



Faculty of Resource Science and Technology

Population Structure of Saltwater Crocodile (*Crocodylus porosus*) from Sarawak River Basin, Sarawak using Microsatellite and mtDNA Approaches

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Population Structure of Saltwater Crocodile (*Crocodylus porosus*) from Sarawak River Basin, Sarawak using Microsatellite and mtDNA Approaches

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

.....

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ABSTRACT

This study is designed to determine the genetic variations and genetic population structure among *Crocodylus porosus* in 13 Sarawak River Basin (RB) using microsatellite and mtDNA approaches. Relationships between saltwater crocodiles in Sarawak RB using 13 microsatellite markers were estimated. Out of the 52 samples amplified, one marker (Cj35) was polymorphic and showed double bands, whereas the other seven markers revealed a single band (Cj127, Cj131, Cj122, Cj101, Cj119, CUD68 and Cj16) and five markers revealed multiple bands. In addition, geographical distances do not influence genetic distances when microsatellite loci were used. DNA microsatellite divides the crocodile samples into four clades: A, B, C and D. Combined sequence data of Cytochrome Oxidase I gene (579 bp) and Cytochrome b gene (913 bp) had shown that *Crocodylus porosus* is monophyletic. Six out of ten haplotype shows unique haplotype (Hap_2, Hap_3, Hap_6, Hap_7, Hap_8 and Hap_10). Further analysis showed that there is gene flow between 12 populations from different RB based on values of nucleotide diversity (π) and number of migrants (N_m) values. Based on AMOVA value, this study also recorded that within population is higher than among population. The findings also revealed that population could be divided into northern, central, and western groupings based on geographical population location rather than RB. Future research should involve more samples representing all 22 RB in Sarawak so that a more comprehensive understanding on the genetic structure of crocodile in Sarawak. The findings from this study is hoped to shed lights on the genetic structure and diversity of saltwater crocodiles in RB and could be used by relevant agencies to carry out sustainable management of wild crocodile population in Sarawak.

Keywords: Saltwater crocodile, genetic distances, microsatellite loci, COI, Cyt b

Analisis Genetik Populasi Buaya Air Masin di Lembangan Sungai Sarawak, Sarawak menggunakan Pendekatan Mikrosatelit dan mtDNA

ABSTRAK

Kajian ini direka untuk menentukan variasi genetik dan struktur populasi genetik di kalangan buaya air masin mewakili 13 Lembangan Sungai (LS) Sarawak menggunakan pendekatan mikrosatelit dan mtDNA. Sebanyak 13 penanda mikrosatelit digunakan dan melibatkan 60 sampel. Satu penanda (Cj35) adalah polimorfik dan menunjukkan jalur berganda, tujuh penanda lain mendedahkan jalur tunggal (Cj127, Cj131, Cj122, Cj101, Cj119, CUD68 dan Cj16) dan lima penanda menunjukkan berbilang jalur. Selain itu, jarak geografi tidak mempengaruhi jarak genetik apabila lokus mikrosatelit digunakan. DNA mikrosatelit membahagikan sampel buaya kepada empat Kumpulan iaitu A, B, C, dan D. Pokok filogenetik gabungan gen Cytochrome Oxidase I (579 bp) dan gen Cytochrome b (913 bp) telah menunjukkan bahawa Crocodylus porosus adalah monofiletik. Enam dari sepuluh haplotaip menunjukkan haplotaip yang unik (Hap_2, Hap_3, Hap_6, Hap_7, Hap_8 and Hap_10). Analisis lanjut menunjukkan terdapat aliran gen antara populasi dari 12 LS yang berbeza berdasarkan nilai kepelbagaian nukleotida (π) dan nilai bilangan migran (Nm). Berdasarkan nilai AMOVA, kepelbagaian genetik populasi dalam kalangan adalah lebih tinggi daripada antara populasi. Penemuan ini juga mendedahkan bahawa populasi boleh dibahagikan kepada kumpulan utara, tengah dan barat berdasarkan lokasi populasi geografi dan bukannya LS. Penyelidikan pada masa hadapan harus melibatkan lebih banyak sampel yang mewakili semua 22 LS di Sarawak supaya pemahaman yang lebih komprehensif dapat dicapai. Penemuan daripada kajian ini diharap dapat memberi penerangan tentang genetik dan kepelbagaian buaya air masin di LS dan boleh digunakan

oleh agensi berkaitan untuk menjalankan pengurusan lestari populasi buaya liar di Sarawak.

