



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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**Master of Science
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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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In fulfillment of the requirements for the degree of Master of Science

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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 (N_m). 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 crocodilians, 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 crocodilian 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.

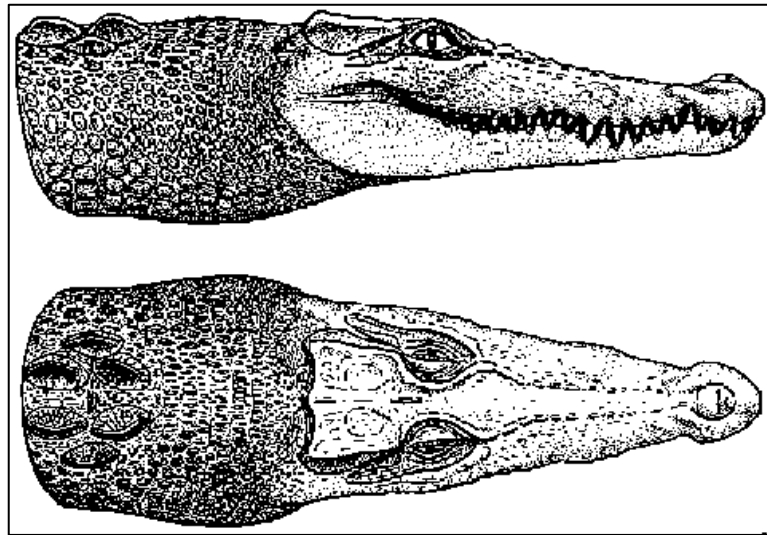


Figure 2.1: Head shape of *Crocodylus porosus* (adapted from Grigg and Gans, 1993).

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

characteristics (Brochu, 2003). *Crocodylus* species share the same morphological, anatomical, and physiological characteristics.

Their nutrition is based on their developmental stage, species, and variety of prospective food because they are nocturnal carnivorous opportunistic hunters (Martin, 2008). The largest crocodilians and reptiles now living are known as *Crocodylus porosus*. Male mature adults can reach lengths of up to 6-7 meters, whereas female *C. porosus* are much shorter, typically not exceeding 3 meters (CITES, 2018). They can be found far from the land and are hardy, powerful swimmers. Over their large heads, are a pair of ridges that run from the orbits of the eyes along the middle of the snout. It can see, hear, and breathe even when nearly entirely submerged because the eyes, ears, and nostrils are situated on the same plane on top of the skull (Kumar et al., 2012).

The sturdy thick jaws have 64–68 teeth. Juveniles are golden in color with black lines and spots. The skin of adults is black with hints of light grey. Near the tip of the tail's underside, it is grey, whereas the belly is golden. Bands of blackness can be seen on the lower flanks. Lacking osteoderms (bony plates), the scales on the flanks and belly skin of the hide are formed like an oval and a square (Kumar et al., 2012) (Figure 2.2).

India, Indonesia, Malaysia, the Philippines, Papua New Guinea, and Australia have all documented sightings of *C. porosus*, which is their southernmost point of distribution (CITES, 2018). Saltwater crocodiles are widely distributed throughout Malaysia. In Sarawak, crocodiles live in every river basin. On its 12 million hectares, Sarawak is home to 22 important river basins (CITES, 2018).

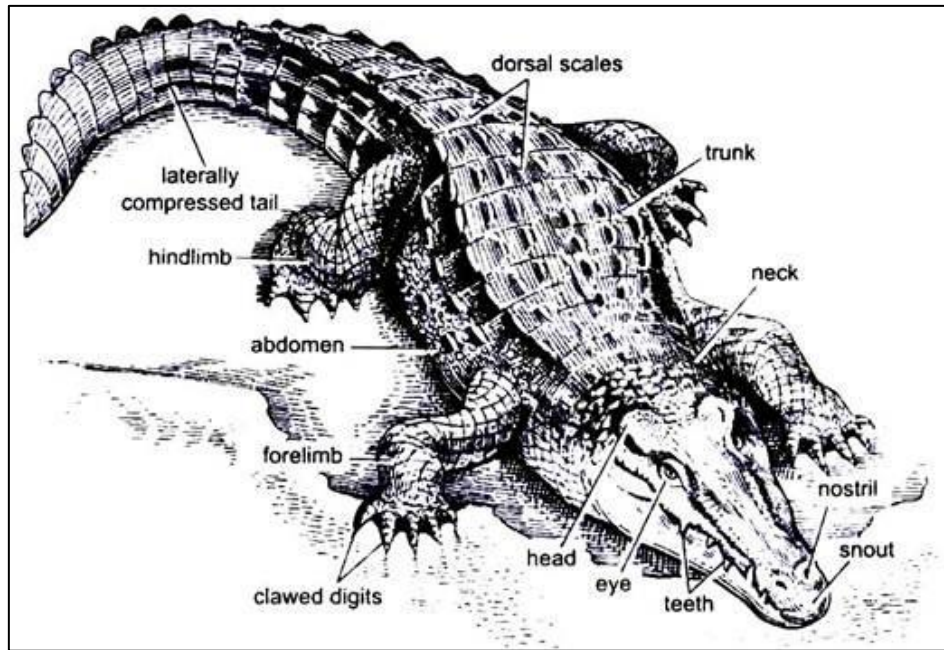


Figure 2.2: Body parts of *C. porosus* (adapted from Bhavya, 2017).

2.2 Threats

2.2.1 Threats to crocodiles

Crocodiles and people have peacefully coexisted in the same area for many years. In contrast, records of human-crocodile clashes have increased over the past three decades, leading to the assumption that crocodile populations are larger presently and spreading to all rivers (Hassan et al., 2018). All crocodilians are heavily impacted, either directly or indirectly, by anthropogenic causes, which are the main factor. In response to public demands for stricter crocodile control in regions close to human habitation, an increased number of crocodiles will be removed locally (Saalfeld et al., 2014).

Apart from humans, other crocodiles are the only significant predator of adult crocodiles, with larger saltwater crocodiles preying on tiny animals of both types. Predators of juvenile hatchlings include fish (like barramundi) and birds (like the Black-

necked Stork), as well as other species like goannas, which can prey on the eggs (Saalfeld et al., 2014).

Due to climate change, drought and floods are common now. Drought can have a significant but not long-lasting impact on *C. porosus* populations unless coupled with other factors. Heavy rainfall and subsequent flooding particularly associated with cyclones can cause localized egg and juvenile mortality (Webb et al., 1991). The likelihood of more frequent and severe dry season wildfires and severe storm occurrences could be detrimental to nesting vegetation, food sources, and survivability rates. But adjustments might also give crocodiles a chance to spread out (Saalfeld et al., 2014).

In Australia, crocodile deaths from net entanglement are a known problem. In the early 1980s, losses of *C. porosus* owing to unintentional capture and drowning in barramundi fishing nets were reported and studied (Saalfeld et al., 2014).

2.2.2 Threats of crocodiles

Crocodiles are apex predators and have a natural instinct to hunt for prey, which can include humans when they venture near water sources. Their powerful jaws, sharp teeth, and exceptional camouflage make them highly effective hunters. Crocodile attacks on humans often occur when people are bathing, washing clothes, or collecting water from rivers or lakes (Abdul Gani et al., 2022). Rural residents mostly rely on natural water sources for drinking water, bathing, and washing clothes. As a result, a lot of people are exposed to the possibility of a crocodile attack virtually every day (Jeremiah & Reniko, 2018). These attacks can result in severe injuries or fatalities due to the immense force of a crocodile's bite and the potential for drowning during an attack.

Small crocodiles are predated, driven out of rivers, and occasionally forced into marginal habitats, such as upstream freshwater regions where people enjoy recreation, which has resulted in an increase in the number of large crocodiles over time (Webb et al., 1991).

Factors such as weather conditions, time of day, and the presence of potential prey can influence their activity levels and behavior. This unpredictability adds to the difficulty of avoiding encounters with crocodiles and staying safe in crocodile habitats (Amarasinghe et al., 2015). Crocodiles are territorial animals and can become aggressive if they perceive any intrusion into their territory. When humans encroach upon their habitats, crocodiles may perceive them as threats or competitors and may attack to defend their territory. This territorial behaviour can lead to dangerous encounters, especially in areas where people rely on natural water sources for daily activities (Najmuddin et al., 2021).

2.3 Importance of crocodiles

According to Sideleau et al. (2017), saltwater crocodiles are known to be dangerous to humans, and understanding their ecology can help in developing strategies for minimizing human-crocodile conflicts. For example, knowing where crocodiles are likely to be found and understanding their behavior can help in developing strategies for avoiding encounters with them.

The socioeconomic importance of saltwater crocodiles refers to the economic and social benefits that can be derived from the species. Here are some ways in which saltwater crocodiles can have socioeconomic importance. In some parts of the world, saltwater crocodiles are farmed for their meat, skins, and other products. This can provide income for local communities and contribute to the local economy (Tosun, 2013). The study of

saltwater crocodiles can lead to scientific discoveries that can benefit a variety of industries. For example, research into the properties of crocodile skin can lead to the development of new materials for use in fashion and other industries (Macgregor, 2002).

2.4 Conservation of Saltwater crocodile in Sarawak

Crocodile assaults have increased recently due to a sharp rise in the number of estuarine crocodiles in Sarawak (Hassan et al., 2013). In the past 30 years, rivers that were never home to crocodiles are now overrun with wild crocodiles, even in their upper reaches where the tides are not present regularly (Abdul Gani et al., 2022). One of the key elements leading to the expansion of the species is regarded to be the application of the Wild Life Protection Ordinance, 1998. Another impact is the clearance of vegetation along river banks, which encourages the establishment of grassy vegetation ideal for crocodile nesting places (Lading, 2013).

Although killing or even "harassing" crocodiles has long been frowned upon, there is a growing perception that these reptiles are a nuisance because they prey on humans and livestock. After fatal assaults on humans, there have frequently been calls and demands for the authorities to kill or remove the crocodiles from the waterways (CITES, 2018). These had led to persistent, intense political and popular appeals for the Sarawakian management authority to act quickly. The crocodile management authority had been instructed by the Sarawak State Cabinet to carry out a state-wide culling of the crocodiles to calm the public (Tisen et al., 2013).

Most of the *C. porosus* populations are covered by the Appendix I listing of the Convention on International Trading in Endangered Species of Wild Fauna and Flora (CITES) in every nation, which implies that trade in necessities is severely constrained.

The populations in Papua New Guinea, Australia, Indonesia, and Malaysia are however included in Appendix II (with the state of Sarawak having a wild harvest restriction and the other states of Malaysia having a zero quota for wild specimens) (Hassan et al., 2018; Abdul-Gani, 2019). The International Trade in Endangered Species Act 2008 [Act 686], as well as local wildlife regulations, serve as the legal framework for CITES implementation in Malaysia (CITES, 2018).

C. porosus has been transferred from Appendix I to Appendix II to enable sustainable use of the wild population in Sarawak, which will benefit communities socio-economically and contribute to the long-term survival of crocodiles in the wild. The current legislation, rules, and ongoing crocodile population monitoring will be used to control and preserve crocodiles (CITES, 2018). The current Appendix II aims to incorporate into the overall management of this species in Sarawak the ability to conduct a strictly controlled sustainable harvest that can benefit local communities who are negatively impacted by the crocodiles economically while maintaining viable wild populations. Crocodiles are increasingly viewed as a pest that needs to be eliminated because they do not produce any financial benefits (CITES, 2018). However, Sarawak state now emphasis on monetary gains to set up conservation of crocodiles as it encourages crocodile aquacultures crocodile harvesting (with permits) and crocodile-based ecotourism (Hassan et al., 2013).

2.5 Molecular markers

2.5.1 Mitochondrial DNA

Most eukaryotic cells contain mitochondria, which are important for the synthesis of ATP (Adenosine triphosphate) molecules through oxidative phosphorylation. In addition, the mitochondria also perform other functions such as the production of steroids and amino acid biosynthesis, FeS metabolism, the induction of apoptosis, and the oxidation of fatty acids. (Lavrov & Pett, 2016). The organelle contains a circular, single-stranded chromosome called mitochondrial DNA (mtDNA) (Figure 2.3), which a creature inherits from its mother (Chinnery & Hudson, 2013). Additionally, due to its tiny molecular size, abundance in animal tissue, and extremely low levels of recombination, mtDNA has been widely utilized as a trustworthy marker for molecular diversity in animals for decades (Galtier et al., 2009; Gissi et al., 2008). In addition, due to the genome's uniparental inheritance and the fact that it does not enter an organism's egg cell, it can be utilized to discover the evolutionary history of an organism (Gissi et al., 2008). Because mtDNA sequences in animals have a higher copy number and a higher mutation rate than nuclear DNA, they are excellent DNA markers for a variety of purposes.

The gene sequences of *Crocodylus acutus* and *Crocodylus novaeguineae* are similar to those of other crocodilians, according to Li et al. (2007), who used data from the complete mitochondrial genome sequence. The monophyly of two crocodilian taxonomic families, Alligatoridae (genera *Alligator*, *Caiman*, and *Paleosuchus*), and Crocodylidae were supported by phylogenetic studies based on the sequences of 12 mitochondrial protein-coding genes (genera *Crocodylus*, *Gavialis*, *Mecistops*, *Osteolaemus*, and *Tomistoma*). They also stated that all crocodilian genera are monophyletic.

According to Russello et al. (2007), saltwater crocodiles from nine river basins in the Northern Territory of Australia were studied to determine their relationships and genetic makeup using mitochondrial regulatory region sequences (in the mtDNA). Sixty-one individuals represented nine river basins, six of unknown origin, and published sequences from additional locations were also used. The findings showed that eight mitochondrial control area haplotypes were found using previously published and brand-new sequencing. Similarly, Gratten (2003), who had employed the same gene, reported that there is no phylogeographic structure for the estuarine crocodile, *C. porosus*, either among populations that were co-distributed with *C. siamensis* and *C. novaeguineae* or at a larger geographic scale encompassing the Western Pacific Ocean and Indo-Malay Archipelago. If there were more samples, it is conceivable to resolve the phylogeography of crocodiles. Genetic studies of the mtDNA regulatory region in saltwater crocodiles conducted by Luck et al. (2012) indicated that there is little to no genetic structure within and between populations in Australia's Northern Territory. It gives the Northern Territory's management and monitoring of wild populations of saltwater crocodiles some basic genetic background data.

Gavialis gangeticus, *Crocodylus siamensis*, *Crocodylus palustris*, *Caiman crocodilus*, *Crocodylus porosus*, and *Crocodylus niloticus* were the six crocodile species whose partial cytochrome b gene sequences were amplified by Meganathan et al. (2009) using two novel sets of primers. After being edited, the incomplete sequences were combined to create a complete cyt b gene sequence, which can be used to determine crocodile species. This sequence can also be utilized for forensic authentication. Using these data, a crocodile species tree was created, and the identification was further validated using the phylogenetic species concept. According to the study, forensic crocodile species

identification primers have a lot of potential and can benefit the effective enforcement of the law and the conservation of endangered species. Meganathan et al. (2009) work did not proceed to the next level because other researchers proposed a more accurate gene marker, namely DNA microsatellite analysis.

Selected mtDNA genes had been used in several investigations on the population structure of *C. porosus* samples taken from rivers in Sarawak. Due to the small number of samples examined, preliminary research by Shoon (2009) and Abdullah (2010) using the Cytochrome b and 12S Ribosomal gene was unable to distinguish the genetic structure of *C. porosus* populations from various parts of Sarawak. In contrast, Abdullah (2010) used a combination of Cyt b and COI genes analysis, partially resolving the phylogenetic relationships among samples taken from several rivers in Sarawak. The author then suggests another accurate gene marker, for example, a DNA microsatellite, to get an accurate picture of Sarawak's wild crocodile genetic structure.

Tomistoma schlegelii had been studied in Sarawak using a DNA sequencing approach by Md Adzhar & Hassan (2017). They discovered that *T. schlegelii* populations have substantial genetic diversity, with values ranging from 0.16 percent to 3.34 percent, by using Cytochrome b-control region mtDNA gene analysis. When the Sunda Shelf was revealed, the analysis suggested that there had once been a river system between West Borneo and Peninsular Malaysia, demonstrating gene flow between crocodile in Western Sarawak and other areas that were consistent with the Sundaland theory and those in Peninsular Malaysia. Given that both Northern Sarawak and Sumatera have observed distinct haplotypes, each *T. schlegelii* species needed its management approaches to ensure the species' conservation (Md Adzhar & Hassan, 2017).

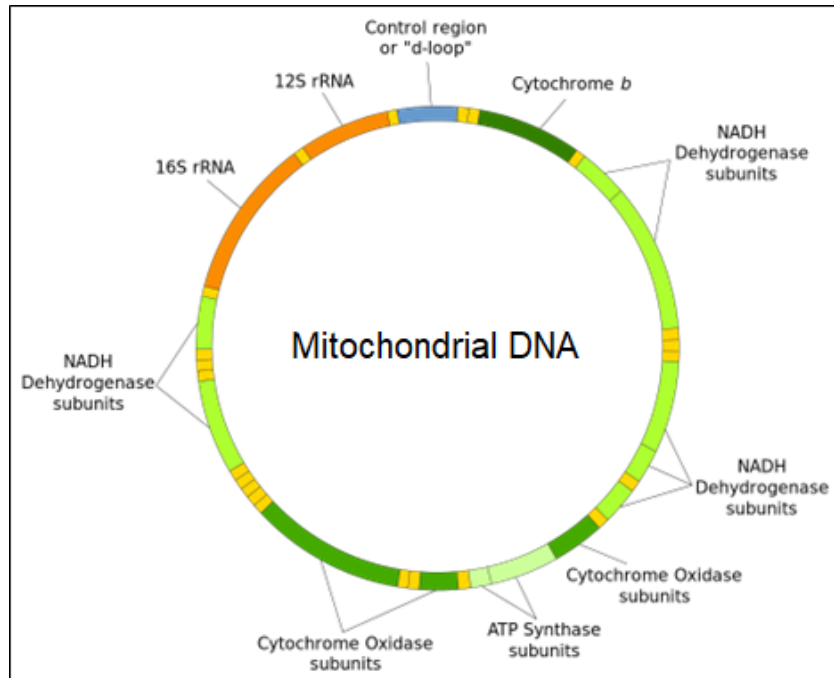


Figure 2.3: Structure of mitochondrial DNA (adapted from Raju et al., 2011).

