Ed.), *Molecular evolutionary genetics* (pp. 95–130). New York: Plenum Press.

Berra, T. (2001). *Freshwater Fish Distributions*. San Diego, CA: Academic Press.

Bhattacharjee, M. J., Laskar, B. A., Dhar, B., & Ghosh, S. K. (2012). Identification and re-evaluation of freshwater catfishes through DNA barcoding. *PloS One, 7*(11).

Bleeker, H.M. (1851a). Bijdrage tot de kennis der ichthyologische fauna van Borneo, met beschijving van 16 nieuwe soorten van zoetwatervisschen. *Nat. Tijdschr. Nederl.-Ind. 1*, 1-16.

Bleeker, P. (1851b). Vierde Bijdrage tot de kennis der ichthyologische fauna van Borneo, met beschrijving van eenige nieuwe soorten van zoetwatervisschen. *Nat. Tijdschr. Nederl.-Ind. 2,* 193-208.

Bleeker, P. (1858a). *Ichthyologiae Arcipelagi Indici Prodromus. Vol. I. Siluri*. Batavia, Nederland-Ind: Lange et Co.

Bleeker, P. (1858b). Zesde bijdrage tot de kennis der vischfauna van Sumatra. Visschen van Padang, Troessan, Priaman, Sibogha en Palembang. *Act. Soc. Sci. Indo-neerl. 3*, 1-50.

Bleeker, P. (1862). *Atlas ichthyologique des Indes Orientales Néerlandaises, publié sous le auspices du Gouvernement colonial néerlandais. Siluroïdes, Chacoïdes et Heterobranchoïdes*. 112 p., pls. 49-101.

Bookstein, F. L. (1982). Foundations of morphometrics. *Annual Review of Ecology and Systematics, 13*, 451-470.

Bookstein, F. L. (1991). *Morphometric tools for landmark data: Geometry and Biology*, Cambridge, England: Cambridge University Press.

Bookstein, F. L., Chernoff, B., Elder, R. L., Humphries, J. M., Smith, G. R., & Strauss, R. E. (1985). Morphometrics in evolutionary biology: the geometry of size and shape change, with examples from fishes. *Special Publication Academy of Natural Sciences Philadephia, Special publication, 15*, 1-277.

Bornbusch, A. H., & Lundberg, J. G. (1989). A new species of *Hemisilurus* (Siluriformes, Siluridae) from the Mekong River, with comments on its relationships and historical biogeography. *Copeia*, 434-444.

Botti, S., & Giuffra, E. (2010). Oligonucleotide indexing of DNA barcodes: identification of tuna and other scombrid species in food products. *BMC biotechnology, 10*, 60. Retrieved from http://www.biomedcentral.com/1472-6750/10/60

Burgess, W. (1989). *An atlas of freshwater and marine catfishes: A preliminary survey of the Siluriformes*. Neptune City, NJ: T.F.H. Publications.

Byrne, M., Rowe, F., & Uthicke, S. (2010). Molecular taxonomy, phylogeny and evolution in the family Stichopodidae (Aspidochirotida: Holothuroidea) based on COI and 16S mitochondrial DNA. *Molecular Phylogenetics and Evolution*, *56*(3), 1068-1081.

Cacot, P., Eeckhoutte, P., Trieu, N. V., Legendre, M., Mariojouls, C., & Lazard, J. (2003). Induced spermiation and milt management in *Pangasius bocourti* (Sauvage, 1880). *Aquaculture*, *215*(1), 67-77.

Campton, D. E. (1987). Natural hybridization and introgression in fishes: methods of detection and genetic interpretations. In N. Ryman & F. Utter (Eds.), *Population genetics and fishery management* (pp. 161-92). Seattle: University of Washington Press.

Carvalho, D. C., Neto, D. A., Brasil, B. S., & Oliveira, D. A. (2011). DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil. *Mitochondrial DNA*, *22*(S1), 97-105.

Castro, J.A., Picornell, A., & Ramon, M. (1998). Mitochondrial DNA: a tool for population genetics studies. *International Microbiology*, 327-332.

Cawthorn, D.-M., Steinman, H. A., & Witthuhn, R. C. (2012). Evaluation of the 16S and 12S rRNA genes as universal markers for the identification of commercial fish species in South Africa. *Gene*, *491*(1), 40-8.

Chakrabarty, P., Warren, M., Page, L. M., & Baldwin, C. C. (2013). GenSeq: An updated nomenclature and ranking for genetic sequences from type and non-type sources. *ZooKeys*, *346*, 29-41.

Chan, D. C. (2006). Mitochondria: Dynamic Organelles in Disease, Aging, and Development. *Cell*, *125*, 1241-1252.

Chardon, M. (1968). Anatomie compare de l'appareil de Weber et des structures connexes chez les Siluriformes. *Annales du Musée Royal de l'Afrique Centrale 169*, 1-277.

.

Chaudhry, S. (2010). *Pangasius myanmar*. The IUCN Red List of Threatened Species 2010: e.T168477A6499500. http://dx.doi.org/10.2305/IUCN.UK.2010-4.RLTS.T168477A6499500.en. Downloaded on **14 November 2016**.

Chong, V. C., Lee, P. K. Y., & Lau, C. M. (2010). Diversity, extinction risk and conservation of Malaysian fishes. *Journal of Fish Biology, 76*(9), 2009-2066.

Chu, C., Rizman-Idid, M., & Ching, C. V. (2013). Phylogenetic relationships of selected genera of Lutjanidae inferred from mitochondrial regions, with a note on the taxonomic status of *Pinjalo pinjalo*. *Ciencias Marinas, 39*(4), 349-361.

Costa, C., & Cataudella, S. (2006). Relationship between shape and trophic ecology of selected species of Sparids of the Caprolace coastal lagoon (Central Tyrrhenian Sea). *Environmental Biology of Fishes, 78,* 115-123.

Crumlish, M., Dung, T. T., Turnbull, J. F., Ngoc, N. T., N. & Ferguson, H. W. (2002). Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. *Journal of Fish Diseases, 25*(12), 733-736.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G. R., & Thorpe, R. S. (2007). Validation of the barcoding gene COI for use in forensic genetic species identification. *Forensic Science International, 173*(1), 1-6.

Department of Fisheries Malaysia. (2000-2015). *Annual Fisheries Statistics*. Jabatan Perikanan Malaysia, Putrajaya: Malaysia.

Department of Standards Malaysia (2007). *Malaysian Standard - Good Aquaculture Practice (GAqP) – Aquaculture Farm – General Guidelines MS 1998:2007*. 8 pp. Jabatan Standard Malaysia, Putrajaya: Malaysia.

De Salle, R. (2006). Species discovery versus species identification in DNA barcoding efforts: response to Rubinoff. *Conservation Biology, 20,* 1545-1547.

DeSalle, R., Egan, M.G., & Siddall, M. (2005). The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society of London, B., 360,* 1905–1916.

De Silva, S. S., Nguyen, T. T., Abery, N. W., & Amarasinghe, U. S. (2006). An evaluation of the role and impacts of alien finfish in Asian inland aquaculture. *Aquaculture Research, 37*(1), 1-17.

De Silva, S. S., Nguyen, T. T., Turchini, G. M., Amarasinghe, U. S., & Abery, N. W. (2009). Alien species in aquaculture and biodiversity: a paradox in food production. *Ambio: a journal of the human environment*, *38*(1), 24-28.

Diogo, R. (2003). Higher level phylogeny of Siluriformes: an overview. In G. Arratia, B. G. Kapoor, M. Chardon & R. Diogo (Eds), *Catfishes* (Vol. 1. pp. 353-384). Enfield, NH: Science Publishers.