Kata kunci: *Buaya air masin, jarak genetik, lokus mikrosatelit, COI, Cyt b*

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
AMOVA	Analysis of Molecular Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
COI	Cytochrome Oxidase I
CTAB	Cetyltrimethylammonium bromide
Cyt b	Cytochrome b
DNA	Deoxyribonucleic acid
HCC	Human-crocodile conflict
IUCN	International Union for Conservation of Nature and Natural Resources
km	Kilometer
NCBI	National Center for Biotechnology Information
RB	River Basin
SFC	Sarawak Forestry Corporation Sdn. Bhd.
UNIMAS	Universiti Malaysia Sarawak
UPGMA	Unweighted Pair Group with Arithmetic Averages

CHAPTER 1

INTRODUCTION

1.1 Study Background

Three families (Gavialidae, Alligatoridae, and Crocodylidae) make up the order Crocodylia. False gharials and gharials belong to the Family Gavialidae, whereas alligators and caimans belong to the Family Aligatoridae. The Crocodylidae includes the Saltwater or Estuarine Crocodile (*Crocodylus porosus*). The Saltwater Crocodile, which can reach lengths of 6-7 meters, is the biggest living reptile on Earth. It is an opportunistic feeder like other crocodylians, adopting active hunting or "sit and wait" methods. The frequency of various prey items varies greatly depending on the environment and body size (Amarasinghe et al., 2015).

Africa, Asia, Australia, and the New World have tropical and semi-tropical climates are regions where the Crocodylia live (Brochu, 2003). Due to their capacity for extended journeys, saltwater crocodiles have a widespread distribution. Northern Australia, New Guinea, and Indonesian coasts are where this species' individuals are most frequently found. Estuarine crocodiles, *C. porosus* can be found anywhere from Australia in the south to Burma and Southeast Asia in the north, and from Sri Lanka and the eastern coast of India in the west to the Caroline Islands in the east (Kumar et al., 2012).

Compared to the Peninsular Malaysia, Sarawak and Sabah, the two states on the island of Borneo, are home to large populations of *C. porosus* in Malaysia (Sarawak Forestry Corporation, 2018). Populations of *C. porosus* can also be found in Peninsular Malaysia on the east coast of Terengganu (Webb et al., 2010). In Sabah, *C. porosus* live in Kinabatangan River Basin and the nearby wetland (Evans et al., 2017). *C. porosus* are

particularly prevalent in Sarawak's extensive river systems, mangrove floodplains, and inland freshwater swamps (Tisen & Ahmad, 2010). Sarawak typically has the highest crocodile densities in the estuary and mid-river regions of medium-sized to large rivers (Tisen & Ahmad, 2010). Many saltwater and freshwater habitats, such as rivers and creeks, coasts, coastal flood plains, lagoons, swamps, and river and canal outfalls, are home to saltwater crocodiles (Amarasinghe et al., 2015).

The most prevalent crocodile species in Sarawak is the saltwater crocodile (*Crocodylus porosus*). Since the ancient times, people and crocodiles have peacefully coexisted in the same area. However, over the past three decades, there have been more instances of human-crocodile conflicts (HCC), leading to the notion that crocodile populations have grown and are now spreading to all rivers (Hassan et al., 2013).

Crocodile assaults have increased recently due to a sharp rise in the number of estuarine crocodiles in Sarawak. One of the key elements leading to the expansion of the species is regarded to be the implementation of the Wild Life Protection Ordinance, 1998. This law prohibited the killing or hunting of wild crocodiles in Sarawak. Therefore, it is illegal to hunt, kill, or sell wild crocodiles in the state. After a few decades of legal protection, Sarawak's crocodile population is now on the rise. Another impact is the clearance of vegetation along river banks, which encourages the establishment of grassy vegetation ideal for crocodile nesting places (Lading, 2013). From a mean of 0.9/year (1980-1985) to 3.3/year, attacks on humans have increased in Sarawak (1986-2004) (Lading, 2004). With an average of 7.8 incidents per year between 2000 until 2020, Sarawak reported 164 cases of crocodile attacks on humans (Abdul Gani et al., 2022). These had led to numerous and intense political and popular appeals for Sarawak's management authority to act quickly (Tisen et al., 2013).