2.5.2 DNA Microsatellite

The term Microsatellite was first coined by Lit and Luty as reported in Pokhriyal et al. (2012). These are known as the Short tandem repeat (STR) or Simple sequence repeat (SSR). Microsatellite markers are known as the most useful nuclear markers (Pokhriyal et al., 2012). Microsatellite markers have been isolated in several crocodilian species including *Crocodylus johnstoni*, *Crocodylus moreletii*, *Alligator mississippiensis*, *Caiman latirostris*, *Crocodylus porosus*, *Paleosuchus trigonatus*, and *Alligator sinensis* (Oliveira et al., 2010).

Microsatellites are used more frequently in population genetics since they are known to have high mutation rates, which can result in high levels of polymorphism and higher allelic variability. Because they can give hyper-variable sequencing tagged single-locus markers and are present in most eukaryote genomes, microsatellite markers are capable of providing very recent estimations of migration and relatedness among

individuals (Miles et al., 2009). For these reasons, the markers have been widely used by numerous researchers to evaluate population structure, mating behavior, diversity, dispersal systems, as well as hybridization in a range of crocodile species (Isberg et al., 2004; Lewis et al., 2013; Hekkala et al., 2015; Lapbenjakul et al., 2017; Mauger et al., 2017). At the same time, a sufficiently substantial collection of microsatellite primers for different species of crocodiles had been produced for research purposes and had assisted numerous researchers on crocodilians' genetic studies.

Using the saltwater crocodile (*Crocodylus porosus*) as a model, Miles et al. (2009) created 253 new polymorphic microsatellite markers, which were successfully tested for cross-species amplification in 18 other crocodile species (Miles et al., 2009). Since that time, the markers have been employed by researchers for genetic research in much less well-known crocodilian species like *T. schlegelii*, *C. rhombifer*, and *C. intermedius* (Bashyal et al., 2014; Shafiei-Astani et al., 2015). Researchers have been able to shed light on population structure and clarify phylogeography in several crocodilian species by employing microsatellite markers. Mauger et al. (2017) investigated the population genetic structure of American crocodile, *C. acutus*, populations in Pacific Costa Rica. Based on their research, they hypothesized that these populations had moderate levels of genetic variation and were not panmictic. Additionally, it was shown that the Costa Rican crocodile populations were facing a genetic bottleneck, perhaps as a result of the declining numbers of crocodiles brought on by illegal poaching and hunting (Mauger et al., 2017). In populations of the saltwater crocodile, *C. porosus*, from the Western Pacific Ocean and Indo-Malay Archipelago, microsatellites data and mtDNA markers revealed genetic differences (Gratten, 2003).

Samples of saltwater crocodiles collected from Darwin Crocodile Farm in Australia's Northern Territory were analyzed for all of their markers and were designed to be used in the development of a genetic linkage map for *C. porosus* (Miles et al., 2019). About 253 new and polymorphic microsatellite loci were identified and isolated for the *C. porosus* using libraries enriched for microsatellite DNA. Those chosen for mapping had an average polymorphism information content of roughly 253 loci, and there were 4.12 alleles produced per locus on average (Miles et al., 2009).

Kasim (2011) and Abdul-Gani (2014, 2019) used microsatellites to analyze the genetic makeup of wild crocodile populations in Sarawak to improve sustainable resource management. According to Abdul-Gani (2019), DNA microsatellites successfully inferred the relationship among *C. porosus* in Sarawak as five separate clades based on the geographical locations (river basins), which are shown in the phylogenetic trees and median-joining Network analysis. Further evidence of frequent migrations across the populations of *C. porosus* in Sarawak comes from the gene flow among the five groups. Crocodile subpopulations with high gene flow (N_m) indicate that the reptile frequently travels within Sarawak's river basin. The data from the evolutionary neutrality test and mismatch distribution show that populations of *C. porosus* in Sarawak are generally expanding, indicating that these populations are panmictic.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Field trip

A total of 62 crocodile samples were collected in this study, representing 13 river basins (RB) in Sarawak (Table 3.1). The highest number of samples collected was from the Suai RB ($n = 12$) while the lowest number of samples were collected from Sadong RB, Niah RB, Limbang RB, and Baram RB, with one sample each (Table 3.1 & Figure 3.1). All samples were kept and recorded according to their locations (Table 3.2). There were two ways to capture crocodiles, depending on the size of the animal. For crocodiles smaller than 1.5 m in length, they were manually captured using a scoop net or cast net whereas those help was obtained from Swift Wildlife Action Team (SWAT), Sarawak Forestry Corporation (SFC). Larger adult crocodiles were captured using custom-made cage traps. To avoid any incident during the sampling process, crocodiles that were captured alive were appropriately restrained before being sampled (Abdul-Gani, 2019). The crocodile samples from this study were provided from previous researchers and SFC. According to Abdul Gani (2014), tissue samples can be taken from the crocodile's tail, legs, and body. The most common place to obtain crocodile DNA samples is from the scute of the crocodile's tail. In this study, the tissues and scutes of crocodiles were taken using a scalpel to remove either a little portion or the entire scute from the crocodile body. The tissues and scutes samples were then preserved in 70% ethanol solutions, labelled accordingly, and transported back to the Aquatic Molecular laboratory in Faculty Resource Science and Technology (FRST), UNIMAS. The samples were stored in the -20 °C freezer until further molecular work was done.

Table 3.1: Sampling area of crocodiles according to the river basin and number of samples.

District	Sampling area	Number of samples (n)	River basin (Area code)	Region
Samarahan	Batang Samarahan	2	Samarahan (SN)	Western
	Sungai Meranek	2		
	Sungai Tuang	3		
	Kampung Pinang	1		
	Sungai Sabang	2		
Kuching	Sungai Maong Hulu	1	Sarawak River (SR)	Western
	Sungai Maong Hilir	2		
	Semariang	3		
	Sungai Sarawak	1		
	Bako	1		
	Ulu Pengkalan Bako	2		
	Jambatan Satok	2		
Pusa	Sungai DIT, Pusa	2	Saribas (SS)	Western
	Pusa River	1		
Simunjan	Kampung Jeragan	1	Sadong (SG)	Western
Sibu	Batang Oya	3	Oya (OY)	Central
Sibu	Sibu STIC	2	Rajang (RJ)	
	Sungai Awik	2		
	Sungai Undai	1		
	Batang Paloh	1		
Bintagor	Sungai Kawi	1	Rajang (RJ)	Central
Bintulu	Bintulu	5	Kemena (KM)	Central
	Kuala Tatau	3	Tatau (TA)	
Miri	Suai	12	Suai (SU)	Northern
	Sungai Terus	1	Niah (NH)	
	Limbang	1	Limbang (LB)	
	Baram wetland	1	Baram (BM)	
	Miri	4	Miri (MI)	

Example of area code:

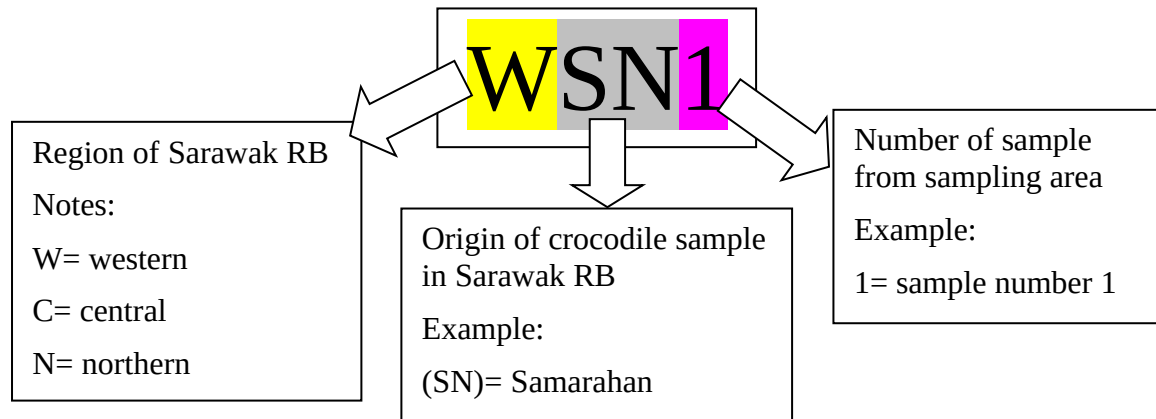


Table 3.2: List of locations and area code of each samples.

Locations	Area code	Locations	Area code
Batang Samarahan	WSN1	Sg Awik B	CRJ4
Batang Samarahan	WSN2	Sg Undai Kg Tambak	CRJ5
Sg Meranek	WSN3	Sg Btg Paloh	CRJ6
Sg Meranek	WSN4	Sg Kawi Bintagor	CRJ7
Sg Tuang Kota Samarahan	WSN5	Kg Hulu Sebauh	CKM1
Sg Tuang Kota Samarahan	WSN6	Sg Labang	CKM2
Sg Tuang Kota Samarahan	WSN7	Kg Sebauh Jepak	CKM3
Sg Pinang	WSN8	Ladang Rajawali	CKM4
Sungai Sabang, Sg btg Samarahan Kiri	WSN9	Samalaju	CKM5
Sungai Sabang, Sg btg Samarahan Kiri	WSN10	Kuala Tatau	CTA1
Sg Maong Hulu	WSR1	Kuala Tatau	CTA2
Sg Maong Hilir	WSR2	Kuala Tatau	CTA3
Sg Maong Hilir	WSR3	Shell, Sg Suai	NSU1
Semariang	WSR4	Kuala Suai estate	NSU2
Sg Sarawak	WSR5	Kuala suai	NSU3

Table 3.2 continued

Bako	WSR6	Sg Suai	NSU4
Ulu Pengkalan Bako	WSR7	Sg Suai	NSU5
Ulu Pengkalan Bako	WSR8	Kg Kuala Suai	NSU6
Tanah Perkuburan islam Semariang	WSR9	Sura Lada	NSU7
Tanah Perkuburan islam Semariang	WSR10	Kg Kuala Suai	NSU8
Sungai Sarawak (Jambatan Satok kuching)	WSR11	Sg suai	NSU9
Sg DIT	WSS1	Sg Terus, suai, good matters plantation	NSU10
Sg DIT	WSS2	Sg Terus, suai, good matters plantation SDN BHD	NSU11
Sg Pusa, saribas	WSS3	Kpg Iran Suai	NSU12
Kg Jeragan	WSG1	Sg Terus, Niah	NNH1
Sg Btg Oya, Kpg Senau	COY1	Limbang River	NLB1
Sg Btg Oya, Kpg Bakong	COY2	Kuala Baram wetland	NBM1
Sg Btg Oya, Kpg Terus	COY3	Promin Jaya Senadin, Miri	NMI1
Sibu STIC	CRJ1	Sungai Rait Miri	NMI2
Sibu STIC	CRJ2	Kpg Ogus Suai Miri	NMI3
Sg Awik A	CRJ3	Sg Sawai, Daro	NMI4

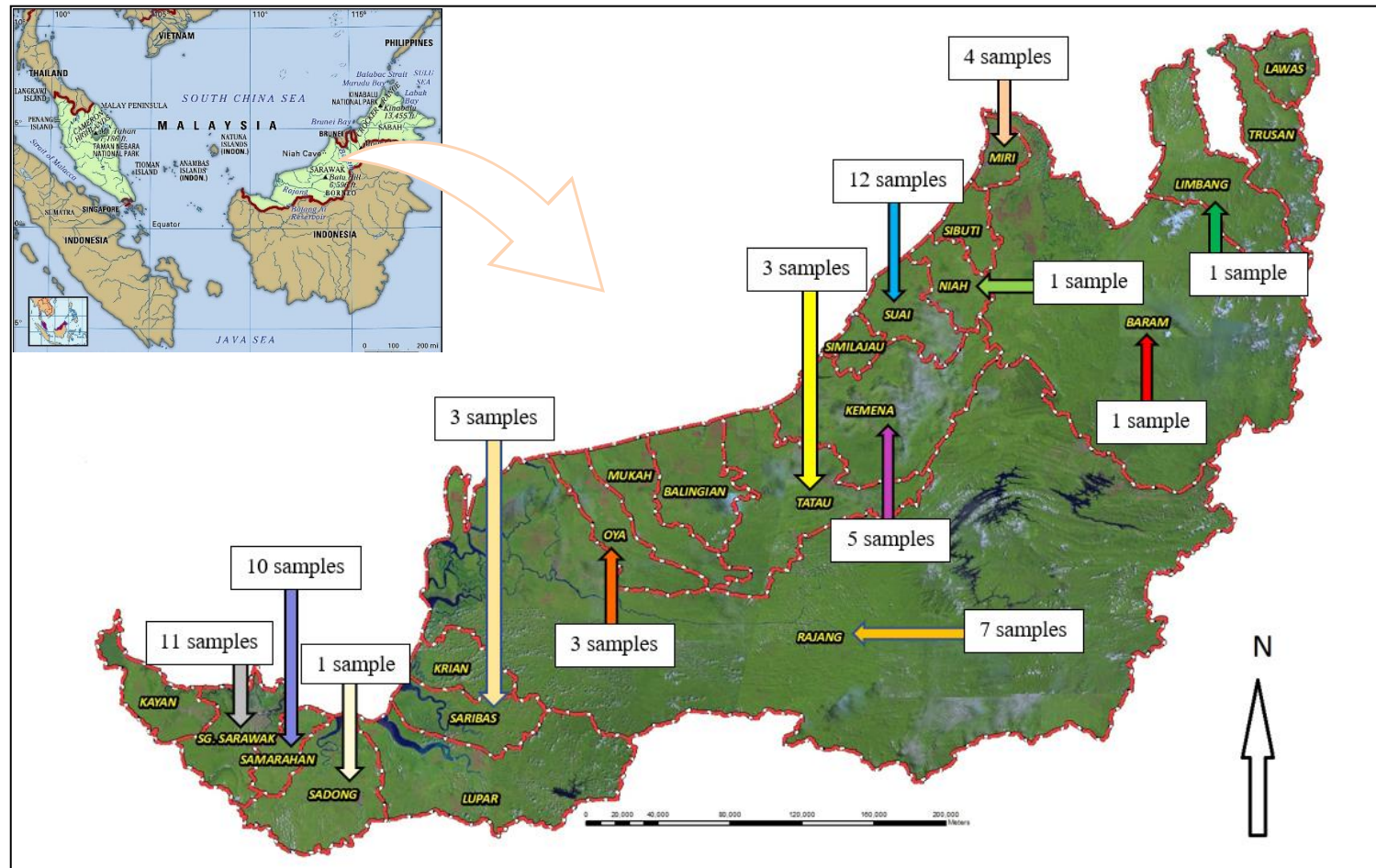


Figure 3.1: Maps of Sarawak River Basin (RB) according to the number of samples taken from each river basin (Sarawak RB map adapted from Official Website of Department of Irrigation and Drainage Sarawak, 2022)

3.2 Laboratory work

The laboratory work includes the total genomic DNA extraction using CTAB protocol and extraction KIT (QIAGEN, Germany), Agarose Gel Electrophoresis (AGE), and Polymerase Chain Reaction (PCR).

3.2.1 Total Genomic DNA extraction using CTAB protocol

Based on the method used by Doyle & Doyle (1987) and some modifications from (Abdul- Gani, 2014), the whole genomic DNA was extracted using the Modified Cetyl-Trimethyl Ammonium Bromide (CTAB) approach. Small-sized less than 10mg tissue samples were mixed with 250 μ l of 2x CTAB buffer on a petri dish. The tissue samples were finely minced before transferred into different 1.5 ml Eppendorf tubes. The tubes were then filled with another 250 μ l of 2x CTAB buffer and 5 μ l of Proteinase K (digests the proteins and nucleases). The samples were then kept in the water bath (WiseBath) at 60 °C for approximately an hour. Then, 700 μ l of chloroform-isoamyl alcohol (CIA) was added to the tubes and the tubes were gently shaken or used pulse-vortex to mix the samples for a few minutes. The tubes were then centrifuged at 13000 rpm for 15 minutes at 4 °C using the high-speed micro centrifuge machine (Himac, CF15RX). After centrifuging the samples, there were two layers of the mixture in the tubes, and the upper aqueous layer was transferred into the new Eppendorf tube without contacting the lower aqueous layer. A micropipette was used to carefully transfer 500-600 μ l of the top aqueous phase into new tubes. Each tube received an equal amount of absolute ethanol (EtOH) (upper aqueous layer = EtOH). After that, the tubes were incubated at room temperature for 20 minutes. The tubes were centrifuged at 13000 rpm for 15 minutes at 4 °C after the 20 minutes incubation period at room temperature. Then, the excess ethanol was removed followed by addition of and 500 μ l of cold 70% EtOH. The sample was then centrifuged at 13000 rpm

for 15 minutes at 4 °C. Again, all excess EtOH was removed before the pellet was dried at room temperature. The dried pellet was then mixed with 50 µl of deionized water and kept in a freezer at -20 °C for further use.