Dodson, J.J., Colombani, F., & Ng P.K. (1995). Phylogeographic structure in mitochondrial DNA of a South-east Asian freshwater fish, *Hemibagrus nemurus* (Siluroidei: Bagridae) and Pleistocene sea-level changes on the Sunda Shelf. *Molecular Ecology*, *4* (3), 331-346.

Dodson, J. J., & Lecomte, F. (2015). A DNA Barcode-Based Evaluation of the Southeast Asian Catfish Genus *Hemibagrus* Bleeker, 1862 (Teleostei: Siluriformes; Bagridae). *Advances in Evolutionary Biology.* 21pp. http://dx.doi.org/10.1155/2015/490158

Esa, Y. B., Siraj, S. S., Daud, S. K., Rahim, K. A. A., Japning, J. R. R., & Tan, S. G. (2008). Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinidae) from five natural populations in Malaysia. *Zoological Studies*, *47*(3), 360-367.

Eschmeyer, W. N. & Fong, J. D. (2016). SPECIES BY FAMILY/SUBFAMILY. (http://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp). **Electronic version accessed 14 Nov 2016.**

FAO. FAO fisheries and aquaculture department (Rome). (2009). *The State of World Fisheries and Aquaculture 2008*. Rome, Italy: Food & Agriculture Organization of the United Nations.

Farias, I. P., Ortí, G., Sampaio, I., Schneider, H., & Meyer, A. (2001). The cytochrome b gene as a phylogenetic marker: the limits of resolution for analyzing relationships among cichlid fishes. *Journal of Molecular Evolution*, *53*(2), 89-103.

Faruk, A. R. (2008). Disease and Health Management of Farmed Exotic Catfish Pangasius hypopthalmus in Mymensingh District of Bangladesh. In M. G. Bondad-Reantaso, C. V. Mohan, M. Crumlish, & R. P. Subashinghe (Eds.), *Diseases in Asian Aquaculture VI. Fish Health Section* (pp. 193-204). Asian Fisheries Society.

Fazimah, A. (2013). *Morphometric, Phylogenetic Analyses and Population Genetics of Asian Arowana (Scleropages formosus) from Bera Lake and Endau River, Peninsular Malaysia*. (Unpublished PhD Thesis). University of Malaya, Kuala Lumpur, Malaysia.

Felsenstein, J. (1985) Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution, 39*(4), 783-791.

Ferdous, S. (2013). *Geometric Morphometrics and Phylogeny of the Catfish genus Mystus Scopoli (Siluriformes: Bagridae) and North American Cyprinids (Cypriniformes).* (Unpublished PhD Thesis). Auburn University, Auburn, Alabama, USA.

Ferguson, H. W., Turnbull, J. F., Shinn, A., Thompson, K., Dung, T. T., & Crumlish, M. (2001). Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage) from the Mekong Delta, Vietnam. *Journal of Fish Diseases, 24*(9), 509-513.

Ferraris, C. J. (2007). *Checklist of catfishes, recent and fossil (Osteichthyes: Siluriformes), and catalogue of siluriform primary types*. Auckland, New Zealand: Magnolia Press.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). *Biology Letters*, *4*, 423-425.

Fowler, H. W. (1938). A list of the fishes known from Malaya. *Fisheries Bulletin 1*, 1-268.

Froese, R., & Pauly, D. (Eds.). (2016). FishBase World Wide Web electronic publication. www.fishbase.org, version (10/2016).

Gerber, A. S., Loggins, R., Kumar, S., & Dowling, T. E. (2001). Does non-neutral evolution shape observed patterns of DNA variation in animal mitochondrial genomes? *Annual Reviews of Genetics, 35*, 539–566.

Gharrett A.J., Gray A.K., & Heifetz J (2001) Identification of rockfish (*Sebastes* spp.) by restriction site analysis of the mitochondrial ND-3/ND-4 and 12S/16S rRNA gene regions. *Fishery Bulletin (Washington D C), 99*, 49–62.

Giles, R. E., Blanc, H., Cann, H. M., & Wallace, D. C. (1980). Maternal inheritance of human mitochondrial DNA. *Proceedings of the National Academy of Sciences*, *77*(11), 6715-6719.

Gozlan, R. E., Britton, J. R., Cowx, I., & Copp, G. H. (2010). Current knowledge on non-native freshwater fish introductions. *Journal of Fish Biology, 76*(4), 751-786.

Granadeiro, P., & Silva, M. A (2000). The use of otoliths and vertebrae in the identification and size-estimation of fish in predator-prey studies. *Cybium*, *24*(1), 383-393.

Greenwood, P.H.., Rosen, D E., Weitzman, S.H., & Myers, G. S. (1966). Phyletic studies of teleostean fishes, with a provisional classification of living forms. *Bulletin of the American. Museum of. National History, 131*, 339-456.

Gross, M. R. (1998). One species with two biologies: Atlantic salmon (*Salmo salar*) in the wild and in aquaculture. *Canadian Journal of Fisheries and Aquatic Sciences*, *55*(S1), 131-144.

Guha, S., Goyal, S. P., & Kashyap, V. K., (2006). Genomic variation in the mitochondrially encoded cytochrome b (Mt–Cyb) and 16S rRNA (MT– RNR2) genes: characterization of eight endangered Pecoran species. *Animal Genetics, 37*, 262–265.

Günther, A. (1864). Catalog of the Physostomi containing families Siluridae, Characinidae, Haplochitonidae, Sternoptychidae, Scopelidae in the collection of the British Museum, London.

Gunz, P., Mitteroecker, P. & Bookstein, F. L. (2004). Semilandmarks in Three Dimensions. In D. E. Slice (Ed.). *Modern Morphometrics in Physical Anthropoly* (pp. 73-98). New York, NY: Kluwer Academics/Plenum Publishers.

Gupta, A. R., Patra, R. C., Das, D. K., Gupta, P. K., Swarup, D., & Saini, M. (2008). Sequence characterization and polymerase chain reaction restriction fragment length polymorphism of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. *DNA Sequence, 19(4)*, 394–400.

Gustiano, R. (2003). *Taxonomy and phylogeny of pangasiidae catfishes from Asia (Ostariophysis, Siluriformes)*. (Unpublished doctoral dissertation). Katholieke Universiteit Leuven, Belgium.

Gustiano, R., Teugels, G. G., & Pouyaud, L. (2004). *Pangasius bedado* Roberts, 1999: a junior synonym of *Pangasius djambal* Bleeker, 1846 (Siluriformes, Pangasiidae). *Cybium, 28 (1),* 13-18.

Gustiano, R. (2009). Pangasiid catfishes of Indonesia. *Buletin Plasma Nutfah*, *15*(2), 91-99.

Hair, J.F., Jr., Anderson, R.E., & Tatham, R.L. (1987). *Multivariate Data Analysis* (2nd ed.) with readings. New York, NY: Macmillan.

Hajibabaei, M., Singer, G. A., Hebert, P. D., & Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, *23*(4), 167-172.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science*, *279*(5350), 509-514.

Hall, A. (2009). The cytoskeleton and cancer. *Cancer and Metastasis Reviews*, *28*(1-2), 5-14.

Hamilton, F. (1822). *An account of the fishes found in the river Ganges and its branches: With a volume of plates in Royal Quarts*. Edinburgh: Constable.

Hanner, R., Becker, S., Ivanova, N. V., & Steinke, D. (2011). FISH-BOL and seafood identification: Geographically dispersed case studies reveal systemic market substitution across Canada. *Mitochondrial DNA*, *22*(S1), 106-122.