Crocodylus is a part of numerous phylogenetic investigations that have determined the fundamental framework of crocodylian phylogeny (Harshman, 2003). Although numerous phylogenetic studies have addressed evolutionary relationships, there is still a lack of a solid, fully established phylogenetic hypothesis between the species at two separate locations (Brochu, 2003). Using Cyt b (Cytochrome-b) and 12S rRNA (ribosomal ribonucleic acid) gene, a preliminary molecular investigation on *C. porosus* in Sarawak has been carried out. Based on geographical locations (river basins), DNA microsatellites successfully inferred the relationship between *C. porosus* in Sarawak as five separate clades, as observed in the phylogenetic trees and network. Additionally, the five populations exhibit gene flow, indicating that regular migrations between the *C. porosus* populations in Sarawak occur (Abdul-Gani, 2019). The genetic population structure of crocodiles in the area is unknown because, as of December 2019, no genetic investigation has been conducted on *C. porosus* that lives in Sarawak RB. Therefore, the purpose of this study is to determine the population structure of *C. porosus* in Sarawak using molecular approach. Additionally, by designating conservation units for many threatened and endangered species, genetic information data can be used to develop management plans (Moritz, 1999).

1.2 Problem Statement

The International Trade in Endangered Species Act 2008 [Act 686] and the local wildlife laws are the instruments used to execute Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Malaysia (CITES, 2018). According to the regional legislation for the states in Peninsular Malaysia and the Federal Territory of Labuan, the Wildlife Conservation Act 2010 [Act 716], the Wildlife Protection

Enactment for Sabah, and the Wildlife Protection Ordinance for Sarawak, crocodiles are either listed as protected or completely protected in Malaysia. In 2016, *C. porosus* was moved from Appendix I to Appendix II to enable the sustainable use of the wild population in Sarawak, which will benefit the local economy and assist ensure the survival of crocodiles in the wild (CITES, 2018). Sarawak is now allowed to hunt saltwater crocodiles but with a permit (quota) to avoid disruption on the river's crocodile population. To maintain sustainable use of this resource, it is crucial to look at the crocodile populations in the Sarawak RB at the genetic level.

As crocodiles now are in Appendix II, human activities including hunting the adults, collecting eggs and juveniles for aquaculture purpose are allowed. In the past, it showed that due to intense local hunting, *C. porosus* in Sarawak was on the verge of extinction in the late 1980s. Farm owners hunt this species largely for its eggs and hatchlings to increase farm productivity, whereas hunters hunt this species mostly for their skin and flesh (Hassan & Abdul-Gani, 2013). Excessive hunting of saltwater crocodiles will decrease the genetic diversity in future (Lapbenjakul et al., 2017). On the other hand, aquaculture industry needs good brood stock in terms of genetic diversity to ensure survival of the animal. To evaluate the level of biodiversity in a species or population, genetic diversity is vital (Ingram & Nguyen, 2015). Without genetic diversity, it is impossible for a population to evolve and adapt to environmental changes.

1.3 Objectives

The objectives of this study are to:

1. To study the population structure of *Crocodylus porosus* based on microsatellite analysis.
2. To study the genetic diversity and population structure of *Crocodylus porosus* based on combined mtDNA COI and Cyt b gene analyses.

1.4 Layout of the thesis

This thesis comprises six chapters. Chapter 1 is the introduction to crocodiles, genes, the objectives, the problem statement, and the hypothesis. Chapter 2 covers literature review on the following topics: (i: overview of saltwater crocodile, ii: threats of saltwater crocodile, iii: importance of saltwater crocodile, iv: conservation of saltwater crocodile in Sarawak, v: mitochondrial data, and vi: DNA microsatellite). Chapter 3 describes general materials and methods which includes field trip, laboratory work (total genomic DNA extraction, and PCR for both microsatellite and mtDNA genes). Chapter 4 is about relationship among wild crocodiles in Sarawak using DNA microsatellite approach whereas Chapter 5 is about genetic diversity and population structure of saltwater crocodile in Sarawak RB using combine mtDNA analysis (COI and Cyt b gene). Chapter 6 contains general discussion and finally Chapter 7 covers conclusion and recommendations.