3.2.2 Total Genomic DNA using QIAamp DNA Investigator Kit (Qiagen. Germany) (following Manufacturer's protocol)

Approximately 10 mg of sample was minced and transferred into a 1.5 ml Eppendorf tube. Immediately 180 µl of Buffer ATL was added and kept at room temperature of about 15–25 °C. Then, 20 µl of proteinase K was added and pulse-vortex was used to mix the sample for 15 seconds. After that, the samples were incubated in water bath at 56 °C overnight or until the samples is lysed. A total of 200 µl of Buffer AL was added into the tube and the mixed for 15 seconds by pulse-vortex. A total of 200 µl of absolute ethanol was added and thoroughly mixed by pulse-vortex for 15 seconds. The sample was incubated at room temperature for 5 minutes. Before centrifugation at 6000 x g (8000 rpm) for one minute, the entire lysate was carefully transferred from the preceding stages to the QIAamp MinElute column without wetting the rim. To get rid of drips from the lid's interior, the 1.5 ml tube was briefly centrifuged. After discarding the collection tubes containing the flow-through, the QIAamp MinElute column was placed in a clean 2 ml collection tube (step 9). The QIAamp MinElute column was opened carefully and 500 µl of Buffer AW1 were added without wetting the rim. Step 9 was repeated after closing the lid and centrifuged at 6000 x g (8000 rpm) for 1 minute. Without wetting the rim, carefully open the QIAamp MinElute column and 700 µl Buffer AW2 were added. Repeat step 9 after closing the lid and centrifuging at 6000 x g (8000 rpm) for 1 minute. The QIAamp MinElute column was carefully opened, and 700 µl of absolute ethanol were added without wetting the rim. The cap was closed, centrifuged for one minute at 6000 x g

(8000 rpm), and step 9 was repeated. To completely dry the membrane, the centrifuge was run at full power (20,000 x g; 14,000 rpm) for 3 minutes. The collection tube with the flow-through was discarded and the QIAamp MinElute column was placed in a clean 1.5 ml Eppendorf tube. The QIAamp MinElute column's lid was carefully opened and incubated for 10 minutes at room temperature. Then, 100 µl of deionized distilled water or buffer ATE were added to the membrane's center. Then, the tube cover was closed and left for a minute of room temperature incubation. The QIAamp MinElute column was spun at full speed (20,000 x g; 14,000 rpm) for 1 minute. Then, The DNA samples were kept in a freezer at -20 °C for future use.

3.2.3 Agarose Gel Electrophoresis

First, the agarose gel was made to distinguish DNA fragments of different lengths. An uncontaminated beaker was filled with 0.5 g of agarose powder, which was weighed using an analytical balance. 50 ml of 1x tris-acetate electrophoresis (TAE) buffer were then added to the beaker. The agarose gel in the beaker was melted in the microwave for one minute. The agarose gel was poured into a contaminated beaker as soon as had melted. Then, a drop of ethidium bromide was added to the beaker. To mix the gel solution, the contaminated beaker was twirled. As soon as the mixture was thoroughly combined, the gel solution was poured into the tray that had been previously prepared, and the comb was set inside the tray to create wells that were then filled with the sample mixture. Once the gel had formed, it was moved into a tank for gel electrophoresis that had been set up and contained 1 x TAE buffer with the well at the negative terminal. 6 µl of 1 kb DNA ladder were then added to the first well. Using a micropipette, 1 µl of 6x loading dye and 5 µl of DNA sample were added to the remaining wells. To evaluate the DNA extraction products,

the gel was run at 90 volts for an hour, and at 30 volts for the evaluation of the PCR products. Under UV illumination, the bands' formation was observed and visualized (Nates et al., 2009).

3.2.4 Polymerase Chain Reaction

Based on the method used by (Isberg et al., 2004; Abdul Gani, 2014) and some modifications from this study. Polymerase Chain Reaction (PCR) was conducted using (MyCycler™) Thermal Cycler. The PCR master mix was prepared first before being carried out PCR. The master mix or PCR cocktail recipes included 5 µl of 5X Green Go Buffer, 0.5 µl of 10 mM dNTP mix, 1 µl of 10 µM of forward primer, 1 µl of 10 µM of reverse primer, and 5 µl of 25 mM MgCl₂, 0.5 µl of *Taq* DNA polymerase, 2 µl of DNA template and 13.5 µl of deionized distilled water. The prepared master mix was labeled and run in the thermocycler. Standard PCR with a total volume of 25 µl was prepared for each batch with a negative control. Optimization had done after a few of trials to get best DNA yield. For further laboratory analysis and PCR thermal profiles were described in chapter 4 and chapter 5.

3.3 Data analysis

3.3.1 Microsatellite analysis

A dendrogram was constructed and the analyses were described in chapter 4.

3.3.2 mtDNA analysis

Data sequencing and alignment analyses were analysed and followed by constructing phylogenetic tree, haplotype data, Network, and population genetic analyses. Detailed analyses were described in chapter 5.

CHAPTER 4

RELATIONSHIP AMONG WILD CROCODILES IN SARAWAK USING DNA MICROSATELLITE APPROACH

4.1 Introduction

The term "microsatellite" is to describe short tandem repeat sequences (STRs) of DNA that are commonly found throughout the genomes of eukaryotic organisms (Pokhriyal et al., 2012). Tandem repeats of 1-6 base pair motifs make up these highly variable sequences, which are generally thought to be distributed randomly and without preference among genomes (Ellegren, 2000; Li et al., 2007). These markers have become the ones for a variety of applications because of their tremendous variability, which has been made possible by modifications in the number of repeated mutations (Bennett, 2000).

Microsatellite markers have characterized several crocodilian species, including *Crocodylus johnstoni*, *Crocodylus moreletii*, *Alligator mississippiensis*, *Caiman latirostris*, *Crocodylus porosus*, *Paleosuchus trigonatus* and *Alligator sinensis* (Oliveira et al., 2010). Microsatellite patterns are often show low levels of polymorphism, leading to challenging interpretation and making fine-scale population structuring besides heterologous markers inefficient for analyses of mating systems in crocodiles (Isberg et al., 2004; Miles et al., 2009). Microsatellite studies of crocodiles are important as it can assess the genetic diversity and structure of crocodile populations in different regions and habitats. Microsatellite also could investigate the mating systems and social behavior of crocodiles, including determining paternity and relatedness among offspring (Isberg et al., 2004).

Previous research had used microsatellite markers on crocodiles which had been successful in resolving relationships among the reptiles. Preliminary study by Kasim (2011) had used a single set of microsatellite marker on a small number of crocodile

samples to investigate the genetic diversity and relationships between crocodiles in three RB in Sarawak. The study revealed monophyletic grouping of *C. porosus* from Miri, Bako, and Sibü. Although only a few samples and primers were utilized, microsatellite loci of around 115 to 122 base pairs (bp) were effectively amplified in this investigation using the Cj 16 primer. Besides that, Abdul Gani (2014, 2019) had carried out studies on wild crocodile populations to assess the genetic structure of the reptiles, aiming that the findings could be used in sustainable resource management. Limited success had been achieved in amplifying microsatellites using Cj127 primer pairs. Microsatellite markers Cj16 revealed different repeat motifs among the population of *C. porosus* from Sarawak (Abdul Gani, 2014). In addition, Abdul Gani (2019) had produced phylogenetic trees showing the geographical locations (only five river basins involved) where *C. porosus* are found in Sarawak thus concluded that microsatellite approach can be used to determine relationships among crocodiles in Sarawak. The study revealed that there is gene flow amongst the five populations suggesting that frequent migrations occur between the populations of *C. porosus* in Sarawak.

The above-mentioned studies used a small number of primers and limited samples. In the current study, a total of 13 sets of microsatellite primers (Table 4.1) were used involving 52 crocodiles from 13 River Basins in Sarawak, hoping to shed light on relationships among wild crocodile populations for future sustainable utilization of the reptile.

4.2 Materials and methods

4.2.1 Laboratory analysis

Polymerase Chain Reaction (PCR) was conducted for 62 crocodile samples and 13 microsatellite markers were used in this study as shown in the Table 4.1. The list of primers was taken from Isberg et al, (2004) studies. Thermal profiles used are as follows: preliminary denaturation at 94 °C for 3 minutes, denaturation at 94 °C for 15 seconds, annealing at 50.1°C (microsatellite) for 30 seconds, extension at 72 °C for 50 seconds, final extension at 72 °C for 5 minutes and soaked at 4 °C. The denaturation, annealing, and extension were repeated for 35 cycles. For microsatellite work, PCR products were only visualized using 1% AGE and the presence of the band were scored and analyzed accordingly. Following the completion of the amplification, 1% AGE was carried out using a 100 bp DNA ladder and viewed by UV light Gel Imaging System.

4.2.2 Data analysis

All positive bands were observed, estimated the size, and scored using binary system accordingly. The positive bands were marked as “1” while negative bands were marked as “0”. A dendrogram was created based on Unweighted Pair Group with Arithmetic Averages (UPGMA) methods after statistical analysis of microsatellite marker data using the NTSYSpc.2.02e program (Jamshidi, 2011).

Table 4.1: Microsatellite primers and sequences used in this study (Isberg et al., 2004)

Microsatellite Primer	Primer sequences (5'-3')
Cj127	CCCATAGTTTCCTGTTACCTG (forward) GTTTCCCTCTCTGACTTCAGTGTTG (reverse)
Cj122	GTTTCATGCTGACTGTTTCTAATCACC (forward) GGAACTACAATTGGTCAACCTCAC (reverse)
Cj131	GTTTGTCTTCTTCCTCCTGTCCCTC (forward) AAATGCTGACTCCTACGGATGG (reverse)
Cj101	ACAGGAGGAATGTCGCATAATTG (forward) GTTTATACCGTGCCATCCAAGTTAG (reverse)
CUD68	GCTTCAGCAGGGGCTACC (forward) TGGGGAAACTGCACTTTAGG (reverse)
Cj119	GTTTGCTGTGGAATGTTTCTAC (forward) CGCTATATGAAACGGTGGCTG (reverse)
Cj105	CAACAGAAAGTGCCACCTCAAG (forward) GTTTGATTATGAGACACCGCCACC (reverse)
Cj16	CATGCAGATTGTTATTCTGATG (forward) TGTCATGGTGTCAATTAAACTC (reverse)
Cj18	ATCCAAATCCCATGAACCTGAGAG (forward) CCGAGTGCTTACAAGAGGCTGG (reverse)
Cj104	TCCTTCCATGCATGCACGTGTG (forward) GTTTCAGTGTCTGGTATTGGAGAAGG (reverse)
Cp10	GATTAGTTTACGTGACATGCA (forward) ACATCAAGTCATGGCAGGTGAG (reverse)
Cj35	GTTTAGAAGTCTCCAAGCCTCTCAG (forward) CTGGGGCAAGGATTTAACTCTC (reverse)
CUJ-131	GTCCCTTCCAGCCCAAATG (forward) CGTCTGGCCAGAAAACCTGT (reverse)

4.3 Results

4.3.1 Band scoring and clustering

The single band, double band, and multiple bands were highlighted as shown in Table 4.2. The green, blue, and light orange colour represented the single bands, multiple bands and double bands, respectively. PCR amplifications utilizing seven markers (Cj127, Cj131, Cj122, Cj101, Cj119, CUD68, and Cj16) showed a distinct single band while one marker (Cj35) exhibited double bands. Multiple bands were visible for five markers (Cj105, Cj18, Cj104, Cp10, and Cu4-121). Based on table 4.2, ten samples out of 62 samples were not successfully amplified and the bands were absent.

Table 4.2: List of band scoring of each sample with 13 sets of primers

List of primers																																							
	A	B	C	D	E	F	G	G	G	H	I	I	I	J	J	J	J	K	K	K	K	K	K	K	K	K	K	K	K	L	L	M	M	M	M	M	M		
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	1	1	1	1	1	1	1	2	3	1	1	2	3	1	2	3	4	1	2	3	4	5	6	7	8	9	1	1	1	2	1	2	3	4	5	6			
																												0	1										
WSN1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	1
WSN2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1
WSN3	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	
WSN6	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1		
WSN7	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	1	1	0	1		
WSN8	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1		
WSN9	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1			
WSN10	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	1			
WSR1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	
WSR2	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1			
WSR3	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1			
WSR4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1			
WSR5	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1			
WSR8	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	0	1		
WSR9	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1	0	0	1		
WSR11	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	0	1		
WSS1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	1	
WSS2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	1			

Table 4.2 continued

WSS3	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1		
WSG1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	
COY1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	1	1	1	0	0	1	0	1	1	0	0	1		
COY3	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	1	1	1		
CRJ1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	1		
CRJ2	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1	
CRJ5	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1	
CRJ6	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1	
CRJ7	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	
CKM1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	
CKM2	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1
CKM3	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	
CKM4	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1	
CKM5	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	
CTA1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	
CTA2	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	
CTA3	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	1		
NSU1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	
NSU2	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	1	1	0	0	0	0	0	1
NSU3	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1
NSU4	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	1	
NSU5	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	0	1
NSU6	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1
NSU8	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	1
NSU9	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	
NSU11	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	1
NSU12	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1

Table 4.2 continued

NNH1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	1
NLB1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NBM1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1
NMI1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NMI2	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NMI3	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NMI4	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	1

(Y-axis = List of primers, X-axis = List of samples)

Notes:

Alphabet	Primers	Alphabet	Primers	Alphabet	Primers
A	Cj127	F	CUD68	K	Cp10
B	Cj131	G	Cj105	L	Cj35
C	Cj122	H	Cj16	M	Cu4-121
D	Cj101	I	Cj18		
E	Cj119	J	Cj104		

4.3.2 Dendrogram

The unweighted pair group with arithmetic mean (UPGMA) clustering algorithm, with coefficients ranging between 0.66 and 1.00, produced a microsatellite-based unrooted phylogenetic tree (Figure 4.1) The dendrogram is divided into 4 clades, ranging from A to D.

Clade A includes samples from the following locations: Samarahan RB (WSN1; WSN2; WSN7), Sungai Sarawak RB (WSR1; WSR2; WSR3; WSR4; WSR5), Saribas RB (WSS1, WSS2), Tatau RB (CTA1), Oya RB (COY1, COY3), Rajang RB (CRJ1; CRJ2; CRJ5; CRJ6), Sadong RB (WSG1) and Kemena RB (CKM5). The highest samples in clade A showed from Sungai Sarawak RB with total of 5 samples and followed by Rajang RB (4 samples), Samarahan RB (3 samples), Saribas RB; Oya RB (2 samples) and a sample from Tatau RB, Sadong RB, Kemena RB respectively. The samples are closely related based on Sarawak RB map in Figure 4.2 where Samarahan RB is close to Sungai Sarawak RB, Sadong RB and Saribas RB while Rajang RB is close to Oya RB, Tatau RB and Kemena RB. The regions covered by clade A are from western and central part of Sarawak RB.

Next, clade B showed the biggest clade in the dendrogram and includes samples from western (Samarahan RB, Sungai Sarawak RB and Saribas RB), northern (Suai RB, Miri RB and Niah RB) and central (Rajang RB, Kemena RB and Tatau RB). The highest samples shown in clade B is from Suai RB with a total of eight samples (NSU1, NSU2, NSU3, NSU4, NSU5, NSU6, NSU8, NSU9) and followed by Samarahan RB with a total of four samples (WSN3, WSN6, WSN8, WSN9). A total of four out of five samples from Kemena RB were found in clade B (CKM1, CKM2, CKM3, CKM4). The samples from Suai RB, Miri RB and Niah RB are from Miri district while Tatau RB and Kemena RB are

from Bintulu district. This indicates a mixture of samples collected from 3 regions which are western (Samarahan RB, Sungai Sarawak RB, and Saribas RB), central (Rajang RB), and northern (Miri district, and Bintulu district). Six sister clades formed and shared in clade B showed the groupings of samples according to the regions in Sarawak RB. Clades B2, B3, B4, B6, and B7 consist of samples from northern, western, northern, northern, and central regions of Sarawak RB, respectively. Clade B1 consists of samples from central and western regions, while clade B5 consists of samples from northern and central regions. This indicates there is a gene flow of saltwater crocodiles across river basins in Sarawak. The samples from clade B6 only consist of samples from Kemena RB.

In addition, clade C consists of all seven samples from Miri district only from the northern region, while clade D comprises four samples from Sungai Sarawak RB (3 samples) and Samarahan RB (one sample) which are closely related and located in western region. The samples in clade C from Limbang RB (one sample), Miri RB (3 samples), Baram RB (one sample), and Suai RB (2 samples).