Hardenberg, J.D.F. (1948). Some new or rare fishes of the Indo-Australian Archipelago. *Treubia*, *19*, 407- 41.

Hardman, M. (2005). The phylogenetic relationships among non-diplomystid catfishes as inferred from mitochondrial cytochrome b sequences; the search for the ictalurid sister taxon (Otophysi: Siluriformes). *Molecular Phylogenetics and Evolution*, *37*(3), 700-720.

Haslawati, B., Rizman-Idid, M., Muniandy, S., & Zakaria-Ismail, M. (2014). The Occurrence of *Pangasius polyuranodon* Bleeker, 1852 (Teleostei: Pangasiidae) in Peninsular Malaysia with Remarks on the Comparative Morphology with *Pseudolais micronemus* (Bleeker, 1847). *Sains Malaysiana*, *43*(11), 1707-1714.

Hebert, P. D., Ratnasingham, S., & de Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences*, *270* (Suppl 1), 96-99.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14812-14817.

Henze, K., & Martin, W. (2003). Evolutionary biology: essence of mitochondria. *Nature*, *426*(6963), 127-128.

Herre, A. W. C. T., & Myers, G. S. (1937). A contribution to the ichthyology of the Malay Peninsula. *Bulletin of the Raffles Museum*, *13*, 5-75.

Hogan, Z. (2011). *Pangasianodon gigas*. The IUCN Red List of Threatened Species 2011: e.T15944A5324699. http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T15944A5324699.en. Downloaded on **03 November 2016**.

Hogan, Z., Na-Nakorn, U., & Kong, H. (2009). Threatened fishes of the world: *Pangasius sanitwongsei* Smith 1931 (Siluriformes: Pangasiidae). *Environmental Biology of Fishes*, *84*(3), 305-306.

Hora S. L. (1938). The game fishes of India II, the pangas catfish, *Pangasius pangasius* (Hamilton). *Journal of Bombay Natural History Society*, 40, 355-356.

Hora, S. L., & Gupta, J. C. (1941). *Notes on Malayan fishes in the Collection of the Raffles Museum, Singapore*.

Hossain, M. Y., Rahman, M. M., & Mollah, M. F. A. (2009). Threatened fishes of the world: *Pangasius pangasius* Hamilton-Buchanan, 1822 (Pangasiidae). *Environmental Biology of Fishes*, *84*(3), 315-316.

Hsieh, H.S., Chai, T., & Hwang, D.F. (2007) Using the PCR-RFLP method to identify the species of different processed products to billfish meats. *Food Contr*ol, *18*, 369–374.

Hubbs, C. L., & Lagler, K. F. (1964). *Fishes of the Great Lakes region*. Ann Arbor: University of Michigan Press.

Hubert, N., Hanner, R., Holm, E., Mandrak, N. E., Taylor, E., Burridge, M., Watkinson, D., ... Ellegren, H. (2008). Identifying Canadian Freshwater Fishes through DNA Barcodes. *Plos One*, *3*, 6.

Humphrey, S. R., & Bain, J. R. (1990). *Endangered animals of Thailand*. Gainesville, Fla: Sandhill Crane Press.

Inger, R. F., & Chin, P. K. (1962). *The freshwater fishes of North Borneo.* Chicago Natural History Museum Chicago, Chicago.

Ismail M. Z. (1989). *Systematics, Zoogeography, and Conservation of the Freshwater Fishes of Peninsular Malaysia.* Doctoral Dissertation. Colorado State University

Jayaram, K. C. (1977). Aid to the identification of Siluroids. 2: Siluroidae, Schilbeidae, Pangasiidae, Amblycipitidae, Akysidae. *Records of the Zoological Survey of India Occasional Paper*, *10*, 1-33.

Jenkins, A., Kullander, F.F., & Tan, H.H. (2009). *Pangasius macronema*. The IUCN Red List of Threatened Species 2009: e.T169550A6646168. http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T169550A6646168.en. Downloaded on **14 November 2016**.

Jenuth, J. P., Perterson, A. C., Fu, K., & Shoubridge, E. A. (1997). Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nature Genetics*, *14*, 146–151.

Johns, G.C., & Avise, J. C. (1998). Test for ancient species flocks based on molecular phylogenetic appraisals of Sebastes rockfishes and other marine fishes. *Evolution*, *52*, 1135-1146.

Karinthanyakit, W., & Jondeung, A. (2012). Molecular phylogenetic relationships of pangasiid and schilbid catfishes in Thailand. *Journal of Fish Biology*, *80*(7), 2549-2570.

Kartavtsev, Y. P., Sharina, S. N., Goto, T., Chichvarkhin, A. Y., Balanov, A. A., Vinnikov, K. A., & Hanzawa, N. (2008). Cytochrome oxidase 1 gene sequence analysis in six flatfish species (Teleostei, Pleuronectidae) of Far East Russia with inferences in phylogeny and taxonomy: *Mitochondrial DNA*, *19*(6), 479-489.

Khan, H. A., Arif, I.A., Al Farhan, A. H., & Al Homaidan, A. A. (2008). Phylogenetic analysis of oryx species using partial sequences of mitochondrial rRNA genes. *Genetics and Molecular Research*, *7*(4), 1150-1155.

Khan, M., Lee, P., Cramphorn, J., & Zakaria-Ismail, M. (1996). Freshwater Fishes of the Pahang River basin, Malaysia. Wetlands International-Asia Pacific Publication No. 112. *Kuala Lumpur: Wetlands International-Asia Pacific*.

Khare, P., Mohindra, V., Barman, A. S., Singh, R. K., & Lal, K. K. (2014). Molecular evidence to reconcile taxonomic instability in mahseer species (Pisces: Cyprinidae) of India. *Organisms Diversity & Evolution*, *14*(3), 307-326.

Khedkar, G. D., Jamdade, R., Naik, S., David, L., & Haymer, D. (2014). DNA Barcodes for the Fishes of the Narmada, One of India's Longest Rivers. *PloS One*, *9*(7), e101460.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*(2), 111-120.

Kitano T, Umetsu K, Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine, 121,* 423– 427.

Kochzius, M., Seidel, C., Antoniou, A., Botla, S. K., Campo, D., Cariani, A., & Blohm, D. (2010). Identifying fishes through DNA barcodes and microarrays. *PLoS One*, *5*(9), e12620.

Kochzius, M., Nölte, M., Weber, H., Silkenbeumer, N., Hjörleifsdottir, S., Hreggidsson, G. O., Marteinsson, V….Blohm, D. (2008) DNA microarrays for identifying fishes. *Marine Biotechnology, 10* (2), :207–217.

Kottelat, M. (1995).The fishes of the Mahakam River, East Borneo: an example of the limitations of zoogeographic analyses and the need for extensive fish surveys in Indonesia. *Tropical Biodiversity, 2*, 401-426.

Kottelat, M. (2013). The fishes of the inland waters of Southeast Asia: A catalogue and core bibliography of the fishes known to occur in freshwaters, mangroves and estuaries. *The Raffles Bulletin of Zoology* (Supplement) *27*, 1-663.

Kottelat, M. (1984). A Review of the species of Indochinese fresh-water fishes described by H.E. Sauvage. *Bulletin du Muséum National.d'histoire Naturelle. Paris, ser. 4*, 6(A), 791-822.

Kottelat, M., Whitten, A.J., Kartikasari, S.R, & Woerjoatmodjo, S. (1993). *Freshwater fishes of Western Indonesia and Sulawesi.* Hongkong: Periplus Edition.