1.5 Hypothesis

At the beginning of this study, the hypotheses suggested were as follows:

Chapter 4

H₀: There is no difference in population structure of *C. porosus* populations, based on microsatellite marker.

H_a: There is a difference in population structure of *C. porosus* populations, based on microsatellite marker.

Chapter 5

H₀: There is no difference in genetic diversity and population structure of *C. porosus* populations, based on mtDNA marker.

H_a: There is a difference in genetic diversity and population structure of *C. porosus* populations, based on mtDNA marker.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Saltwater crocodile

Twenty-four species of alligators, caimans, crocodiles, and gharials are living crocodilians. The tropics and warm temperate regions of the earth are home to several populations of these sizable semi-aquatic ambush predators (Martin, 2008). Within the class of Reptilia, there is a minor order called Crocodylia. There are eight genera and 23 species in the genus Crocodylia (Meganathan et al., 2010) make up of large, cold blooded, carnivorous, semi-aquatic reptiles.

Table 2.1: Taxonomy of *C.porosus* (Oaks, 2007)

Kingdom	Animalia
Phylum	Chordata
Class	Reptilia Laurenti, 1768
Order	Crocodylia
Family	Crocodylidae
Genus	<i>Crocodylus</i> Laurenti, 1768
Species	<i>Crocodylus porosus</i> Schneider, 1801

The feature includes a robust tail, strong jaws, and eyes and nostrils on top of their heads (Figure 2.1). Tropical and semi-tropical regions of Asia, Australia, Africa, and the New World are home to the Crocodylia (Brochu, 2003). Gavialidae, Alligatoridae, and Crocodylidae are the three families that make up the order Crocodylia. Gharial and false gharial are members of the family Gavialidae, whereas alligator and caiman are members of the family Alligatoridae. Real crocodiles belong to the Crocodylidae family. There are

currently two genera of real crocodiles, *Crocodylus* and *Osteolaemus*, which fall under the subfamily Crocodylinae.

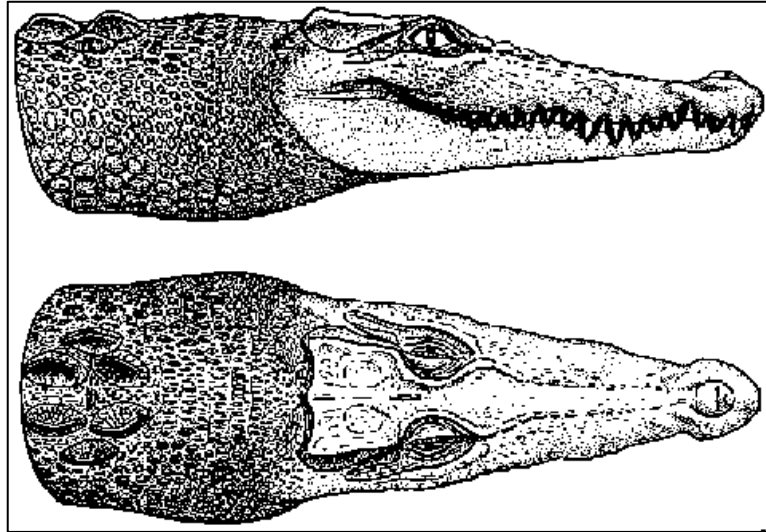


Figure 2.1: Head shape of *Crocodylus porosus* (adapted from Grigg and Gans, 1993).

Crocodylus porosus is also referred to as a saltwater crocodile or an estuarine crocodile. East and West Malaysia are home to this species. Due to its history in Sarawak, the saltwater crocodile is commonly referred to among the locals as "buaya katak" or "bujang senang" (Abdul Gani, 2019). *C. porosus* can live in a variety of aquatic environments, including freshwater rivers, mangrove swamps, woodland streams, and marine. *Crocodylus*, the largest genus, is represented by 11 species (Meganathan et al., 2010). *Crocodylus cataphractus* was previously thought to be a member of the *Crocodylus* genus, however, subsequent investigations have revealed consistent evidence for this species being a non-*Crocodylus* member (Brochu, 2003). Instead, it belongs to a distinct genus called "*Mecistops*." The African slender-snouted crocodile was reclassified and moved to the *Mecistops* genus, reflecting its unique evolutionary lineage and