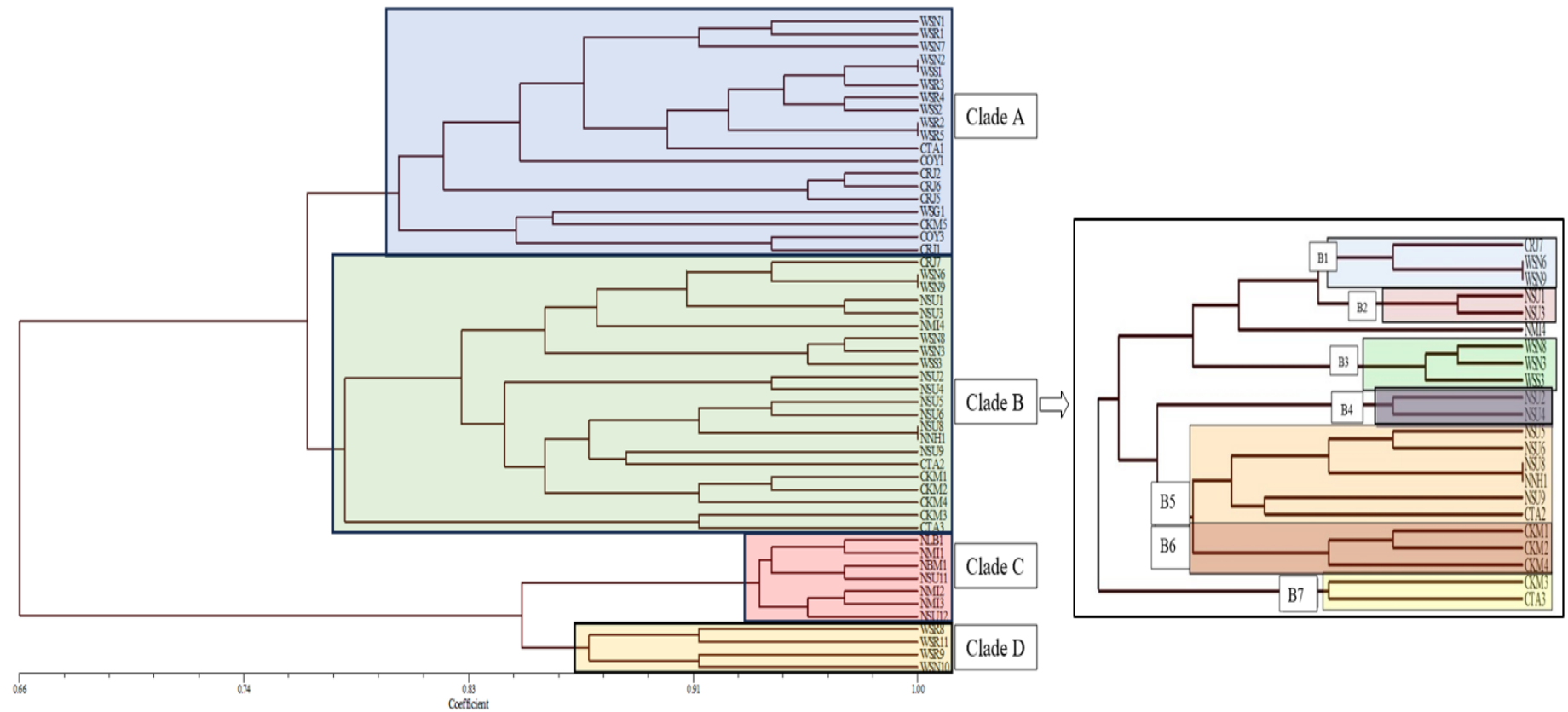


Figure 4.1: Microsatellite-based unrooted phylogenetic tree for 52 Saltwater crocodiles generated by the unweighted pair group with arithmetic mean (UPGMA) clustering method.

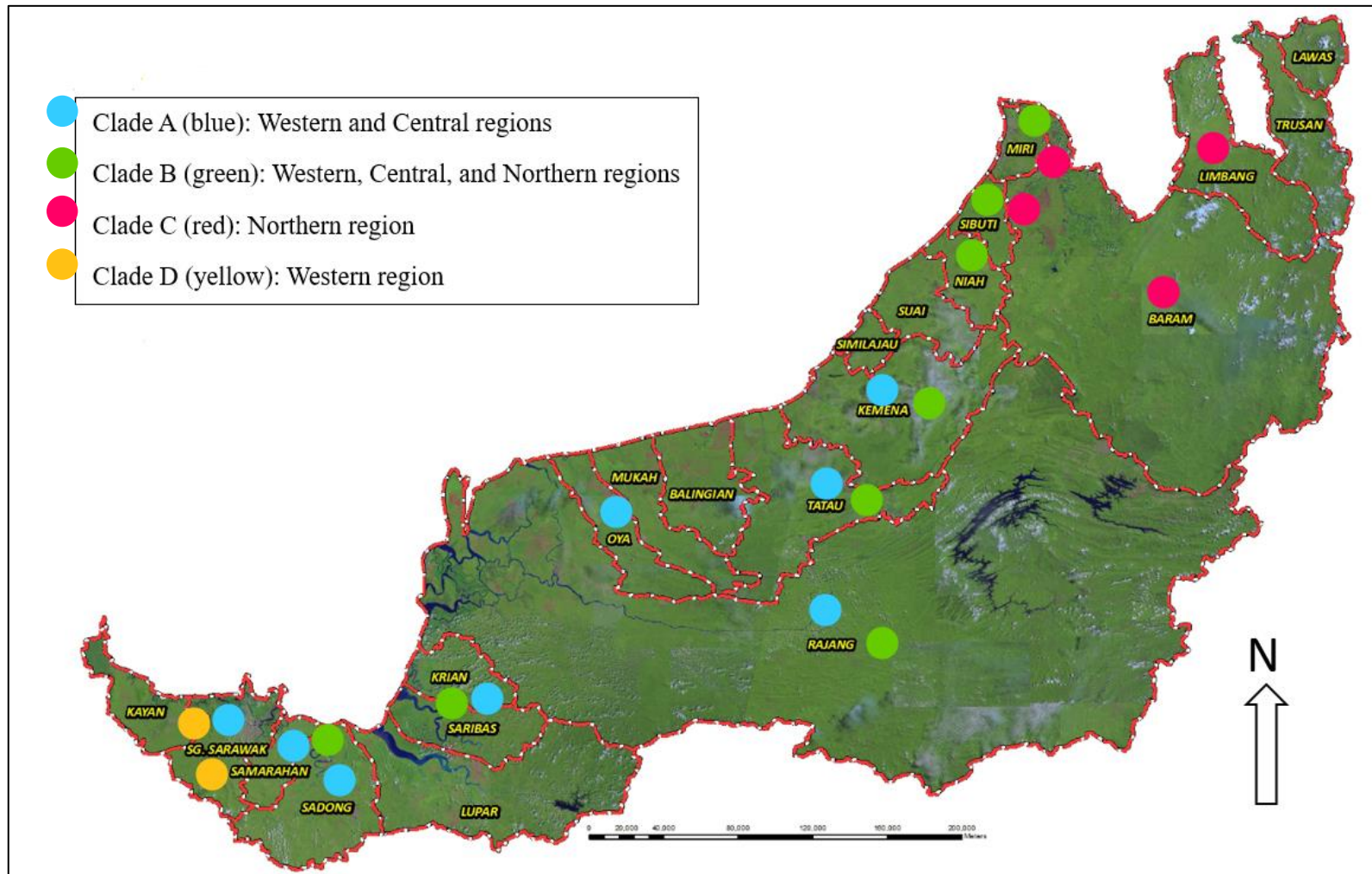


Figure 4.2: Maps of Sarawak River Basin (RB) according to the clades of each site (Map adapted from Sarawak Drainage)

4.4 Discussion

The CTAB procedure (Doyle and Doyle, 1987) and QIAamp DNA Investigator Kit (QIAGEN, Germany) had been successful to extract whole genomic DNA from crocodiles. In other investigations, researchers applied various methods, such as the standard proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (Fitzsimmons et al., 2000; Li et al., 2007), standard salting-out protocol (Lapbenjakul et al., 2017), the DNeasy blood and tissues kit (Rossi et al., 2020; Russello et al., 2007). Techniques to extract DNA had evolved over the years, which allows researchers to choose a much safer and faster as well as suitable with monetary allocation for each project.

Ten samples (WSN4, WSN5, WSR6, WSR7, WSR10, COY2, CRJ3, CRJ4, NSU7, and NSU10) underwent the Kit technique during DNA extraction and showed a faint band, probably due to poor DNA concentration yield. The absence of a band in the samples was most likely due to a low concentration of DNA (Abdul Gani., 2014). According to Asif et al. (2008), Polyacrylamide Gel Electrophoresis (PAGE) is a better method for distinguishing alleles of microsatellite markers than normal Agarose Gel Electrophoresis (AGE). This visualization method, however, needs hazardous radioactive materials and is time-intensive (Asif et al., 2008), therefore PAGE was not used in the current study. The ten samples that could not give any positive PCR products; thus, these samples need further investigation. Palumbi et al. (2002) reported that there are many factors that could influence the success of PCR, therefore systematic optimization of PCR conditions should be carried out accordingly.

Besides that, the crocodile samples in clade A were genetically identical and came from eight different river basins that were geographically connected. According to Gratten (2003), sea barriers do not appear to be a significant deterrent to migration in *C. porosus*,

and migrations of *C. porosus* across the sea can surpass 800 km. Campbell et al. (2013) verified records of the saltwater crocodile, *C. porosus*, traveling a few hundred kilometers across the coastal area using telemetry and a GPS transmitter. This species' high migration rate could result in considerable gene flow and restricted population structure (Abdul Gani, 2014). According to Abdul Gani (2019), phylogenetic trees revealed the geographical locations (river basins) but there is gene flow among the five populations, suggesting that regular migrations occur among the *C. porosus* in Sarawak.

Similarly, clade B contains crocodiles from nine different river basins that were from eight districts in Sarawak which are geographically connected, which again suggests the potential for gene flow among these populations. This genetic connectivity may be facilitated by the crocodiles' ability to move between different river basins, further supporting the notion of regular migrations among *C. porosus* in Sarawak. The distance between the river mouths in numerous adjacent river basins in Sarawak is minimal (they are close to one another). For example, the distance between the river mouth in Samarahan/Sadong RB is less than 60 km, so it is highly likely that crocodiles will cross the sea to the next river basin (Abdul Gani, 2019).

The distribution of clades showed clade A is present in a range spanning from central to western while clade B is more widespread, covering a larger territory within the region. Clade C only comprised samples from the northern and clade D from the western of Sarawak RB. The presence of gene flow among populations is essential for maintaining genetic diversity and reducing the risk of inbreeding. Regular migrations between river basins may help to prevent the isolation of local populations and promote a more interconnected crocodile population across the region (Kenney et al., 2014).

The sister clades in clade B are fully resolved which is clade B6 is composed of samples exclusively from the Kemena RB. The genetic analysis fully resolved in this clade, indicating that crocodiles from the Kemena RB form a distinct and well-defined group. Interestingly, Clade C is also fully resolved, with all samples originated from Miri district (northern region) which consists of four river basins that share the same geographical area and clade D also entirely resolved from Samarahan RB and Sungai Sarawak RB of western region of Sarawak RB. Clade C comprised samples from Miri and clade D are from Kuching and Samarahan (these two areas are adjacent to each other). The findings imply that microsatellite data, which provides information on genetic variation within populations, can be used to distinguish individual crocodiles from different river basins. This ability to assign individuals to specific river basins based on their genetic profiles is similar to the findings of Abdul Gani (2019). Understanding the genetic diversity and structure of these populations is essential for effective conservation and management efforts (Tikendra et al., 2021).

4.5 Conclusion

Crocodile populations in Sarawak RB have been divided into four clades (A, B, C, and D) using DNA microsatellite analysis. Clades C and D are specifically linked to the northern and western parts of Sarawak RB, respectively. DNA microsatellite has proven to be a valuable genetic marker for understanding relationships among *C. porosus* populations in the state of Sarawak. Clades A and B consist of crocodile samples from different regions of Sarawak RB. The fact that samples from different geographical regions cluster together in these clades suggests that there is genetic connectivity or gene flow among crocodile populations in Sarawak. Future research will require more samples and

microsatellite primers to determine the relationships between the crocodiles in Sarawak. DNA sequencing should be performed on all positive PCR results so that more rigorous data analysis can be done in the future. Researchers in the next generations can obtain reliable and informative data on the genetics of crocodile populations which require careful selection of markers, optimization of PCR conditions, genotyping, data analysis, and interpretation of results.

CHAPTER 5

GENETIC DIVERSITY AND POPULATION STRUCTURE OF SALTWATER CROCODILE IN SARAWAK RB USING COMBINE MTDNA ANALYSIS (COI AND CYT B GENE)

5.1 Introduction

Cytochrome oxidase subunit 1 (CO1) gene is a widely used genetic marker due to its conserved characteristics, making it suitable for DNA barcoding exercise in animal species. Besides, this gene is highly variable among species and it has higher evolutionary rate compared to nuclear genes, making it useful to distinguish closely related species (Roldan et al. 2009). Similar to COI gene, Cytochrome b (Cyt b) gene is a protein coding gene found in the mitochondria of eukaryotic cells. Cyt b is commonly used to determine phylogenetic relationships between organisms, within family and genera, due to its sequence variability.

Meganathan et al. (2010) had sequenced a complete mitochondrial genome of *C. porosus*. This information was later combined with other gene information and concluded that their phylogenetic study supported the well-established intergeneric crocodilian phylogenetic relationships. Bloor et al. (2015) had sequenced three captive populations of *C. acutus* in Colombia (mtDNA cytochrome b and cytochrome oxidase I gene sequences) for sequence variation. The two separate lineages *C. acutus*-I and *C. acutus*-II were found to match to the remaining Colombian haplotypes and closely similar Central American haplotypes, respectively.

Mc Aliley et al. (2006) had analysed two nuclear genes and two mitochondrial genes and they managed to support the hypothesis that *C. cataphractus* is not a member of *Crocodylus* or *Osteolaemus*. Preliminary molecular work using Cyt b and 12S rRNA gene

were unable to resolve relationships among crocodiles from different areas in Sarawak by Shoon (2009) probably due to small number of samples used. On the other hand, Abdullah (2010) had used combination of Cyt b and COI genes analysis and managed to partially resolve phylogenetic relationships among samples obtained from different rivers in Sarawak. For *Tomistoma*, Cyt b - control region mtDNA gene analysis, revealed that the genetic diversity among *T. schlegelii* ranging from 0.16% to 3.34%, with unique haplotypes in Sumatera as well as in Northern Sarawak; thus each *T. schlegelii* population deserved its own management strategies to ensure the conservation of the species (Md Adzhar & Hassan, 2017).

Although Sarawak supports a high population of crocodiles, limited studies on the genetics of crocodiles had been carried out, thus understanding of the population at the genetic level is still at its infancy stage. Abdullah (2010), had analyzed combine COI and Cyt b gene analysis but involved limited number of samples, eventually, not all relationships among crocodiles of Sarawak were resolved. This study aims to sequence and analyze population structure of COI gene and Cyt b gene from crocodiles in Sarawak River Basins. Findings from this study could be used in formulating sustainable utilization of crocodile hunting and aquaculture in the rivers, for the benefit of local people.

5.2 Materials and Methods

5.2.1 Laboratory works

Total genomic DNA was extracted from crocodile samples (tissues and scutes) using 2% modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) and followed by 1% Agarose Gel Electrophoresis (AGE) as described in Chapter 3.

PCR was conducted using thermocycler (MyCycler™) to amplify COI gene using with forward primer COIf (5'- CCT GCA GGA GGA GGA GAY CC-3'), and reverse primer COIa (5'-AGT ATA AGC GTC TGG GTA GTC-3'). As for Cyt b gene, forward primer LGL765 (5'-GGA AAA CCA TCG TTG TWA TTC AAC-3'), and reverse primer LGL766 (5'-GTT TAA TTA GAA TYT YAG CTT TGG G-3'). Standard PCR with total volume of 25µl was prepared for each batch together with a negative control. Thermal profile used are as follow: preliminary denaturation at 94 °C for 15 seconds, annealing at 50 °C (COI gene), 48 °C (Cyt b gene) for 30 seconds and extension at 72 °C for 50 seconds (35 cycles) and followed by a final 4 minutes extension step at 72 °C. Once the amplification completed, all the PCR products were subjected to 1% AGE with 100bp DNA ladder. All PCR products were then sent to a commercial company namely as Apical Scientific Sdn. Bhd., Seri Kembangan, Selangor, Malaysia or known as First Base Laboratories Sdn Bhd for PCR product cleaning and single pass DNA sequencing. Ten samples were excluded in this study as the PCR products had not been successful to amplify the whole genomic DNA after a few trials. This chapter involving 52 samples of crocodiles and only comprises samples from 12 RB as a sample from Baram RB was excluded.

5.2.2 Data sequencing and alignment

The raw DNA sequences were observed under CHROMAS PRO software to view the quality of the chromatogram. Then, the DNA sequences were subjected to nucleotide Basic Local Alignment Search Tool (BLAST) to find the similarities and confirmed the species (Altschul et al., 1990). All 52 sequences from this study and 24 sequences from The National Center for Biotechnology Information (NCBI) Genbank database (Table 5.1) were uploaded in Multiple Sequence Comparision by Log- Expectation (MUSCLE) and set

the output format as Pearson/FASTA. All sequences were loaded in ClustalX version 2.1 and the alignment were trimmed (Larkin et al., 2007). MEGA 11 program was used to calculate the nucleotide base composition together with nucleotide richness (Kumar et al., 2016).

Table 5.1: Accession number of *Crocodylus* samples from NCBI Genbank database.

Samples	Accession number
<i>C. siamensis</i> 1	MT554035.1
<i>C. siamensis</i> 2	EF581859.1
<i>C. siamensis</i> 3	MT727015.1
<i>C. palustris</i> 1	HM488087.1
<i>C. palustris</i> 2	NC_014706.1
<i>C. palustris</i> 3	MT554049.1
<i>C. intermedius</i> 1	NC_015648.1
<i>C. intermedius</i> 2	MT554044.1
<i>C. intermedius</i> 3	HM636895.1
<i>C. acutus</i> 1	NC_015647.1
<i>C. acutus</i> 2	MT554043.1
<i>C. acutus</i> 3	HM636894.1
<i>C. moreletti</i> 1	NC_015235.1
<i>C. moreletti</i> 2	HQ585889.1
<i>C. rhombifer</i> 1	MT727026.1
<i>C. rhombifer</i> 2	MT727025.1
<i>C. rhombifer</i> 3	MT727024.1
<i>C. suchus</i> 1	MT727011.1
<i>C. suchus</i> 2	MT727033.1
<i>C. suchus</i> 3	MT727008.1
<i>C. novaeguineae</i> 1	MT727020.1
<i>C. novaeguineae</i> 2	MT727023.1
<i>C. johnsoni</i> 1	MT727018.1
<i>C. johnsoni</i> 2	MT727019.1

5.2.3 Phylogenetic tree

Three methods of constructing phylogenetic tree were used for this study. Neighbour- Joining (NJ) were analyzed in the MEGA 11 (Kumar et al., 2016) while Maximum- Parsimony (MP) and Maximum- Likelihood (ML) were determined using PAUP 4.0a169 software. The substitution model test that was used to estimate ML was TVM plus gamma distributed that were selected based on Akaike Information Criterion (AIC), calculated using PAUP 4.0a 169 software (Swofford, 2002). All phylogenetic trees used Bootstrap Method (1000 replicates). Based on the best-fit substitution model in Mega 11, Tamura- Nei model and gamma distributed were applied to construct NJ. *Crocodylus siamensis*, *Crocodylus palustris*, *Crocodylus intermedius*, *Crocodylus acutus*, *Crocodylus moreletti*, *Crocodylus rhombifer*, *Crocodylus suchus*, *Crocodylus novaeguineae*, *Crocodylus johnsoni* and *Tomistoma schlegelii* sequences were taken from NCBI GenBank database were used as outgroup in all phylogenetic trees analyses of combine gene. Details as in Table 5.1.