Lakra, W. S., Goswami, M., Gopalakrishnan, A., Singh, D. P., Singh, A., & Nagpure, N. S. (2010). Genetic relatedness among fish species of Genus Channa using mitochondrial DNA genes. *Biochemical Systematics and Ecology, 38*(6), 1212-1219.

Laskar, B. A., Bhattacharjee, M. J., Dhar, B., Mahadani, P., Kundu, S., Sankar, S., & Ghosh, K. (2013). The species dilemma of northeast Indian mahseer (Actinopterygii: Cyprinidae): DNA barcoding in clarifying the riddle. *PLoS One, 8* (1).

Lei, R., Jian, Z., Hu, Z., & Yang, W. (2003). Phylogenetic relationships of Chinese antelopes (Subfamily Antilopinae) based on mitochondrial Ribosomal RNA gene sequences. *Journal of Zoology, 261*, 227–237.

Lim, K.K.P., & Zakaria-Ismail, M. (1995). The occurrence of the catfish *Helicophagus waandersii* (Pisces: Pangasiidae) in Peninsular Malaysia. *Malayan Nature Journal. 49*, 37-40.

Lin W–F., & Hwang D-F. (2007) Application of PCR-RFLP analysis on species identification of canned tuna. *Food Control, 18*, 1050–1057.

Lockley, A. K., & Bardsley, R. G. (2000). DNA-based methods for food authentication. *Trends in Food Science & Technology*, *11*(2), 67-77.

Loh, K-H., Muhammad Ali, S. H., Chong, V. C., & Sasekumar, A. (2015). Notes on the Moray Eels (Anguilliformes: Muraenidae) of Malaysia with two new records. *Sains Malaysiana, 44* (1), 41-47.

López-Calleja, I., González, I, Fajardo, V, Rodriguez, M. A., Harnández, P. E., Garcia, T., & Martin, R. (2004). Rapid detection of cows' milk in sheeps' and goats' milk by a species-specific polymerase chain reaction technique. *Journal of Dairy Science*, 87, 2839–45.

Lowenstein, J. H., Burger, J., Jeitner, C. W., Amato, G., Kolokotronis, S. O., & Gochfeld, M. (2010). DNA barcodes reveal species-specific mercury levels in tuna sushi that pose a health risk to consumers. *Biology Letters*, *6*(5), 692-5.

Lowenstein, J. H., Amato, G., & Kolokotronis, S. O. (2009). The real maccoyii: identifying tuna sushi with DNA barcodes–contrasting characteristic attributes and genetic distances. *PLoS One*, *4*(11).

Lundberg, J. G., Sullivan, J. P., Rodiles-Hernández, R., & Hendrickson, D. A. (2007). Discovery of African roots for the Mesoamerican Chiapas catfish, *Lacantunia enigmatica*, requires an ancient intercontinental passage. *Proceedings of the Academy of Natural Sciences of Philadelphia*, *156*(1), 39-53.

Lundberg, J. G., & Friel, J. P. (2003). *Siluriformes. Catfishes*. Retrieved from http://tolweb.org/Siluriformes/15065/2003.01.20

Malakar, A. K., Lakra, W. S., Goswami, M., Singh, M., & Mishra, R. M. (2012). Molecular identification of three Ompok species using mitochondrial COI gene. *Mitochondrial DNA*, *23*(1), 20-24.

Martîn, I. García, T, Fajardo, V., López-Calleja, I, Rojas, M., Pavón, M.A., Hernández, P.E., González, I.., & Martin, R. (2007) Technical note: detection of chicken, turkey, duck, and goose tissues in feedstuffs using species-specific polymerase chain reaction. *Journal of Animal Science, 85*, 452–8.

McConnell, S. K. J. (2004). Mapping aquatic faunal exchanges across the Sunda shelf, South-East Asia, using distributional and genetic data sets from the cyprinid fish *Barbodes gonionotus* (Bleeker, 1850). *Journal of Natural History, 38*(5), 651-670.

McGinnity, P., Stone, C., Taggart, J. B., Cooke, D., Cotter, D., Hynes, R., ... Ferguson, A. (1997). Genetic impact of escaped farmed Atlantic salmon (Salmo salar L.) on native populations: use of DNA profiling to assess freshwater performance of wild, farmed, and hybrid progeny in a natural river environment. *ICES Journal of Marine Science*,*54*: 998e1008

McGinnity, P., Prodöhl, P., Ferguson, A., Hynes, R., Maoiléidigh, N. O., Baker, N. … Cross, T. (2003). Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo sala*r, as a result of interactions with escaped farm salmon. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1532), 2443-2450.

Medina, M., Collins, T. M., & Walsh, P. J. (2001). mtDNA ribosomal gene phylogeny of sea hares in the genus Aplysia (Gastropoda, Opisthobranchia, Anaspidea): implications for comparative neurobiology. *Systematic Biology, 50*, 676–88.

Meier, R., Shiyang, K., Vaidya, G., & Ng, P. K. (2006). DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Systematic Biology*, *55*(5), 715-728.

Meng, H. J., & Stocker, M. (1984). An evaluation of morphometrics and meristics for stock separat of Pacific herring (*Clupea harengus pallasi*). *Canadian. Journal of Fisheries and Aquatic Sciences, 41*, 414-422.

Meyer, A. (1994). Shortcomings of the cytochrome b gene as a molecular marker. *TREE* 9(8), 278–280.

Meyer, C. P., & Paulay, G. (2005) DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology, 3*, 2229–2238.

Meusnier, I., Singer, G. A., Landry, J. F., Hickey, D. A., Hebert, P. D., & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC genomics*, *9*(1), 214.

Mitsuhiro, N., Yasuyuki, I., & Syuiti, A. (2004). Novel primer sets for species-specificamplification of the mitochondrial 12S rRNA genes in four Japanese woodpeckers (Picidae, Piciformes). *Meat Science 66*, 551–556.

Mohd-Shamsudin, M.I., Zahedi-Fard, M., Mather, P.B., Suleiman, Z., Hassan, R, Othman, R.Y., & Bhassu, S. (2011). Molecular characterization of relatedness among colour variants of Asian arowana (*Scleropages formosus*). *Gene*, *490(*1-2*)*, 47-53.

Mo, T. (1991). Anatomy and systematics of Bagridae (Teleostei), and siluroid phylogeny. *Theses Zoologicae* 17, 1-216.

Monteiro, L. R., & Abe, A. S. (1999). Functional and historical determinants of shape in the scapula of Xenarthran mammals: Evolution of a complex morphological structure. *Journal of Morphology, 241*, 251-263.

Moritz, C., Dowling, T.E., & Brown, W.M. (1987). Evolution of Animal mitochondrial DNA-revelence for population biology and systematic. *Annual Review of Ecology and Systematics, 18*, 269-292.

Moyle, P. B., & Cech, J. J. Jr. (2000). *Fishes: An introduction to Ichthyology* (4th ed.). NJ. USA: Prentice Hall.

Nagashima, Y., Matsumoto, T., Kadoyama, K., Ishizaki, S., & Terayama, M. (2011) Toxicity and molecular identification of green toadfish *Lagocephalus lunaris* collected from Kyushu Coast, Japan. *Journal of Toxicology*, Volume *2011*.

Na-Nakorn, U., Sukmanomon, S., Nakajima, M., Taniguchi, N., Kamonrat, W., Poompuang, S., & Nguyen, T. T. T. (2006). MtDNA diversity of the critically endangered Mekong giant catfish (*Pangasianodon gigas* Chevey, 1913) and closely related species: implications for conservation. *Animal Conservation, 9*(4), 483-494.

Naylor, G.J., Collins, T.M., & Brown, W.M. (1995) Hydrophobicity and phylogeny. *Nature, 373,* 565–566.

Nei, M. (1987). *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Nei, M., & Kumar, S. (2000). *Molecular Evolution and Phylogenetics.* New York: Oxford University Press.

Nelson, J. S. (2006). *Fishes of the World*. New Jersey: John Wiley & Sons.

Ng, H.H. (2002). The identity of *Mystus nigriceps* (Valenciennes in Cuvier and Valenciennes, 1840) with the description of a new Bagrid catfish (Teleostei: Siluriformes) from Southeast Asia. *The Raffles Bulletin of Zoology, 50*(1), 161-168.

Ng, H.H. (2003). A review of the *Ompok hypophthalmus* group of silurid catfishes with the description of a new species from South-East Asia. *Journal of Fish Biology, 62,* 1296-1311.

Ng, H.H. (2004). *Wallago micropogon*: a new species of Silurid catfish (Teleostei: Siluridae) from mainland Southeast Asia. *Copeia,* (1), 92-97.

Ng, H. H., & Kottelat, M. (2000). *Helicophagus leptorhyncus*, a new species of molluscivorous catfish from Indochina (Teleostei: Pangasiidae). *Raffles Bulletin of Zoology, 48,* 55-58.

Nguyen, V. H. (2008) Sustainability issues in Vietnam's catfish industry. *Aquaculture Asia Pacific, 4,* 15-17.

Nguyen, T. T. T., & Na-Nakorn, U. (2004). Genetic impacts of translocations on biodiversity of aquatic species with particular reference to Asian countries. *Aquaculture Asia, 9,* 4-7.

Nguyen, T. T. T., Ingram, B., Sungan, S., Gooley, G., Sim, S. Y., Tinggi, D., & De Silva, S. S. (2006). Mitochondrial DNA diversity of broodstock of two indigenous mahseer species, *Tor tambroides* and *T. douronensis* (Cyprinidae) cultured in Sarawak, Malaysia. *Aquaculture*, *253*(1-4), 259-269.

Nukwan, S., Lawanyawut, K., Tangtrongpiros, M., & Veerasidth, P., (1990). Backcrossing experiment of hybrid between *Clarias macrocephalus* and *Clarias gariepinus*. Proceedings of the 28th Kasetsart University Conferences (pp. 529–544). Bangkok, Thailand: Kasetsart University (In Thai).

Pal, M. (2010). *Pangasius pangasius*. The IUCN Red List of Threatened Species 2010: e.T166404A6201771.http://dx.doi.org/10.2305/IUCN.UK.2010-4.RLTS.T166404A6201771.en. Downloaded on **14 November 2016**.

Palumbi, S., Martin, A., Romano, S., McMillan, W., Stice, L., & Grabowski, G. (1991). The Simple Fool's Guide to PCR, Version 2.0, privately published document compiled by S. Palumbi. Dept. *Zoology, Univ. Hawaii, Honolulu, HI, 96822*.

Pascoal, A., Barros-Velázquez, J., Cepeda, A., Gallardo, J. M., & Calo-Mata, P. A. (2008) polymerase chain reaction-restriction fragment length polymorphism method based on the analysis of a 16S rRNA/tRNA(Val) mitochondrial region for species identification of commercial penaeid shrimps (Crustacea: Decapoda: Penaeoidea) of food interest. *Electrophoresis, 29,* 499–509.

Phan L. T., Bui T. M., Nguyen T. T. T., Gooley G. J., Ingram B. A., Nguyen H. V., P.T. Nguyen, P. T., & De Silva, S.S. (2009). Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus* in the Mekong Delta. Vietnam. *Aquaculture, 296,* 227–236.

Pierce, G. J., & Boyle, P. R. (1991). A review of methods for diet analysis in piscivorous marine mammals. *Oceanography and. Marine. Biology, An. Annual. Review, 29,* 409-486.

Pierce, S. E., Angielczyk, K. D., & Rayfield, E. J. (2008). Patterns of morphospace occupation and mechanical performance in extant crocodilian skulls: A combined geometric morphometric and finite element modeling approach. *Journal of Morphology*, *269*(7), 840-864.

Pimentel, R. A. (1979). *Morphometrics, the multivariate analysis of biological data*. Dubuque, Iowa: Kendall/Hunt Pub. Co.

Pook, C. E., & McEwing, R. (2005). Mitochondrial DNA sequences from dried snake venom: a DNA barcoding approach to the identification of venom samples. *Toxicon*, *46*, 711–5.

POSMA, FSPS II. (2009) Briefing document on the current status of Vietnam's fishery sector. Prepared by the Post-Harvest and Marketing Component of the Fishery Sector Programme Support Phase II. June 2009. 50 pp. Unpublished report prepared for the FSPS II national steering committee meeting, June 2009. http://www.ambhanoi.um.dk/en/menu/Developmentpolicy/SectorProgrammes/Fisheries/.

Poulsen, A. F., Hortle, K. G., Valbo-Jorgensen, J., Chan, S., Chhuon, C. K., Viravong, S., & Tran, B. Q. (2004). Distribution and ecology of some important riverine fish species of the Mekong River Basin. *MRC technical paper*, *10*, 116pp. ISSN: 1683-1489

Pouyaud, L., Teugels, G. G., & Legendre, M. (1999). Description of a new pangasiid catfish from South-East Asia (Siluriformes). *Cybium*, *23*(3), 247-258.

Pouyaud, L., & Teugels, G.G. (2000). Description of a new pangasiid from Northeast Kalimantan, Indonesia (Siluriformes, Pangasiidae). *Ichthyological Exploration in Freshw*ater, *11*, 193-200.

Pouyaud, L., Teugels, G. G., Gustiano, R., & Legendre, M. (2000). Contribution to the phylogeny of pangasiid catfishes based on allozymes and mitochondrial DNA. *Journal of Fish Biology*, *56*(6), 1509-1538.

Pouyaud, L., Gustiano, R., & Teugels, G. G. (2002). Systematic revision of *Pangasius polyuranodon* (Siluriformes, Pangasiidae) with description of two new species. *Cybium*, *26*(4), 243-252.

Pouyaud, L., Gustiano, R., & Teugels, G.G. (2004) Contribution to the phylogeny of the Pangasiidae based on mitochondrial 12S rDNA. *Indonesian Journal of Agricultural Science,* 5(2), 45-62.

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, *21*(8), 1864-1877.

Rahman, M.A., Bhadra, A., Begum, N., Islam. M.S., & Hussain, M.F., (1995). Production of hybrid vigor cross breeding between *Clarias batrachus* Lin. & *Clarias geriepinus* Bur. *Aquaculture, 138*, 125-130.

Rainboth, W.J. (1991). Cyprinids of Southeast Asia. In I. J. Winfield and J.S. Nelson (Eds.), *Cyprinid Fishes: Systematics, biology and exploitation* (pp: 156-210). London: Chapman & Hall.

Rainboth, W.J. (1996). *FAO Species identification field guide for fishery purposes. Fishes of the Cambodian Mekong.* Rome, Italy: Food & Agriculture Organization of the United Nations.

Ramlah, Z. (2009). *Ecology and Molecular Phylogenetics of Frogs from the Genus Rana Linnaeus 1758 in Sarawak*. (Unpublished PhD Thesis), Universiti Kebangsaan Malaysia, Bangi. Malaysia.

Ratnasingham, S., & Hebert, P. D. (2007). BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Notes, 7*(3), 355-364.