5.2.4 Haplotype data and Network analysis

Based on the mtDNA haplotype data produced using DNA Sequence Polymorphism (DNASP) version 6.12.03 software, a median-joining (MJ) network was created for *C. porosus* by NETWORK 10.2 in order to calculate the species' dispersion (Bandelt et al., 1999; Rozas et al., 2017).

5.2.5 Population genetic analyses

Using DNASP 6.12.03, estimates of population genetic characteristics such nucleotide diversity (π) and nucleotide divergence (Da) were calculated using mtDNA data sequences. DNASP 6.12.03 was used to construct measures of gene flow (number of

migrants, Nm), population subdivision (Fst), and nucleotide subdivision (Nst) among populations of *C. porosus*. Lynch and Crease (1990) were used to estimate the Nst value, whilst Hudson et al. (1992) were used to estimate the Fst and Nm values. Analysis of Molecular Variance (AMOVA) was used to analyze demographic history and the differentiation of regional populations. Arlequin 3.5.1.2 was used to do AMOVA, and 1000 permutations were used to evaluate for statistical significance (Rozas et al., 2017; Lynch and Crease, 1990; Excoffier & Lischer, 2010).

5.3 Results

5.3.1 BLAST and nucleotide composition

Approximately 579 bp of COI gene and 913 bp of Cyt b genes from 52 individuals had been successfully sequenced. Table 5.2 shows the BLAST results, all COI and Cyt b gene sequences from this study matched with two *C. porosus* DNA sequences from NCBI Genbank database, Accession No.: DQ273698.1 and NC008143.1 with average of 92.21% to 99.68% similarity. The E-value for all samples matched Genbank data were zero.

Table 5.2: BLAST results of Crocodile samples in this study and NCBI GenBank database of *Crocodylus porosus*. Combined sequence data of COI gene (579 bp) and Cyt b gene (913 bp) was used in this study. The length of final alignment was 1492 bp.

Species/ Accession no.	Genbank data (<i>Crocodylus porosus</i>)			
	DQ273698.1		NC008143.1	
	E-value	Identities (%)	E-value	Identities (%)
CKM1	0	97.54	0	95.79
CKM2	0	97.37	0	95.61
CKM3	0	97.84	0	96.08
CKM4	0	97.86	0	96.28

Table 5.2 continued

CKM5	0	96.44	0	94.92
NLB1	0	97.19	0	95.59
NMI1	0	97.07	0	95.48
NMI2	0	97.52	0	95.92
NMI3	0	96.96	0	95.40
WSN1	0	97.00	0	95.17
WSN2	0	96.45	0	94.75
WSN3	0	98.12	0	96.31
WSN4	0	98.03	0	96.23
WSN5	0	98.18	0	96.45
WSN6	0	96.72	0	95.00
WSN7	0	97.86	0	96.11
WSN8	0	97.52	0	95.95
WSN9	0	98.14	0	96.36
WSN10	0	96.36	0	94.58
WSS1	0	96.14	0	94.44
WSS2	0	97.54	0	95.79
WSS3	0	97.66	0	95.82
WSR1	0	97.78	0	96.03
WSR2	0	98.19	0	96.30
WSR3	0	97.58	0	95.79
WSR4	0	98.21	0	96.42
WSR5	0	98.39	0	96.53
WSR6	0	97.45	0	95.70
WSR7	0	97.29	0	95.54
WSR8	0	97.45	0	95.65
WSR9	0	96.81	0	95.08

Table 5.2 continued

WSR10	0	96.76	0	94.98
WSR11	0	98.05	0	96.26
CTA1	0	96.67	0	95.05
CTA3	0	98.36	0	96.73
WSG1	0	97.53	0	95.78
CRJ1	0	97.69	0	96.02
CRJ2	0	98.57	0	96.80
CRJ3	0	98.85	0	97.05
CRJ5	0	97.03	0	95.51
COY1	0	97.71	0	95.97
NNH1	0	98.31	0	96.69
NSU1	0	96.68	0	95.06
NSU2	0	97.54	0	95.95
NSU3	0	96.69	0	95.12
NSU4	0	96.83	0	95.24
NSU5	0	98.26	0	96.61
NSU6	0	98.31	0	96.70
NSU8	0	98.29	0	96.73
NSU9	0	98.45	0	96.81
NSU11	0	98.12	0	96.49
NSU12	0	98.01	0	96.43

From a total of 1492 aligned bases, 1471 sites (99%) were conserved while 20 sites (1%) were variable. From the variables sites, 14 sites were singleton sites and 6 were parsimonious informative sites.

Table 5.3 shows the nucleotide base composition of fifty-two *C. porosus* based on combination of COI and Cyt b gene. The average of the nucleotide base composition is as follow: thymine (T) = 26.3%, cytosine (C) = 29.5%, adenine (A) = 28.7% and guanine (G) = 15.5%. Cytosine showed the highest values of nucleotide composition and guanine showed the lowest values which the range from 28.6% to 28.8% and 15.4% to 15.6%, respectively. Higher proportion of A+T bases were observed compared to G+C bases which the values are 55% and 45%, respectively.

Table 5.3: The average of nucleotide base composition of *C. porosus* samples (Sarawak) with the combination gene of COI and Cyt b

Sample	T	C	A	G
WSN1	26.4	29.4	28.8	15.4
WSN2	26.3	29.5	28.7	15.5
WSN3	26.4	29.4	28.8	15.4
WSN4	26.4	29.4	28.8	15.4
WSN5	26.4	29.4	28.8	15.4
WSN6	26.4	29.4	28.8	15.4
WSN7	26.4	29.4	28.8	15.4
WSN8	26.2	29.4	28.8	15.5
WSN9	26.4	29.4	28.8	15.4
WSN10	26.4	29.4	28.8	15.4
WSR1	26.3	29.5	28.7	15.5
WSR2	26.3	29.5	28.7	15.5
WSR3	26.4	29.5	28.7	15.4
WSR4	26.3	29.5	28.7	15.5
WSR5	26.3	29.5	28.7	15.5
WSR6	26.3	29.5	28.7	15.5
WSR7	26.3	29.5	28.7	15.4
WSR8	26.3	29.5	28.7	15.5

Table 5.3 continued

WSR9	26.4	29.5	28.7	15.4
WSR10	26.3	29.5	28.7	15.5
WSR11	26.4	29.4	28.8	15.4
NSU1	26.4	29.5	28.6	15.4
NSU2	26.3	29.5	28.7	15.5
NSU3	26.3	29.5	28.7	15.5
NSU4	26.3	29.5	28.7	15.5
NSU5	26.3	29.5	28.7	15.5
NSU6	26.3	29.5	28.7	15.5
NSU8	26.3	29.5	28.7	15.5
NSU9	26.3	29.5	28.7	15.5
NSU11	26.3	29.5	28.7	15.5
NSU12	26.3	29.5	28.7	15.5
WSS1	26.3	29.5	28.7	15.5
WSS2	26.4	29.4	28.8	15.4
WSS3	26.3	29.5	28.7	15.5
CKM1	26.3	29.5	28.6	15.6
CKM2	26.3	29.5	28.6	15.6
CKM3	26.3	29.5	28.6	15.6
CKM4	26.3	29.5	28.7	15.5
CKM5	26.3	29.5	28.7	15.5
NNH1	26.3	29.5	28.7	15.4
NMI1	26.3	29.5	28.7	15.5
NMI2	26.3	29.5	28.7	15.5
NMI3	26.3	29.5	28.7	15.5
CTA1	26.3	29.4	28.7	15.6
CTA3	26.3	29.5	28.7	15.5
COY1	26.3	29.4	28.8	15.5
WSG1	26.3	29.5	28.7	15.5

Table 5.3 continued

NLB1	26.3	29.5	28.7	15.5
CRJ1	26.3	29.5	28.7	15.5
CRJ2	26.3	29.4	28.7	15.6
CRJ3	26.3	29.5	28.7	15.5
CRJ5	26.3	29.5	28.7	15.5
Average	26.3	29.5	28.7	15.5

5.3.2 Phylogenetic tree

5.3.2.1 Cytochrome oxidase I

All fifty-two samples in this study are closer to each other and grouped into one clade namely Clade A, with bootstrap values of 62%, 73%, and 66%, for NJ, ML, and MP, respectively (Figure 5.1), thus *Crocodylus porosus* is monophyletic. There is no grouping of *C. porosus* according to the RB using cytochrome oxidase 1.

5.3.2.2 Cytochrome b

The Cyt b phylogenetic tree (Figure 5.2) shows that *C.porosus* is monophyletic which is supported by low bootstrap value. Clade A consists of samples from the western, central and northern region. Clade B with bootstrap values of 40%, 59%, and 60% consists of samples from northern and central regions of Sarawak RB while clade C comprised samples from western and central region. The Cyt b tree showed there is no samples that share the same geographical clade.

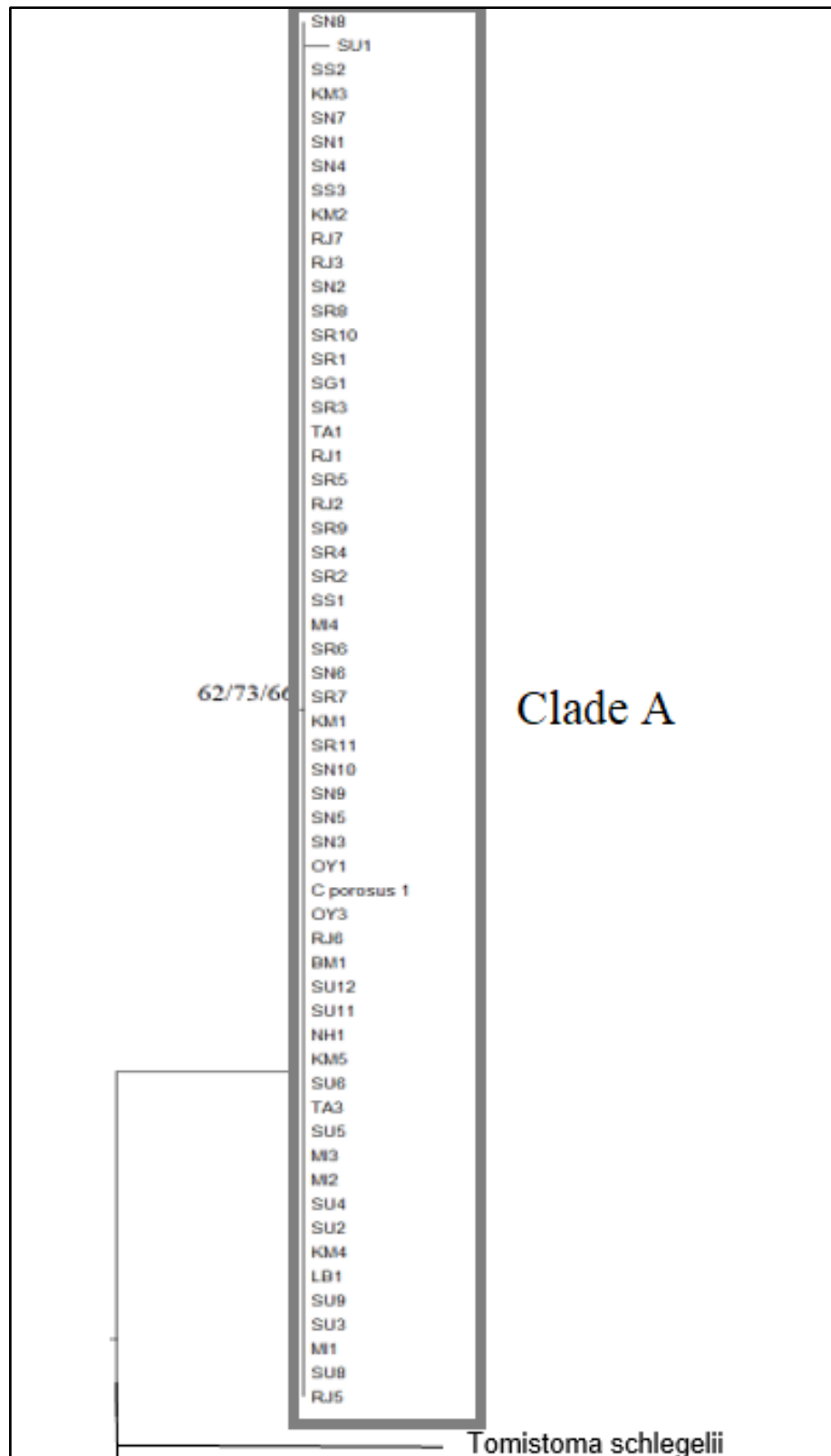


Figure 5.1: Phylogenetic tree constructed using COI gene sequences showing the relationship among *Crocodylus porosus* with *Tomistoma schlegelii* as outgroup.

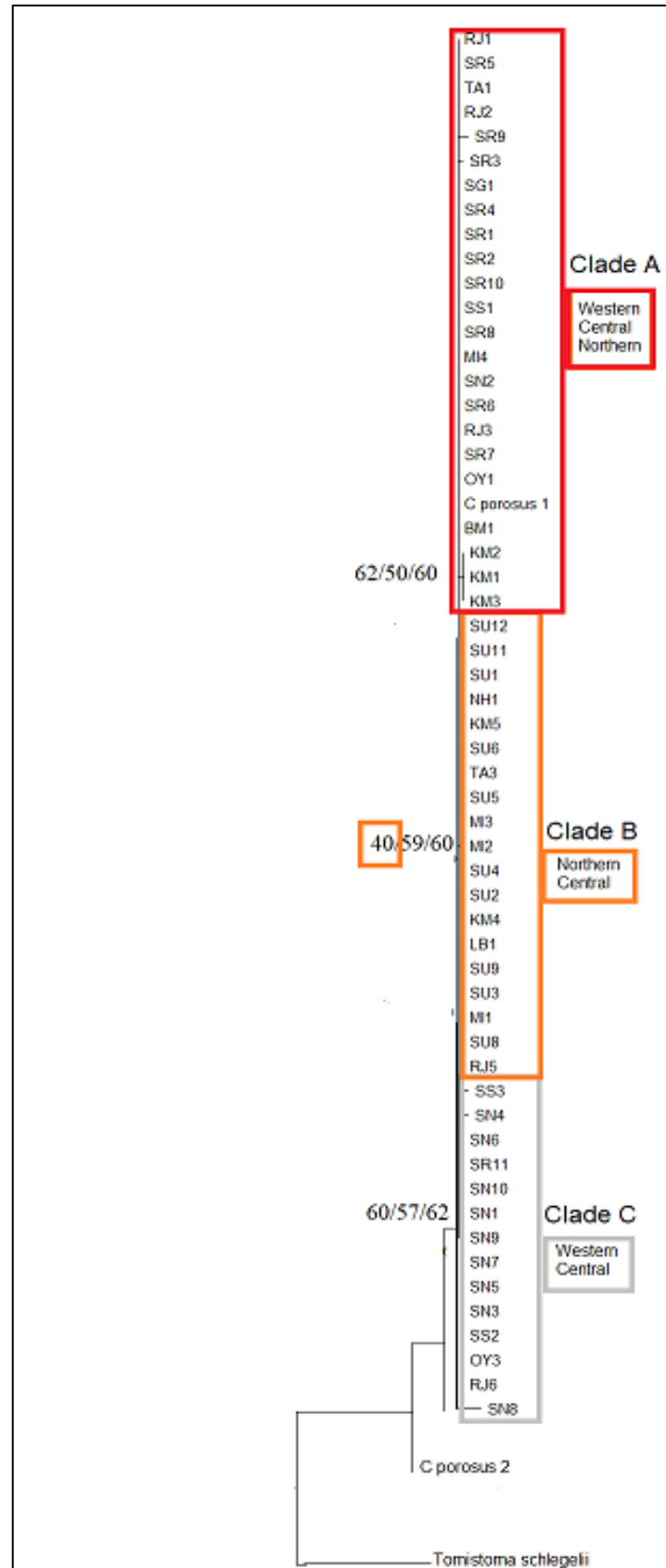


Figure 5.2: Phylogenetic tree constructed using Cyt b gene sequences showing the relationship among *Crocodylus porosus* with *Tomistoma schlegelii* as outgroup.

5.3.2.3 Combine gene of COI gene and Cyt b gene

All phylogenetic trees are in agreement in terms of monophyletic of *Crocodylus porosus* with the outgroup species, *Tomistoma schlegelii*. All fifty-two samples in this study are closer to each other and grouped into four clades namely Clade A, Clade B, Clade C, and Clade D (Figure 5.3).