Reed, C. (2008). Import risk analysis: Frozen, skinless and boneless fillet meat of Pangasius spp. fish from Vietnam for human consumption. Biosecurity New Zealand Ministry of Agriculture and Forestry Wellington New Zealand Ministry of Agriculture and Forestry Te Manatu Ahuwhenua, Ngaherehere Pastoral House 25 The Terrace P O Box 2526 Wellington New Zealand . Internet: http://www.maf.govt.nz

Rojas, M., González, I., Fajardo, V., Martîn, I., Hernández, P.E., García, T., & Martin, R. (2008). Polymerase chain reaction-restriction fragment length polymorphism authentication of raw meats from game birds. *Jounal of AOAC International, 91(6)*, 1416–22.

Roberts, T. R. (1989). The freshwater fishes of Western Borneo (Kalimantan Barat, Indonesia*). Memoirs of the California Academy of Sciences, 14*, 1-210.

Roberts, T. R., & Vidthayanon, C. (1991). Systematic revision of the Asian catfish family Pangasiidae, with biological observations and descriptions of three new species. *Proceedings of the Academy of Natural Sciences of Philadelphia, 143*, 97-143.

Roberts, T. R., & Jumnongthai, J. (1999). Miocene fishes from Lake Phetchabun in north-central Thailand, with descriptions of new taxa of Cyprinidae, Pangasiidae and Chandidae. *Natural History Bulletin of the Siam Society, 47*, 153-189.

Romer, A. S. (1947). *Vertebrate paleontology*. Illinois, USA: The University of Chicago Press.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., & Rozas, R. (2009). *DNA Sequence Polymorphism* Version 5.10.00.

Ryan, J.R.J., & Esa, Y. B. (2006). Phylogenetic analysis of hampala fishes (Subfamily Cyprininae) in Malaysia inferred from partial mitochondrial cytochrome b DNA sequences. *Zoological Science, 23,* 893-901.

Saidin, T., Othman, A.A., & Mohamad-Zaini, S. (1988). Induced spawning techniques practised at Batu Berendam, Melaka, Malaysia. *Aquaculture, 74*(1), 23-33.

Saini, M. Das, D. K., Dhara, A., Swarup, D., Yadav, M. P., & Gupta, P. K. (2007). Characterisation of peacock (*Pavo cristatus*) mitochondrial 12S rRNA sequence and its use in differentiation from closely related poultry species. *British Poultry Science, 48 (2),* 162–166

Sanders, M (1934). Die fossilen Fische der altertiaren Susswas serablagerungen aus Mittel-Sumatra. *Verhandelingen van het Geologish Mijnbouwkundig Genoootschap, Den Hague, 11,* 1-143.

Santos, S., Schneider, H., & Sampaio, I. (2003). Genetic differentiation of Macrodon ancylodon (Sciaenidae, Perciformes) populations in Atlantic coastal waters of South America as revealed by mtDNA analysis. *Genetics and Molecular Biology, 26*(2), 151–161.

Scotland, R. W., Olmstead, R. G., & Bennett, J. R. (2003). Phylogeny reconstruction: the role of morphology. *Systematic Biology, 52*(4), 539-548.

Senanan, W., Kapuscinski, A.R., Na-Nakorn, U., & Miller, L. (2004). Genetic impacts of hybrid catfish farming (*Clarias macrocephalus vs C. gariepinus*) on native catfish populations in central Thailand. *Aquaculture, 235,* 167–184.

Serrao, N. R., Steinke, D., & Hanner, R. H. (2014). Calibrating snakehead diversity with DNA barcodes: expanding taxonomic coverage to enable identification of potential and established invasive species. *PLoS One, 9*(6), e99546.

Simons, A. M., & Mayden, R. L. (1998). Phylogenetic relationships of the western North American phoxinins (Actinopterygii: Cyprinidae) as inferred from mitochondrial 12S and 16S ribosomal RNA sequences. *Molecular Phylogenetics and Evolution, 9,* 308–29.

Singh, A. K., & Lakra, W. S. (2012). Culture of *Pangasianodon hypophthalmus* into India: impacts and present scenario. *Pakistan Journal of Biological Sciences, 15*(1), 19-26.

Siti-Zahrah, A., Zamri-Saad, M., Firdaus-Nawi, M., Hazreen-Nita, M. K., & Nur-Nazifah, M. (2014). Detection of channel catfish virus in cage-cultured *Pangasius hypophthalmus* (Sauvage, 1878) in Malaysia. *Journal of Fish Diseases, 37*(11), 981-983.

Slaney, T. L., Hyatt, K. D., Northcote, T. G., & Fielden, R. J. (1996). Status of anadromous salmon and trout in British Columbia and Yukon. *Fisheries, 21*(10), 20-35.

Smith, H. M. (1931). Description of new genera and species of Siamese fishes. *Proceedings of the. United States National Museum, 9,* 1-48.

Smith, H. M. (1945). The freshwater fishes of Siam or Thailand. *Bulletin of the United States National Museum, 188,* 1-622.

Sneath, P. H. A., & Sokal, R. R. (1973). *Numerical taxonomy: the principles and practice of numerical classification.* San Francisco: Freeman.

Song, L. M., Munian, K., Abd Rashid, Z., & Bhassu, S. (2013). Characterisation of Asian Snakehead Murrel *Channa striata* (Channidae) in Malaysia: An insight into molecular data and morphological approach. *The Scientific World Journal, 2013*.

Sridphairoj, K., Klinbunga, S., Kamonrat, W., & Na-Nakorn, U. (2010). Species identification of four economically important Pangasiid catfishes and closely related species using SSCP markers. *Aquaculture, 308*(SUPPL.1) S47-S50.

Strauss, R. E. (1987). The importance of phylogenetic constraints in comparisons of morphological structure among fish assemblages. In W.J. Matthews and D. C. Hines (Eds.) *Community and evolutionary ecology of North American stream fishes*, (pp. 136-143). Norman, Oklahoma: University of Oklahoma Press.

Strauss, R. E., & Bookstein, F. L. (1982). The truss: body form reconstructions in morphometrics. *Systematic Zoology, 31,* 113-135.

Strauss, R. E., & Bond, C. E. (1990). Taxonomic methods: morphology. In C. B. Schreck & P. B. Moyle (Eds.) *Methods for Fish Biology* (pp. 109-140). Bethesda: American Fisheries Society.

Steinke, D., Zemlak, T. S., & Hebert, P. D. (2009). Barcoding Nemo: DNA-based identifications for the ornamental fish trade. *PLoS One, 4*(7), e6300.

Subagja, J, Slembrouck, J., Hung, L.T., & Legendre, L. (1999). Larval rearing of an Asian catfish *Pangasius hypophthalmus* (Siluroidei Pangasiidae): analysis of precocious mortality and proposition of appropriate treatments. *Aquatic Living Resources, 12* (I), 37- 44.

Sullivan, J. P., Z.-G. Peng, J. G. Lundberg, J.-L. Peng, & S.-P. He. (2008). Molecular evidence for di-phyly of the Asian catfish family Amblycipitidae (Teleostei: Siluriformes) and exclusion of the South American Aspredinidae from Sisoroidea. *Proceedings of the Academy of Natural Sciences of Philadelphia 157,* 51–65.

Sullivan, J. P., Lundberg, J. G., & Hardman, M. (2006). A phylogenetic analysis of the major groups of catfishes (Teleostei: Siluriformes) using *rag1* and *rag2* nuclear gene sequences. *Molecular Phylogenetics and Evolution, 41*(3), 636-662.

Swofford, D.L. (2002) PAUP*: *phylogenetic analysis using parsimony*, version 4.10. Sinauer Associates, Sunderland, MA

Székely, C., Shaharom-Harrison, F., Cech, G., Mohamed, K., & Molnár, K. (2009). Myxozoan pathogens of Malaysian fishes cultured in ponds and net-cages. *Diseases of Aquatic Organisms*, *83*(1), 49-57.

Tabachnik, B.G., & Fidell, L.S. (1989). *Using Multivariate Statistics*. New York: Harper and Row.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., ….Willerslev, E. (2007). Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research, 35*(3).

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725-2729.

Tan, H.H., & Ng. H.H. (2000). The catfishes (Teleostei: Siluriformes) of Central Sumatra: *Jornal of Natural History, 34,* 267-303.

Tan, S. H., Rizman-Idid, M., Mohd-Aris, E., Kurahashi, H., & Mohamed, Z. (2010). DNA-based characterisation and classification of forensically important flesh flies

(Diptera: Sarcophagidae) in Malaysia. *Forensic Science International, 199*(1), 43-49.

Teugels, G. G. (1996). Taxonomy, phylogeny and biogeography of catfishes (Ostariophysi, Siluroidei): An overview. *Aquatic Living Resources, 9*, 9-34.

Teletchea, F. (2009). Molecular identification methods of fish species: Reassessment and possible applications. *Reviews in Fish Biology and Fisheries*, *19*(3), 265-293.

Teletchea, T., Maudet, C., & Hänni, C. (2005) Food and forensic molecular identification: update and challenges. *Trends in Biotechnology, 23,* 359–366.

Teletchea, F., Bernillon, J., & Duffraisse, M. (2008) Molecular identification of vertebrate species by oligonucleotide microarray in food and forensic samples. *Journal of Applied Ecology, 45*, 967–975

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 24*, 4876–4882.

Tilak, R. (1963). Studies on the Nematognathine pectoral girdle in relation to taxonomy. *The Annals and. Magazine of. Natural History, 13,* 145-155.

Tweedie, M. W. F. (1936). A list of the fishes in the collection of the Raffles Museum. *Bulletin of the Raffles Museum 12*, 16-28.

Vaillant, L. (1902). *Résultats zoologiques de l'expedition scientifique néerlandaise au Borneo Central. Not. Leiden Mus. 24,* 1-166.

Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology & Evolution*, *24*(2), 110-117.

Van Zalinge, N. P., Lieng S., Ngor P.B., Heng K., & Valbo-Jørgensen J. (2002). Status of the Mekong *Pangasianodon hypophthalmus* resources, with special reference to the stocks shared between Cambodia and Vietnam. *MRC Technical Paper No 1*. Phnom Penh: Mekong River Commission.

Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y., & Vieites, D.R. (2005) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology, 2*(5).

Vidthayanon, C. (1993). *Taxonomic revision of the catfish family Pangasiidae*. (Unpublished doctoral dissertation), Tokyo University of Fisheries, Tokyo, Japan.

Vidthayanon, C. (2012a). *Helicophagus leptorhynchus*. The IUCN Red List of Threatened Species 2012: e.T180838A1668380. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T180838A1668380.en. Downloaded on **11 December 2016**.

Vidthayanon, C. (2012b). *Pangasius bocourti*. The IUCN Red List of Threatened Species 2012: e.T180848A1669669. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T180848A1669669.en. Downloaded on **11 December 2016**.

Vidthayanon, C. (2012c). *Pangasius conchophilus*. The IUCN Red List of Threatened Species 2012: e.T181218A1710343. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T181218A1710343.en. Downloaded on **28 November 2016**.

Vidthayanon, C. (2012d). *Pangasius elongatus*. The IUCN Red List of Threatened Species 2012: e.T180765A1660461. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T180765A1660461.en. Downloaded on **28 November 2016**.

Vidthayanon, C. (2012e). *Pangasius mekongensis*. The IUCN Red List of Threatened Species 2012: e.T181260A1714731. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T181260A1714731.en. Downloaded on **28 November 2016**.

Vidthayanon, C. (2012f). *Pseudolais micronemus*. The IUCN Red List of Threatened Species 2012: e.T181073A1695516. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T181073A1695516.en. Downloaded on **28 November 2016**.

Vidthayanon, C. (2012g). *Pseudolais pleurotaenia*. The IUCN Red List of Threatened Species 2012: e.T181078A1696005. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T181078A1696005.en. Downloaded on **28 November 2016**.

Vidthayanon, C. (2013a). *Pangasius nasutus*. The IUCN Red List of Threatened Species 2013: e.T180981A1684954. http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T180981A1684954.en. Downloaded on **15 November 2016**..

Vidthayanon, C. (2013b). *Pangasius polyuranodon*. The IUCN Red List of Threatened Species 2013: e.T180650A1647680. http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T180650A1647680.en. Downloaded on **15 November 2016**.

Vidthayanon, C. & Hogan, Z. (2011). *Pangasianodon hypophthalmus*. The IUCN Red List of Threatened Species 2011: e.T180689A7649971. http://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T180689A7649971.en. Downloaded on **03 November 2016**.

Vidthayanon, C., & Roonthongbaisuree, S. (1993) Taxonomy of Thai riverine catfishes family Schilbeidae amd Pangasiidae. *National Inland. Fisheries Dept. Fisheries. Technical Paper, 150*, 1-57 (in Thai with English abstract)

Vinson, C., Grazielle, G., Schneider, H., & Sampaio, I. (2004). Sciaenidae fish of the Caete river estuary, Northern Brazil: Mitochondrial DNA suggests explosive radiation for the Western Atlantic assemblage. *Genetics and Molecular Biology, 27(2)*, 174–180.

Voris, H. K. (2000). Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *Journal of Biogeography, 27*(5), 1153-1167.

Vrijenhoek, R. C. (1998). Conservation genetics of freshwater fish. *Journal of Fish Biology, 53*, 394–412.

Wallace, A. R. (1863). On the physical geography of the Malay Archipelago. *Journal of the Royal Geographical Society of London, 33*, 217-234.

Wallace, A. R. (1876). *The geographical distributions of animals*.. (2 Vols.) London: Macmillan.

Wang, B., Jiang, J., Xie, F., Chen, X., Dubois, A., Liang, G., & Wagner, S. (2009). Molecular phylogeny and genetic identification of populations of two species of *Feirana* frogs (Amphibia: Anura, Ranidae, Dicroglossinae, Paini) endemic to China. *Zoological Science, 26*, 500–9.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences, 360*(1462), 1847-1857.

Ward, R. D., & Holmes, B. H. (2007). An analysis of nucleotide and amino acid variability in the barcode region of cytochrome c oxidase I (cox1) in fishes. *Molecular Ecology Notes, 7*(6), 899-907.

Weber, M., & De Beaufort, L. F. (1912). Die Fische. In Durch Zentral-Sumatra Band II. (A. Maass, Ed.). Leipzig, Germany.

Weder, J.K.P., Rehbein, H., & Kaiszer, K.P. (2001). On the specificity of tuna-directed primes in PCR-SSCP analysis of fish and meat. *European Food Research and Technology, 13,* 139–144.

Welcomme, R.L., & Vidthayanon, C. (2003). The impacts of introduction and stocking of exotic species in the Mekong basin and policies for their control. *MRC Technical Paper*. Mekong River Commission, Cambodia, 65 p

Więcaszek, B., Keszka, S., Sobecka, E., & Boeger, W. A. (2009). Asian pangasiids - an emerging problem for European inland waters? Systematic and parasitological aspects. *Acta Ichthyologica et Piscatoria, 39*(2), 131-138.