Clade A comprises samples from 2 regions that are closely related region 1 are from Sungai Sarawak RB, Sadong RB, Saribas RB, and Samarahan RB, and region 2 from Rajang RB, Tatau RB and Oya RB. A total of ten samples out of eleven samples from Sungai Sarawak RB were found in clade D (WSR1-WSR10), followed by Rajang RB with a total of 3 samples (CRJ1, CRJ2, CRJ3,). A sample from Samarahan RB (WSN2), Sadong RB (WSG1), Saribas RB (WSS1), Tatau RB (CTA1) and Oya RB (COY1). Clade A also comprises sample from NCBI Genbank database (*C. porosus* 1).

Next, samples from Suai RB (NSU1, NSU2, NSU3, NSU4, NSU5, NSU6, NSU8, NSU9, NSU11, NSU12), Miri RB (NMI1, NMI2, NMI3), Kemena RB (CKM4, CKM5), Niah RB (NNH1), Limbang RB (NLB1), Tatau RB (CTA3), and Rajang (CRJ5) were closer to each other and form clade B with bootstrap values of 64%, 79%, and 70%, for NJ, ML, and MP, respectively. In clade B, most of the samples are from Suai RB (10 samples) and followed by Miri RB (3 samples), Kemena RB (2 samples) and the lowest number of samples were from Niah RB, Limbang RB, Tatau RB and Rajang RB (a sample). Based on Sarawak River Basin map in Figure 5.1, samples from clade B are closely related to each other where Suai RB, Niah RB, Miri RB and Limbang RB are from Miri district, Kemena RB and Tatau RB from Bintulu district, and Rajang RB from Sibu district, respectively. In addition, a total of three out of five samples from Kemena RB were found in clade C (CKM1, CKM2, CKM3).

Next, clade D includes samples Samarahan RB, Saribas RB, and Sungai Sarawak RB. The highest samples shown in clade D is from Samarahan RB with total of nine samples (WSN1, WSN3, WSN4, WSN5, WSN6, WSN7, WSN8, WSN9, WSN10) and followed by Saribas RB with total of 2 samples (WSS2, WSS3) and a sample from Sungai Sarawak RB (WSR11). This indicates samples collected from the western region which are (Samarahan RB, Saribas RB, and Sungai Sarawak RB). There is a subclade in clade D which comprising sample from NCBI Genbank database (*C. porosus* 2) which form sister clade relationship with other crocodile samples. Based on the phylogenetic trees, there is a geographical clade (clade C) from Kemena RB observed in this study.

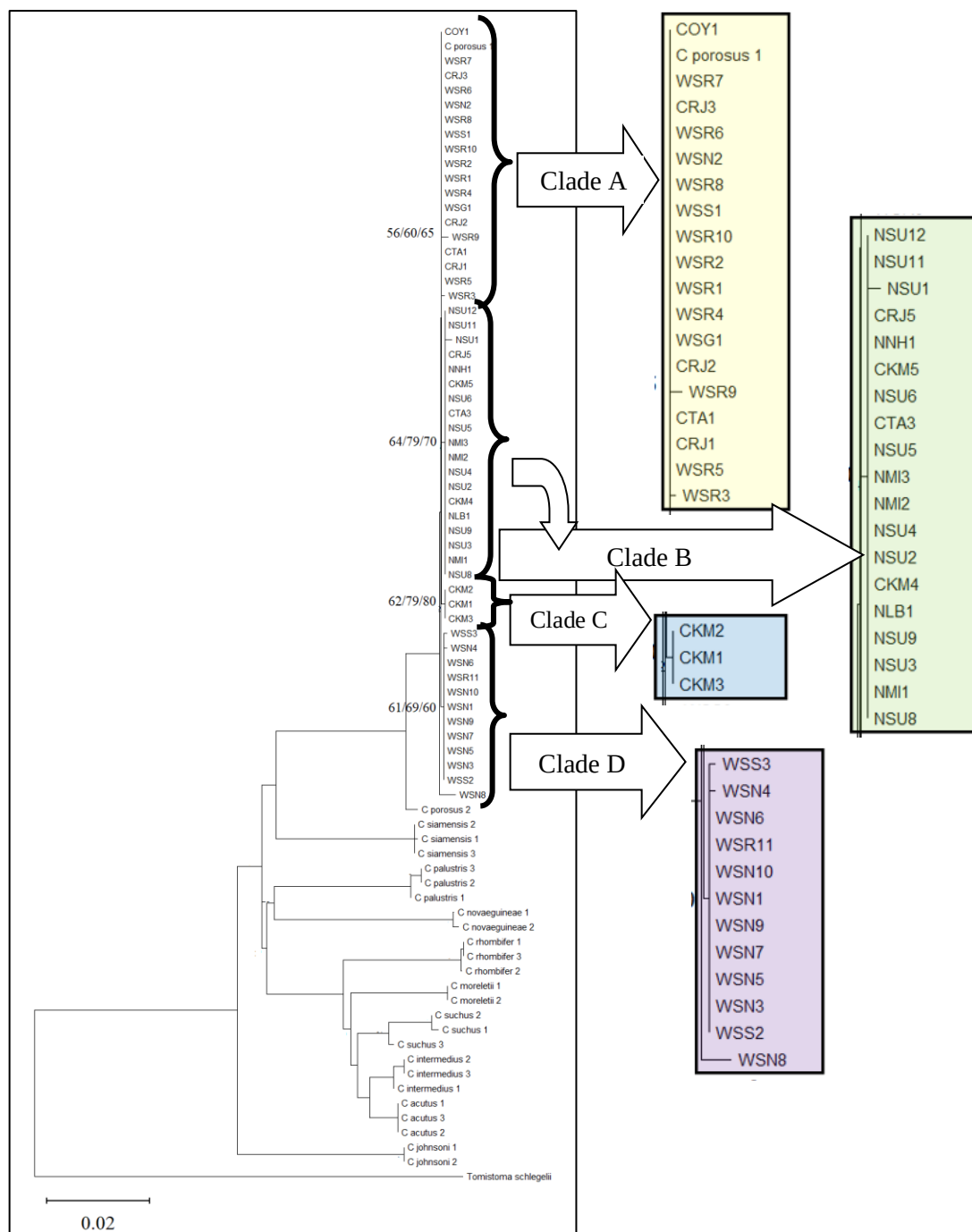


Figure 5.3: Phylogenetic tree constructed using COI and Cyt b gene sequences showing the relationship among *Crocodylus porosus* with *Tomistoma schlegelii* as outgroup. Bootstrap values next to the node were Neighbour-joining (NJ), Maximum likelihood (ML) and Maximum Parsimony (MP) analysis.

5.3.3 Haplotype build and network analyses

Out of 52 sequences in this study, a total of 10 haplotypes was identified (Table 5.4). Each haplotype consists of 18 nucleotides. Based on Table 5.4, haplotype 11 shows the highest varieties compared to other haplotypes with differences of ten nucleotide positions. Haplotype 5 has the least differences in the sequences (one nucleotide difference).

Table 5.4: List of haplotype sequences and the differences of nucleotide position.

Haplotype	Haplotype sequences
Hap_1	GATCCTTCAAGGCTAGAG
Hap_2	GATCCTTCAAGGCTA ATG
Hap_3	GATCCTTCAAGGCT TGAA
Hap_4	GATCCTTCAAG AT TAGAG
Hap_5	GATCCTTCA G GGCTAGAG
Hap_6	GATCCTTCAAGG TC AGAG
Hap_7	GATCCTT AAAGAT TAGAG
Hap_8	GA ATTAC CAAGGCTAGAG
Hap_9	GATCCTTC GAA GCTAGAG
Hap_10	TC TCCTTC GAA GCTAGAG

Referring to Table 5.5, a total of six out of ten haplotype shows unique haplotype and only a crocodile sample appeared in the specific haplotype. The unique haplotype are haplotype 2 (WSR3), haplotype 3 (WSR9), haplotype 6 (WSS3), haplotype 7 (WSN4), haplotype 8 (WSN8), and haplotype 10 (NSU1).

Hap_1 consists of samples from Sungai Sarawak RB (WSR1, WSR2, WSR4, WSR5, WSR6, WSR7, WSR8, WSR10), Rajang RB (CRJ1, CRJ2, CRJ3), Oya RB (COY1), Tatau RB (CTA1), Samarahan RB (WSN2), Sadong RB (WSG1), and Saribas RB (WSS1) total of sixteen samples which are shared same haplotype (Hap_1). Nine

samples of crocodiles from Samarahan RB (WSN1, WSN3, WSN5, WSN6, WSN7, WSN9, WSN10), Saribas RB (WSS2) and Sungai Sarawak RB (WSR11) shared same haplotype (Hap_4). Next, eighteen samples from Suai RB (NSU2, NSU3, NSU4, NSU5, NSU6, NSU8, NSU9, NSU11 and NSU12), Kemena RB (CKM4; CKM5), Miri RB (NMI1, NMI2, NMI3), Niah RB (NNH1), Rajang RB (CRJ5), Limbang RB (NLB1) and Tatau RB (CTA3) also shared same haplotype (Hap_9). Hap_5 consists only of samples from Kemena RB with a total number of three samples (CKM1, CKM2, CKM3).

Table 5.5: Haplotype identity for 58 mtDNA sequences of *C. porosus*.

Haplotype	Haplotype frequency	Voucher samples	River Basin (RB)
Hap_1	16	WSR1	Sungai Sarawak
		WSR2	
		WSR4	
		WSR5	
		WSR6	
		WSR7	
		WSR8	
		WSR10	
		WSS1	Saribas
		WSN2	Samarahan
		WSG1	Sadong
		COY1	Oya
Hap_2	1	CTA1	Tatau
		CRJ1	Rajang
		CRJ2	
Hap_3	1	CRJ3	
Hap_2	1	WSR3	Sungai Sarawak
Hap_3	1	WSR9	Sungai Sarawak

Table 5.5 continued

Hap_4	9	WSN1	Samarahan
		WSN3	
		WSN5	
		WSN6	
		WSN7	
		WSN9	
		WSN10	
Hap_5	3	WSS2	Saribas
		WSR11	Sungai Sarawak
		CKM1	Kemena
		CKM2	
Hap_6	1	CKM3	
Hap_7	1	WSS3	Saribas
Hap_8	1	WSN4	Samarahan
Hap_9	18	WSN8	Samarahan
		NSU2	Suai
		NSU3	
		NSU4	
		NSU5	
		NSU6	
		NSU8	
		NSU9	
		NSU11	
		NSU12	
		CKM4	Kemena
		CKM5	
		NMI1	Miri
		NMI2	
		NMI3	
Hap_10	1	NNH1	Niah
		NLB1	Limbang
		CTA3	Tatau
		CRJ5	Rajang
		NSU1	Suai

Based on median-joining network for 10 haplotypes, there is a gene flow among crocodiles in Sarawak (Figure 5.4). Four groups of haplogroup were determined, which is congruent with the phylogenetic trees topologies (Figure 5.3). Four groupings or clade had been identified namely clade A to D.

Clade B consists of haplotype 9 and haplotype 10 which mixture of samples from Suai RB, Miri RB, Kemena RB, Niah RB, Limbang RB, Rajang RB and Tatau RB while clade A consists of haplotype 1 (Samarahan RB, Tatau RB, Oya RB, Rajang RB, Saribas RB, Sungai Sarawak RB), haplotype 2 (Samarahan RB) and haplotype 3 (Samarahan RB).

Clade C shows haplotype 3 which the samples are from Kemena RB with total of three out of five samples formed a cluster. Clade D consists of haplotype 4, haplotype 6, haplotype 7, and haplotype 8 which formed one cluster. The samples are from Sungai Sarawak RB, Samarahan RB, and Saribas RB. Haplotype 6, haplotype 7, and haplotype 8 have unique haplotype in clade D. Haplotype 1, haplotype 4 and haplotype 6 were connected to a median vector (mv1) by 1 mutation step.

According to the locality region haplotype diagram, Hap_2, Hap_3, Hap_4, Hap_6, Hap_7, and Hap_8 comprises samples only from the western region while Hap_5 and Hap_10 consist of samples from the central region and northern region of Sarawak RB, respectively. Hap_1 and Hap_9 comprise a mixture of samples from the central region to the western region and central region and northern region, respectively.

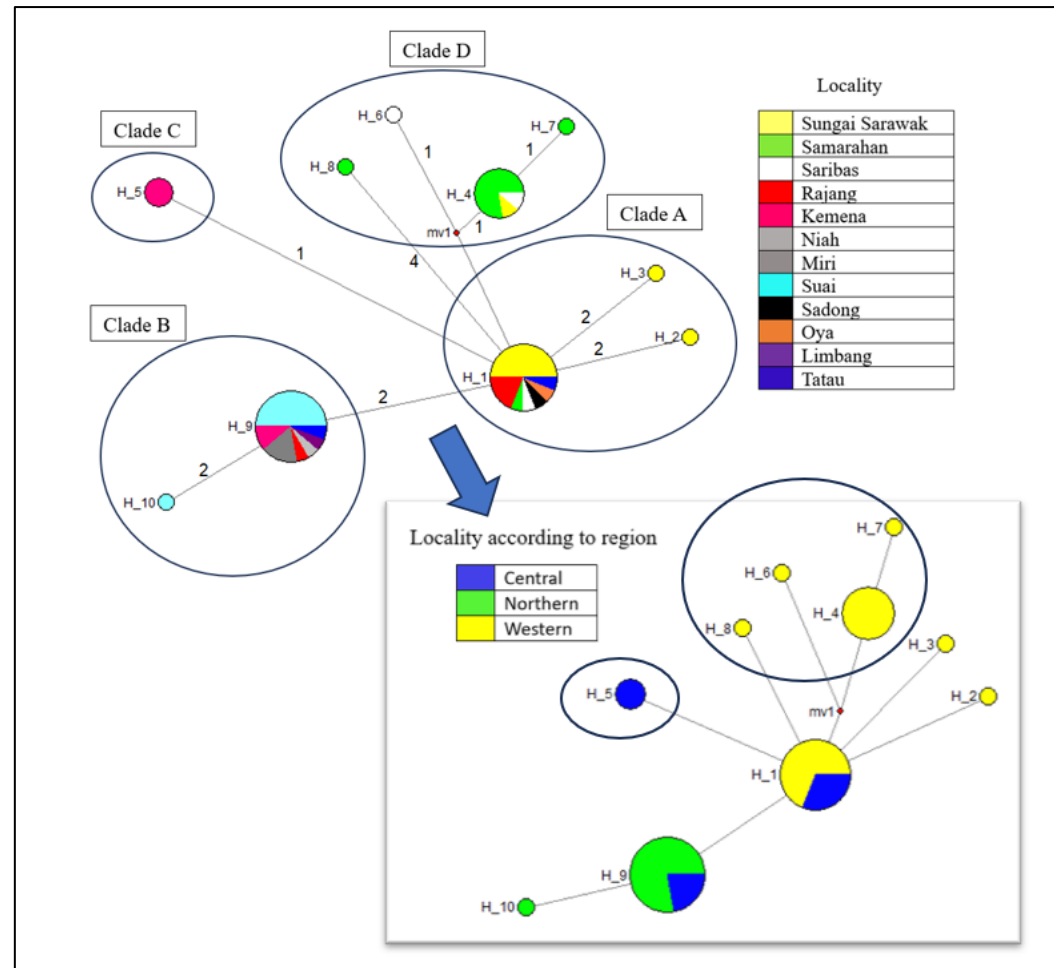


Figure 5.4: The median-joining Network generated by NETWORK software version 10.2 illustrating the relationship of the saltwater crocodile, *C. porosus* from different localities in Sarawak. Each circle represents a haplotype and the diameter of the circle is scale to the haplotype frequency. Different colours in the circle represent different localities. Bold number next the lines connecting the haplotypes indicate number of mutation step(s).

5.3.4 Population Genetic Analyses

Nucleotide diversity (π) among 12 populations of saltwater crocodiles in Sarawak was modest, with values ranging from 0.0% to 0.2%, while net nucleotide divergence (Da) ranged between 0.0% and 0.3%, indicating minimal genetic differentiation among the groups (Table 5.6).

Nucleotide diversity values were lower in northern region (Niah RB/Limbang RB), (Niah RB/Miri RB), (Niah RB/ Suai RB) and (Suai RB/ Limbang RB) with values 0.00000, 0.00000, 0.00037 and 0.00037, respectively. In addition, Niah RB and Limbang RB show a high nucleotide diversity value to Oya RB with the value of 0.00201.

Net Nucleotide Divergence (Da) has been recorded in this study (Table 5.6). The lowest Da value are from Niah RB to Limbang RB and Niah RB to Miri RB with a value of 0.00% while the highest value of Da is from Samarahan RB to Suai RB with a value of 0.30%. There is no nucleotide diversity between Niah RB to Limbang RB and Niah RB to Miri RB.

According to Table 5.7, the highest nucleotide diversity and Da according to the region from Sarawak RB is from the western region to the northern region with values of 0.16% and 0.23%. The lowest nucleotide diversity and Da is from the central region to the northern region of Sarawak RB with values of 0.1% and 0.14%, respectively. The nucleotide diversity values range from 0.1% to 0.16% and Da values range from 0.14% to 0.23%.