Wong, L. L., Peatman, E., Lu, J., Kucuktas, H., He, S., Zhou, C., & Liu, Z. (2011). DNA barcoding of catfish: species authentication and phylogenetic assessment. *PLoS One*, *6*(3), e17812.

Yang, J. X., Chen, X. Y., & Chen, Y. R. (2007). On the population status and migration of Pangasiid catfishes in Lancangjiang River Basin, China. *Zoological Research*. *28*(1), 63-67.

Yang, L., Tan, Z., Wang, D., Xue, L., Guan, M.-X., Huang, T., & Li, R. (2014). Species identification through mitochondrial rRNA genetic analysis. *Scientific Reports*, *4*, 4089. 11 p.

Yancy, H. F., Zemlak, T. S., Mason, J. A., Washington, J. D., Tenge, B. J., Nguyen, N. L. T., & Hebert, P. D. (2008). Potential use of DNA barcodes in regulatory science: applications of the Regulatory Fish Encyclopedia. *Journal of Food Protection*, *71*(1), 210-217.

Zakaria-Ismail, M. (1992). Notes on the catfish *Laides sinensis* (Pisces: Pangasiidae) from Peninsular Malaysia. *Malayan Nature Journal, 46,* 111–114.

Zakaria-Ismail, M. (1994). Zoogeography and biodiversity of the freshwater fishes of Southeast Asia. *Hydrobiologia*, *285*(1-3), 41-48.

Zakaria-Ismail, M., & Sabariah, B. (1995). Lake and river water quality as determinants of fish abundance at Temengor, Hulu Perak, Malaysia. *Malayan Nature Journal, 48,* 333-345.

Zardoya, R., & Meyer, A. (1996). Phylogenetic performance of mitochondrial protein-coding genes in resolving realtionships among vertebrates. *Molecular Biology and Evolution, 13*, 933-942.

Zeng, Q., Ye, H., Peng, Z., & Wang, Z. (2012). (Teleostei, Siluriformes). *Mitochondrial DNA. 23*(5), 355-357.

Zhang, Y. P., & Ryder, O. A. (1994). Phylogenetic relationships of bears (the Ursidae) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution, 3*, 351–9.

Zhao, H., Kong, X., & Zhou, C. (2013). The mitogenome of *Pangasius sutchi* (Teleostei, Siluriformes: Pangasiidae). *Mitochondrial DNA, 25*(5), 342-344.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. **Haslawati, B.**, Khairul-Nizam, A., Rizman-Idid, M & Zakaria-Ismail, M (2007). Towards phylogenetics analysis of Peninsular Malaysian Pangasiids: a comparison of phylogenetic trees of Pangasiid (Teleostei: Pangasiidae) estimated from three mitochondrial DNA sequences. Paper presented at the 12th Biological Sciences Graduate Congress. Institute of Biological Sciences, University of Malaya, Kuala Lumpur. 17th-19th December 2007. **(Oral presentation)**

2. **Haslawati, B.**, Zulkafli, A. R., Rizman-Idid, M and Zakaria-Ismail, M. (2008). Fishes of the family Pangasiidae (Teleostei) from the lower reaches of Pahang River Basin, Malaysia. Presented in the 4th Life Sciences Postgraduate Congress. University Science Malaysia, Penang. 18-20th June 2008. **(Oral presentation)**

3. **Haslawati, B.,** Rizman-Idid, M., Zulkafli, A. R., Muniandy, S., and Zakaria-Ismail, M. (2010a). Identification of potential threat to the indigenous Peninsular Malaysian Pangasiids (Teleostei: Pangasiidae) using partial mitochondrial 16S ribosomal RNA gene sequences. Oral paper presented in the Annual Seminar on Marine Science & Aquaculture. Universiti Malaysia Sabah. 10-12th March 2010. **(Oral presentation)**

4. **Haslawati, B.,** Rizman-Idid, M., Zulkafli, A. R., Muniandy, S., and Zakaria-Ismail, M. (2010b). Morphometric and mitochondrial DNA analysis of Pangasiid catfish species (Teleostei: Pangasiidae) from Peninsular Malaysia: implications on potential aquaculture impacts on the natural indigenous Pangasiid populations. Paper presented in the 2nd Moscow International Conference "Molecular Phylogenetics MolPhy-2". Moscow State University, Moscow, Russia. 18-21st May 2010. **(Poster presentation)**

5. **Haslawati, B.,** Rizman-Idid, M., Zulkafli, A. R., Muniandy, S., and Zakaria-Ismail, M. (2010c). Haplotype distribution of the Pangasiids (Teleostei: Pangasiidae) in Peninsular Malaysia. Oral paper presented in the Seminar Nasional Ikan VI dan Kongres Masyarakat Iktiologi Indonesia III. Gedung Widyasawaloka, Bidang Zoologi, Pusat Penelitian Biologi – Lembaga Ilmu Pengetahuan Indonesia (LIPI), Cibinong – Bogor, Indonesia. 8-9 Jun 2010. **(Oral presentation)**

6. Zulkafli A. R., Mustafa A. & **Haslawati B.** (2010). *Ikan-ikan Air Tawar Sungai Pahang*. Penerbitan Jabatan Perikanan Malaysia (The Freshwater Fishes of Pahang River. Publication of the Department of Fisheries Malaysia) (in Malay) . 61p.

7. **Haslawati, B**., Rizman-Idid, M., Muniandy, S., and Zakaria-Ismail, M. (2011a) Mitochondrial DNA haplotype variation in Pangasiid catfish species (Teleostei: Pangasiidae) of Peninsular Malaysia. Presented in the 22nd Pacific Science Congress. Asia Pacific Science in the 21st Century: Meeting the Challenges of Global Change. Kuala Lumpur Convention Centre. 14th -17th June 2011. **(Poster presentation)**

8. **Haslawati, B.**, Rizman-Idid, M., Muniandy, S., and Zakaria-Ismail, M. (2011b) MtDNA variation in Peninsular Malaysian Pangasiid catfish: application of DNA barcoding in fisheries management. Presented in the 4th International Barcode of Life Conference. University of Adelaide, Australia. 28thNovember – 3rd December 2011. **(Poster presentation)**

9. **Haslawati, B.**, Rizman-Idid, M., Muniandy, S., and Zakaria-Ismail, M. (2012). Stock structure and migratory pattern of *Pteropangasius micronemus* (Teleostei: Pangasiidae) in Pahang River as inferred using mitochondrial gene region 16S rRNA. Presented in Malaysia International Biological Symposium (SIMBIOMAS): Sustainable management of bioresources. Residence Hotel, UNITEN Selangor. 11-12 July 2012. **(Oral presentation)**

10. **Haslawati, B.**, Rizman-Idid, M., Muniandy, S., and Zakaria-Ismail, M. First record of *Pangasius polyuranodon* Bleeker, 1852 (Teleostei: Pangasiidae) in Peninsular Malaysia with remarks on the comparative morphology with *Pseudolais micronemus* (Bleeker, 1847). Presented in the Biodiversity of Asian Freshwater Fishes Conference. University Brunei Darussalam, Brunei Darussalam. 10th -13th January 2013. **(Oral presentation)**

11. **Haslawati, B**., Rizman-Idid, M., Sekaran, M. and Zakaria-Ismail, M. (2014). The occurrence of *Pangasius polyuranodon* Bleeker 1852 (Teleostei: Pangasiidae) in Peninsular Malaysia with remarks on the comparative morphology with *Pseudolais micronemus* (Bleeker 1847). *Sains Malaysiana*. 43 (11) 1707-1714.