Table 5.6: Nucleotide Diversity (π) and Net Nucleotide Divergence (Da) among populations of saltwater crocodile analysed by River Basin (RB)

River basin	Distance (km)	Nucleotide diversity (π) ^{a,b}	Net nucleotide divergence (Da)
Samarahan- Oya	344	0.00144	0.00215
Samarahan - Rajang	370	0.00157	0.00198
Samarahan - Saribas	148	0.00129	0.00130
Samarahan – Sungai Sarawak	25.3	0.00133	0.00165
Samarahan - Kemena	519	0.00199	0.00268
Samarahan - Baram	728	0.00144	0.00215
Samarahan – Niah	628	0.00156	0.00282
Samarahan- Suai	590	0.00199	0.00302
Samarahan - Miri	716	0.00182	0.00282
Samarahan- Limbang	870	0.00156	0.00282
Samarahan - Tatau	452	0.00166	0.00248
Samarahan - Sadong	38.4	0.00132	0.00148
Oya - Rajang	142	0.00101	0.00101
Oya - Saribas	200	0.00145	0.00156
Oya – Sungai Sarawak	370	0.00067	0.00037
Oya - Kemena	188	0.00174	0.00174
Oya- Niah	297	0.00201	0.00201
Oya- Suai	259	0.00072	0.00215
Oya- Miri	384	0.00101	0.00201
Oya- Limbang	539	0.00201	0.00201
Oya – Tatau	121	0.00156	0.00134
Oya - Sadong	315	0.00067	0.00067
Rajang – Saribas	241	0.00128	0.00140
Rajang – Sungai Sarawak	397	0.00072	0.00070
Rajang – Kemena	318	0.00142	0.00137
Rajang – Niah	427	0.00107	0.00117
Rajang – Suai	389	0.00086	0.00134
Rajang – Miri	514	0.00096	0.00117
Rajang – Limbang	669	0.00107	0.00117
Rajang – Tatau	257	0.00107	0.00101
Rajang - Sadong	341	0.00080	0.00050
Saribas – Sungai Sarawak	175	0.00090	0.00114
Saribas – Kemena	391	0.00189	0.00210
Saribas – Niah	500	0.00179	0.00224
Saribas – Suai	502	0.00122	0.00244
Saribas – Miri	587	0.00161	0.00224
Saribas – Limbang	742	0.00179	0.00224
Saribas – Tatau	330	0.00174	0.00190
Saribas – Sadong	120	0.00112	0.00089
Sungai Sarawak – Kemena	543	0.00104	0.00131
Sungai Sarawak – Niah	723	0.00090	0.00171
Sungai Sarawak – Suai	743	0.00122	0.00184

Table 5.6 continued

Sungai Sarawak – Miri	828	0.00106	0.00171
Sungai Sarawak – Limbang	982	0.00090	0.00171
Sungai Sarawak – Tatau	476	0.00083	0.00104
Sungai Sarawak – Sadong	112	0.00067	0.00037
Kemena – Baram	239	0.00161	0.00134
Kemena – Niah	142	0.00143	0.00134
Kemena – Suai	142	0.00104	0.00152
Kemena – Miri	227	0.00141	0.00148
Kemena – Limbang	382	0.00165	0.00148
Kemena – Tatau	68.3	0.00166	0.00154
Kemena – Sadong	491	0.00156	0.00121
Niah – Suai	33	0.00037	0.00020
Niah – Miri	110	0.00000	0.00000
Niah – Limbang	265	0.00000	0.00000
Niah – Tatau	184	0.00134	0.00101
Niah – Sadong	582	0.00134	0.00134
Suai – Miri	143	0.00031	0.00020
Suai – Limbang	297	0.00037	0.00020
Suai – Tatau	164	0.00065	0.00114
Suai – Sadong	561	0.00061	0.00154
Miri – Limbang	163	0.00054	0.00034
Miri- Tatau	272	0.00080	0.00101
Miri – Sadong	685	0.00067	0.00134
Limbang – Tatau	429	0.00134	0.00101
Limbang – Sadong	842	0.00134	0.00134
Tatau - Sadong	429	0.00134	0.00101

^aEstimated using the Kimura 2-parameter distance (Kimura, 1980). ^bsites with gaps were completely excluded.

Table 5.7: Nucleotide Diversity (π) and Net Nucleotide Divergence (Da) among populations of saltwater crocodiles analysed by region of Sarawak RB.

Regions in Sarawak RB	Nucleotide diversity (π) ^{a,b}	Net nucleotide divergence (Da)
Central – Western	0.00133	0.00148
Cenral – Northern	0.00104	0.00139
Western - Northern	0.00162	0.00229

^aEstimated using the Kimura 2-parameter distance (Kimura, 1980). ^bsites with gaps were completely excluded.

The AMOVA analysis (Table 5.8) revealed that most of the molecular variance was founded within the populations (50.73%) than among the populations (49.27%), which both were significantly differentiated ($p = 0.00$).

Table 5.8: Measures of geographical population differentiation in *Crocodylus porosus* based on an analysis of Molecular Variance approach using combine mtDNA sequences data

Source of variation	Variance component	Percent (%) variation	Fixation index, ϕ	p^a
Among populations	0.58	49.27	0.49	0.00*
Within populations	0.60	50.73	0.49	0.00*
Total	1.18		0.49	0.00±0.00

*Significant ($p < 0.05$). ^aProbability of finding a more-extreme variance component of the ϕ index than that observed by chance alone after 1000 permutations.

All 12 populations according to river basins of *C. porosus* in Sarawak showed low nucleotide (N_{st}) and population (F_{st}) subdivision with a high number of migrants per generation (N_m), indicating there is gene flow among the populations. The population of Sungai Sarawak RB and Miri RB had higher N_{st} and F_{st} values ($N_{st} = 0.79$, $F_{st} = 0.79$) compared to other areas (Table 5.9). Values in F_{st} and N_m do not correlate with the distance between river basins.

Table 5.9: Measures of Nucleotide Subdivision (N_{st}), Population Subdivision (F_{st}) and Gene Flow (Number of Migrants, N_m) among populations of *C. porosus* analysed by locations

Locality (n)	Locality (n)	Approx. Distance (km)	Nucleotide Subdivision (N_{st}) ^a	Estimate of Population Subdivision (F_{st}) ^b	Number of Migrants per generation (N_m) ^b
Samarahan	Rajang	370	0.46070	0.46091	0.34
Samarahan	Saribas	148	-0.01157	-0.01149	-22.00
Samarahan	Kemena	519	0.43612	0.43611	0.32

^a Estimated using Lynch and Crease (1990). ^b Estimated using Hudson et al. (1992).

Table 5.9 continued

Samarahan	Sungai_Sarawak	25.3	0.38822	0.38848	0.39
Samarahan	Miri	716	0.77238	0.77249	0.07
Samarahan	Suai	590	0.72099	0.72099	0.10
Samarahan	Tatau	452	0.33638	0.33634	0.49
Rajang	Saribas	241	0.18209	0.18182	-15
Rajang	Kemena	318	0.06640	0.06667	4.82
Rajang	Sungai_Sarawak	397	0.00014	0	3.39
Rajang	Miri	514	0.66667	0.66667	0.19
Rajang	Suai	389	0.58830	0.58824	0.28
Rajang	Tatau	257	-0.50000	-0.50000	-0.75
Saribas	Kemena	391	0.26618	0.26596	0.69
Saribas	Sungai_Sarawak	175	0.08946	0.08929	2.55
Saribas	Miri	587	0.70022	0.70000	0.11
Saribas	Suai	502	0.64250	0.64220	0.14
Saribas	Tatau	330	0.11790	0.11765	1.88
Kemena	Sungai_Sarawak	543	0.25691	0.25701	0.72
Kemena	Miri	227	0.50000	0.50000	0.36
Kemena	Suai	142	0.38069	0.38053	0.41
Kemena	Tatau	68.3	-0.09808	-0.21739	-1.40
Sungai Sarawak	Miri	828	0.78576	0.78571	0.07
Sungai Sarawak	Suai	743	0.72858	0.72848	0.09
Sungai Sarawak	Tatau	476	0.00025	0.00000	0.00
Miri	Suai	143	0.00000	0.00000	0.00
Miri	Tatau	272	-0.00000	-0.00000	0.00
Suai	Tatau	164	-0.05877	-0.05882	-4.50

^a Estimated using Lynch and Crease (1990). ^b Estimated using Hudson et al. (1992).

According to Table 6.1, nucleotide (N_{st}) and population (F_{st}) subdivision with number of migrants per generation (N_m) according to the regions in Sarawak RB, the population of western region and northern region showed the highest values of N_{st} and F_{st} ($N_{st} = 0.70$, $F_{st} = 0.70$) and lowest N_m value (0.12). The highest N_m value was recorded in western and central region with a value of 0.73.

Table 6.1: Measures of Nucleotide Subdivision (N_{st}), Population Subdivision (F_{st}) and Gene Flow (Number of Migrants, N_m) among populations of *C. porosus* analysed by regions in Sarawak RB

Regions in Sarawak RB	Nucleotide Subdivision (N_{st}) ^a	Estimate of Population Subdivision (F_{st}) ^b	Number of Migrants per generation (N_m) ^b
Western and central	0.26	0.26	0.73
Western and northern	0.68	0.68	0.12
Central and northern	0.52	0.52	0.31

^a Estimated using lynch and Crease (1990). ^b Estimated using Hudson et al. (1992).

5.4 Discussion

A total of 579 bp of COI gene and 913 bp of Cyt b gene had been successfully sequenced from 58 samples. Li et al. (2017) had reported 1557 bp of COI gene and 1165 bp for Cyt b gene from saltwater crocodiles which approximately 50% longer than this study. This differences in length may be due to set of primers used during PCR experiment. This study used universal primers by Palumbi et al. (2002) whereas Li et al. (2017) made specific design primers. Milián-García et al. (2018) had conducted a study of American crocodile (*C. acutus*) used same primers to amplify COI gene and different Cyt b gene as in this study. They reported the length of COI and Cyt b gene is 665 bp and 1320 bp, respectively.

According to Kerfeld and Scott (2011), by comparing a query sequence to sequences in a database, the Basic Local Alignment Search Tool (BLAST) could determine how similar the sequences are. Sequence homology detection enables us to locate potential genes in novel sequences as cited in Syngai et al. (2013). In this study, LGL 765 and LGL 766 primers were used to amplify Cyt b gene of *C. porosus* had been successful, and the sequences are able to determine the similarities of *C. porosus* sequences in NCBI GenBank database. According to Meganathan et al. (2009), specific primers can be very useful, and genus-specific primers are crucial for good identification tools (Jayanto et al., 2015).

Similar to Li et al. (2017), the nucleotide composition in this analysis revealed that A and T were more frequent than C and G. Although Li et al. (2017), recorded nucleotide composition as $A > C > T > G$, this study recorded nucleotide bases in the following order: $C > A > T > G$. Borrow (2003) reported that the AT content of mammals and reptiles is lower than the GC content, however Meister and Borrow (2005) claimed that the same species tend to have the same nucleotide composition.

In this study, COI gene phylogenetic tree has not been successful to group the *C. porosus* samples according to the regions of Sarawak RB while the Cyt b gene phylogenetic tree showed there is no samples that share the same geographical clade and supported by low bootstrap values of each clade. The combined gene of COI and Cyt b phylogenetic trees showed that *C. porosus* is monophyletic, similar to those reported by Meganathan et al. (2010). Milián-García et al (2018) combined COI and Cyt b gene sequences for further analysis as no phylogenetic conflict between mitochondrial genes and no variation among samples of *C. acutus* and *C. rhombifer* were observed in their studies. This study suggested that combine analysis of COI and Cyt b gene could resolve

phylogenetic relationships within Order Crocodylia, as all species form reciprocally monophyletic groupings. This finding is coherent with the current classification using morphological characters. According to the results, network analysis is congruent to the phylogenetic tree in this study showing the cluster of samples according to regions (northern, central, and western region of Sarawak). Clade A, clade B, clade C, and clade D in this study correspond to samples collected in the western and central region, northern and central region, central region, and western region, respectively.

A total of 10 haplotypes were identified from the 52 crocodile samples of *C. porosus* sequences in this study. Hap_2, Hap_3, Hap_6, Hap_7, Hap_8, and Hap_10 have unique haplotypes except for Hap_1, Hap_4, Hap_5, and Hap_9, where there is a mixture of some river basins of Sarawak in each haplotype. The uniqueness of each haplotype in the current study may also imply a high level of genetic variation among the *C. porosus* population in Sarawak (Abdul Gani, 2019). In this study, using combine mtDNA genes of COI and Cyt b gene could determine each unique haplotype in Sarawak River Basins. Bloor et al. (2015), using mtDNA cytochrome b and cytochrome oxidase I gene sequences from captive *C. acutus* from Colombia, found two different lineages: first, that is distinctive to Colombian *C. acutus* and the other that is closely related to Central American haplotypes. The results support such data and emphasize the value of further investigation to comprehend haplotype linkages in South American *C. acutus* (Bloor et al., 2015). One possibility is that contemporary patterns of gene flow may be responsible for the sharing of haplotypes. For example, saltwater crocodiles from one river basin may migrate to a neighboring basin and breed with individuals from that population, introducing new haplotypes into the area. Over time, these haplotypes may become more common in the population, leading to the sharing of haplotypes between the two river basins (Lapbenjakul

et al., 2017). It is also possible that the sharing of haplotypes in Sarawak RB may be due to a combination of historical and contemporary factors. Further research, including population genetic studies and analysis of the historical and ecological factors that have influenced the distribution and movement of saltwater crocodiles in the region, would be needed to determine the specific mechanisms underlying the sharing of mtDNA haplotypes in these populations (Luck et al., 2012).

According to the findings in this study, the samples from western of Sarawak, Samarahan, and Saribas had low levels of nucleotide ($N_{st} = -0.012$) and population subdivision ($F_{st} = -0.011$) and high levels of migration per generation ($N_m = -22.00$), indicating either ongoing gene flow or historical relationships between them (Zainudin et al., 2010). Low levels of nucleotide (N_{st}) and population subdivision (F_{st}) were also found in the samples from central Sarawak (Rajang) and central (Kemena) populations, whereas significant levels of migrants per generation were found (N_m). According to Abdul Gani. (2019), the population expansion hypothesis is further supported by the fact that a high number of migrants per generation (N_m) indicated a high rate of gene flow or migration across populations of *C. porosus*. Because of the numerous migrations of *C. porosus* in Sarawak, population genetic data analysis suggests that there is population expansion. *C. porosus* has spread throughout the state of Sarawak in large part due to the intricate river systems and huge web-like shapes in several main river basins in Sarawak (Abdul Gani., 2019).

In Sarawak, saltwater crocodiles are under threats due to habitat loss, hunting, and illegal trade (Stuebing et al., 1994). The conversion of wetland and coastal habitats for human development, agriculture, and aquaculture has led to the loss and fragmentation of crocodile habitat, reducing the availability of suitable nesting, and feeding sites (Stuebing

et al., 1994). Hunting and illegal trade have also had a significant impact on crocodile populations, with individuals being killed for their meat, skins, and other body parts. However, Abdul Gani et al. (2022), reported that the crocodile population showed an increasing trend which coincides with a high number of human crocodile conflict (HCC) cases. The number of crocodile attacks on humans documented in Sarawak between 2000 and 2020 was 164 cases, with an average of 7.8 occurrences per year (Abdul Gani et al., 2022). Western region and central region had low levels nucleotide ($N_{st} = 0.26$) and population subdivision ($F_{st} = 0.26$) and high levels of migration per generation ($N_m = 0.73$). The results in this study indicates there is a frequent movement of *C. porosus* from the western region to central region in Sarawak RB. The rapid development taking place in Sarawak, driven by an increasing human population, can have a range of effects on crocodile populations and their movements (Abdul Gani, 2019).

According to Mauger et al. (2017) who had done studies on *C. acutus* populations in Pacific Costa Rica stated that 81% of molecular variance occurred within populations while 19% occurred between populations based on AMOVA analysis which is similar to this study. In this study, 50.73% were within populations (interspecific) and 49.27% were among populations (intraspecific). The analysis showed that there is gene flow between populations from different RB. Genetic studies have found evidence of gene flow between saltwater crocodile populations in Australia and Southeast Asia, indicating that the species has the ability to disperse over long distances and maintain genetic connectivity (Russello et al., 2007). However, other studies have also highlighted the potential impact of habitat loss and fragmentation on gene flow, particularly in areas where human activities have caused the separation of crocodile populations (Russello et al., 2007).

5.5 Conclusion

C. porosus is monophyletic, according to the combined sequencing data of the COI gene (579 bp) and Cyt b gene (913 bp). Based on data, nucleotide diversity (π) and the quantity of migrants (N_m), revealed that there is gene flow across populations from various RB. According to the results of this study, the AMOVA value is higher in within population than among populations. The results also showed that although the population could be grouped into northern, central, and western regions, samples were unable to characterize according to RB. In this study, Kemena RB samples had clustered together and fully resolved. In addition, there is no nucleotide diversity between Niah RB/ Limbang RB and Niah RB/ Miri RB. In the future, large samples are required to allow for the examination of patterns of genetic variation within and between populations. This information can be used to infer the evolutionary history and population structure of crocodiles, which can have important conservation implications.

CHAPTER 6

GENERAL DISCUSSION

6.1 General discussion

Microsatellite (Chapter 4) and mtDNA (Chapter 5) studies are both important tools for studying the genetic diversity and population structure of saltwater crocodiles (*Crocodylus porosus*), and they can provide complementary information about different aspects of the species' evolutionary history and ecology. Microsatellite and mtDNA studies can provide a more complete picture of the genetic diversity and structure of saltwater crocodile populations (Luck et al., 2012). For example, microsatellite data can be used to estimate contemporary gene flow and population structure, while mtDNA data can provide insights into historical gene flow and colonization events (Bos et al., 2008). In addition, the combination of microsatellite and mtDNA data can help to identify areas of genetic diversity hotspots or refugia, and to prioritize conservation efforts based on genetic uniqueness or evolutionary distinctiveness (Taylor et al., 2011). Overall, the integration of microsatellite and mtDNA studies is an important approach for understanding the genetic diversity and evolutionary history of saltwater crocodiles, and can help to aid conservation management and promote the long-term survival of the species.

One factor that contributes to increase of crocodile population density is that the species adaptability, which allows them to persist in a range of habitat types, including modified habitats such as aquaculture ponds and reservoirs (Macgregor, 2002). Additionally, conservation efforts such as protected areas, captive breeding programs, and community-based conservation initiatives have helped to mitigate some of the threats facing crocodile populations in Sarawak (CITES, 2018). This may involve measures such

as habitat protection and restoration, regulation of hunting and trade, and community engagement and education programs to promote conservation awareness and support.

In Chapter 4, the genetic linkages between individuals and the population genetic structure across several river basin (RB) were clarified by cluster analysis. The population is divided according to northern, central, and western regions. However, using microsatellite loci, genetic distances were not affected by geographic distances. When assessing genetic distances using microsatellite loci, it is possible that some populations that are geographically close to each other show high genetic distance due to barriers to gene flow, such as rivers or mountain ranges resulting in allopatric speciation (Cooke et al., 2016). Conversely, some populations that are geographically distance from each other may be genetically similar if there is a history of gene flow between the populations (Pacheco-Sierra et al., 2016).

According to Chapter 5, combined sequence data of COI gene and Cyt b gene had shown that *C. porosus* is monophyletic. Further analysis showed that there is gene flow between populations from different RB based on values of nucleotide diversity (π), net nucleotide divergence (D_a) and number of migrants per generation (N_m) values. This study also recorded that within populations is higher than among populations based on AMOVA values. This may suggest that the crocodile populations have high survival rates in Sarawak RB. The results could support the proposal in harvesting wild crocodiles by local communities to generate income. The harvesting of saltwater crocodiles in Sarawak is an important economic activity and employment opportunity for many local communities (Webb et al., 2010).

This study found that genetic diversity and population structure data are important for aquaculture purposes and could sustain the management of wild crocodile populations.

According to Loughnan et al. (2019), who have done studies on barramundi (*Lates calcarifer*) mentioned that to improve genetically of aquaculture programs and to exploit fisheries sustainably, it is crucial to understand the population structure of fish stocks.

Genetic studies of population structure and diversity can also provide valuable information about the genetic health of a brood stock population. By analyzing the genetic makeup of individuals within a population, researchers can determine how much genetic diversity exists within the population and identify any genetic bottlenecks or inbreeding that may be occurring (Hsu et al., 2020). Harvesting adults from the wild can be one way to introduce new genetic material into a brood stock population. By selecting individuals from different populations or regions, breeders can increase the genetic diversity of their brood stock and help prevent inbreeding (Chacón et al., 2021).

The survival rates of saltwater crocodile populations can be increased through responsible management practices, such as the conservation of natural habitats and the implementation of sustainable harvesting practices for eggs and adults. This can also provide economic benefits for local communities through the sale of crocodile eggs and adults as crocodile eggs are in high demand for consumption and for use in the production of cosmetics and other products (Bernama, 2021). In some areas, crocodile farming has been successful in providing a sustainable source of income for local communities while also promoting the conservation of wild crocodile populations (Tisdell et al., 2007).

Crocodile farming involves the captive breeding and rearing of crocodiles for their meat, skin, and other products. This can provide economic benefits to communities while reducing the demand for wild-caught crocodiles (Tosun, 2013). However, it is important to ensure that harvesting is done in a sustainable manner to prevent negative impacts on the wild populations. Effective management and conservation strategies, such as monitoring

population levels, implementing harvest quotas, and enforcing regulations, are necessary to ensure the long-term sustainability of this activity and the survival of this species.

COI gene and Cyt b gene could resolve the monophyly of crocodiles and managed to group the crocodiles in northern, central, and western regions. The findings from this study showed gene flow among RB based on the existence of haplotype 1 and haplotype 9 among 12 RB. The presence of unique haplotypes representing 12 RB implies that that is some degree of difference among wild crocodile populations, so this information is important for the future management of crocodiles in Sarawak.

The information gathered in this study is hoped can benefit future crocodile aquaculture in Sarawak. Since the nucleotide diversity is high, adult crocodiles can become the brood stock in any aquaculture activity. It is likely inbreeding would occur and reduce genetic diversity; therefore, some adults could be harvested from different rivers to enrich the brood stock population.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

The microsatellite approach had been successful to cluster crocodiles according to regions namely; western region, central region, and northern region. Samples from the Miri district of the northern region (clade C) while samples from the western region (clade D) in Sarawak RB had totally resolved and grouped together. DNA microsatellite is a promising tool to determine the relationships among crocodiles in Sarawak. The findings are useful for the future sustainable utilization of the wild crocodile population. Combine COI and Cyt b gene analysis resolved the monophyly of *C. porosus*. It also showed the clustering of samples obtained from selected regions (northern, central, and western). Samples from the Kemena RB had clustered together and fully resolved. High nucleotide diversity (π) and nucleotide divergence (Da) observed in this study suggest there is gene flow among populations reflecting the frequent movement of crocodiles between river basins. The findings from this study are hoped to shed light on the genetic structure and diversity of saltwater crocodiles in RB and could be used by relevant agencies to carry out sustainable management of the wild crocodile population in Sarawak.

7.2 Recommendations

In future microsatellite studies, the application of a capillary array and fluorescently labeled primer to score alleles should be done, so that basin-specific allele frequency could be characterized as well as estimating genetic relationships i.e., relatedness and F_{is} (inbreeding coefficient), and inferring of recent migration rate (estimated using BayesAss

with microsatellite genotypes and Bayesian clustering). More microsatellite loci are required to determine the relationship between the crocodiles in Sarawak.

Combine gene analysis (COI gene and Cyt b gene) could not resolve the relationship among crocodile samples from nearby river basins. In future, to achieve a more complete understanding of the genetic structure of crocodiles in Sarawak, future research should include more samples that represent all 22 RB in Sarawak.

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APPENDICES

Appendix A: Journal Publications

1. **Nadarajan, R.**, Hassan, R., Abdul Gani, M. I. Z., Hassan, M. A., Ahmad, R., & Ketol, B. (2023). Genetic Analysis of Saltwater Crocodile (*Crocodylus porosus*) from Sarawak River Basin, Sarawak using Cytochrome Oxidase I and Cytochrome b Gene Analysis. *Journal of Survey in Fisheries Sciences*, 10(1), 80–88.

Appendix B: Agarose gel pictures (microsatellite)

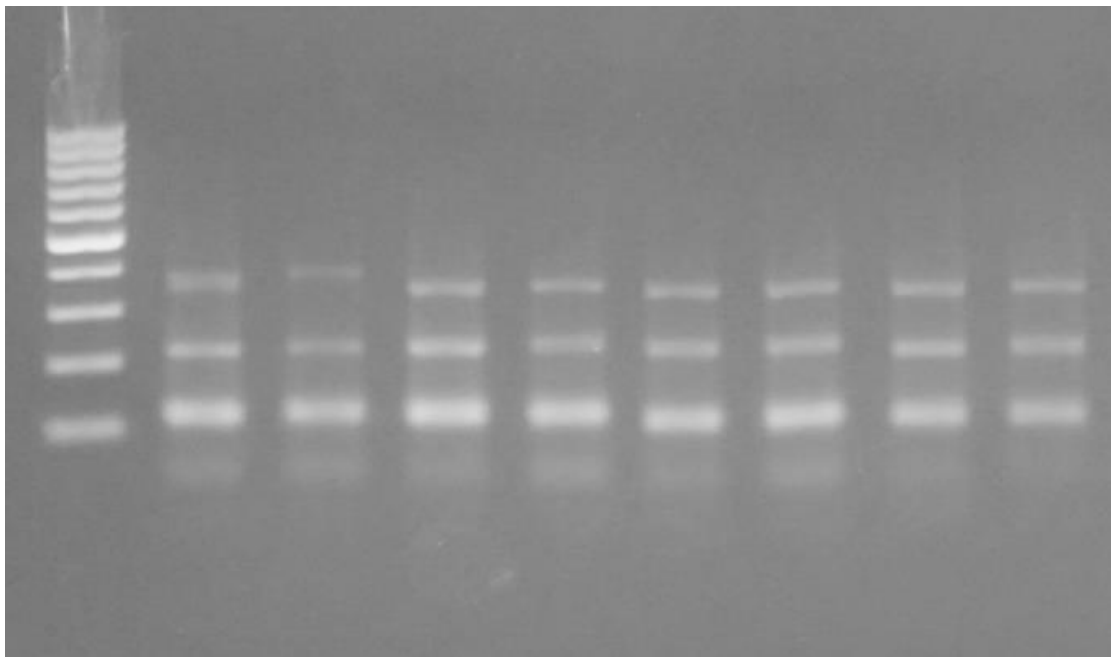


Figure Appendix B1: An example of 1% agarose gel picture of microsatellite amplification using three primers showing single bands.

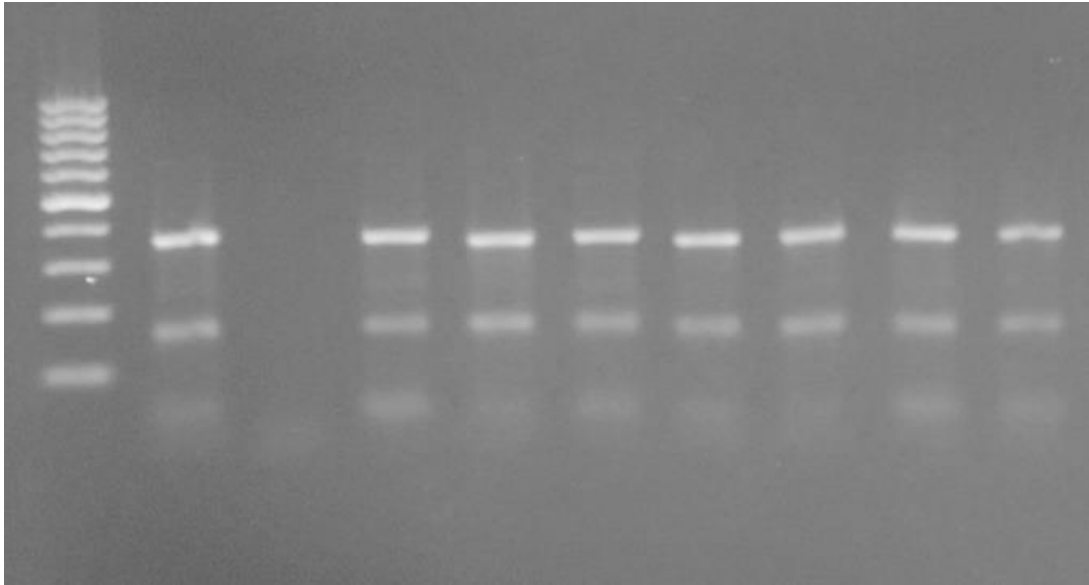


Figure Appendix B2: An example of 1% agarose gel picture of microsatellite amplification using Cj35 primers showing double bands.

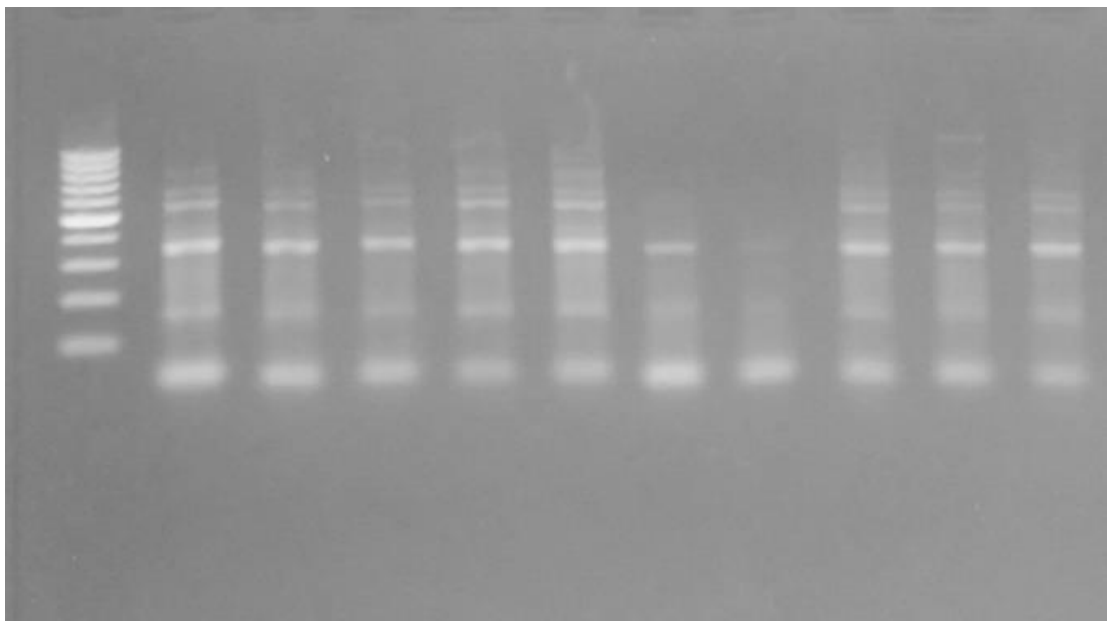


Figure Appendix B2: An example of 1% agarose gel picture of microsatellite amplification using (Cj105, Cj18, Cj104, Cp10 and Cu4-121) primers showing multiple bands.

Appendix C: Agarose gel picture (mtDNA)

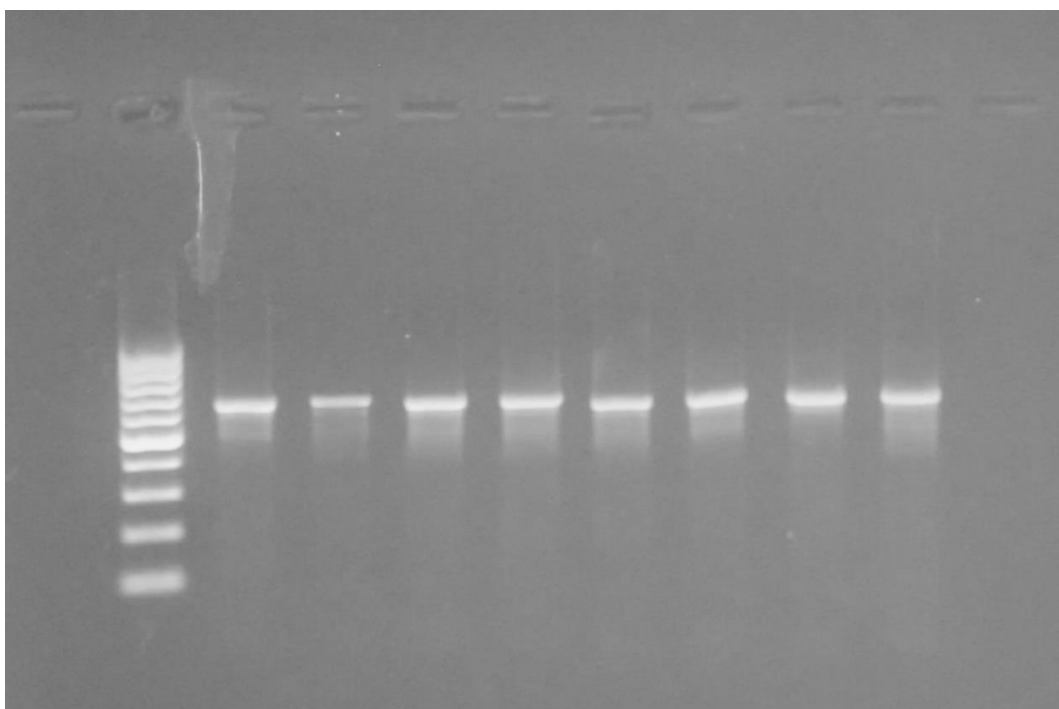


Figure Appendix C1: An example of 1% agarose gel picture of mtDNA amplification using universal COI primers showing single bands.

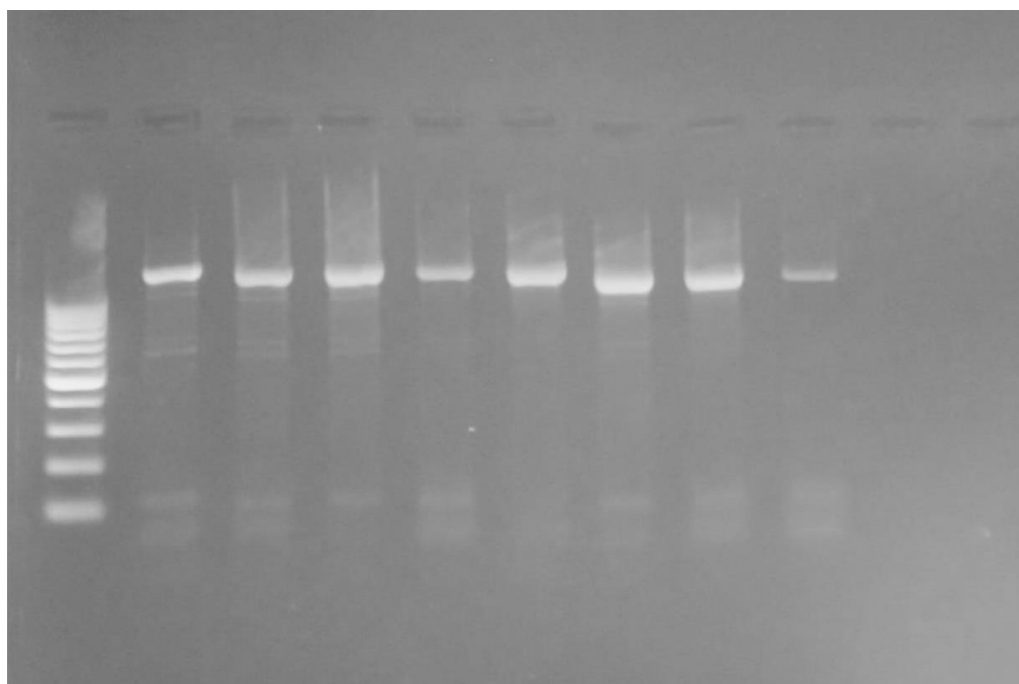


Figure Appendix C2: An example of 1% agarose gel picture of mtDNA amplification using universal Cyt b primers showing single bands.