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Original Article

Microsatellite-based population structure corresponding to the geographic origin of saltwater crocodiles in Sarawak River Basins

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Abstract

Of the recognized 24 crocodilian species, two species are found in Sarawak: the saltwater crocodile (*Crocodylus porosus*) and Malayan false gharial (*Tomistoma schlegelii*). *C. porosus* is the most commonly found crocodile and currently listed in Appendix II of the Convention on International Trading in Endangered Species of Wild Flora and Fauna (CITES), which allows harvesting wild populations for commercial purposes. To strengthen conservation efforts, ecological and genetic data are needed to inform management decisions. Thus, this study was designed to estimate relationship coefficients between crocodiles in thirteen river basins using 13 microsatellite markers. Fifty-eight wild crocodile samples were obtained and finally analysed by clustering of PCR products. Of the 60 samples amplified, one marker (Cj35) was polymorphic and showed double bands, whereas the other seven markers (Cj127, Cj131, Cj122, Cj101, Cj119, CUD68 and Cj16) revealed a single band. Microsatellite loci (Cj105, Cj18, Cj104, Cp10, and Cu4-121) displayed multiple bands. Using the unweighted pair group with the arithmetic mean (UPGMA) clustering method, an unrooted phylogenetic tree was obtained, with coefficients ranging between 0.51 and 1.00. We successfully assessed population genetic structure and resolved genetic relationships among six clades (Clade A to F) out of the total seven clades. DNA microsatellites are a promising resource for determining the relationships among crocodiles in Sarawak. The findings are useful for future sustainable utilization of the wild crocodile population.

Keywords: biodiversity, microsatellites, loci, clustering, crocodile

1. Introduction

Crocodilia is a group of usually giant, predatory semiaquatic reptiles that dwell in various aquatic environments such as rivers, marshes, swamps, forest streams, and elbow lakes. The saltwater crocodile is commonly referred to as "buaya katak" or "buaya tembaga" (translated as "frog crocodile" or "copper crocodile," respectively) by the locals in Sarawak (Abdul Gani, 2019). The species has a huge head, and a pair of ridges run from the eye along the middle of the snout. They are found in over 90 nations and islands worldwide (Martin, 2008). Sarawak is habitat to just two species of crocodiles: the Malayan false gharial (*Tomistoma schlegellii*) and the saltwater crocodile (*Crocodylus porosus*) (Hassan *et al*., 2016, 2018).

C. porosus can be found in nearly all of Sarawak's river basins and is frequently involved in human-crocodile conflicts (Abdul Gani, 2019; Tisen & Ahmad, 2010). All crocodilians are heavily impacted, either directly or indirectly, by anthropogenic causes, which are the main factor. In response

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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).

In Sarawak, saltwater crocodiles live in the wild, widely dispersed across the twenty-two river basins, with a concentration in the mangrove areas near the coastline zone (Abdul Gani, 2019; Hassan, Adzhar, Abdul-Gani, & Ahmad, 2018). In the early 1980s, crocodile studies conducted throughout Sarawak revealed uniformly low populations, according to Cox and Gombek (1985). However, more recent surveys have shown a dramatic increase in crocodile numbers in major rivers in Sarawak (Webb, Manolis, & Brien, 2010). As a result, Sarawak State has requested that the *C. porosus* population be transferred from Appendix I to Appendix II (CITES) and was successful in 2016. The goal is to benefit local communities socio-economically as well as sustainably manage Sarawak's wild crocodile population (Hassan *et al*., 2018).

The term "microsatellite" was first coined by Lit and Lutty, as reported in Pokhriyal, Thorat, and Dubey (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, Wu, Ji, Yan, & Amato, 2007). These markers have become the ones used in 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 isolated several crocodilian species, including *Crocodylus johnstoni, Crocodylus moreletii, Alligator mississipiensis, Caiman latirostris, Crocodylus porosus, Paleosuchus trigonatus* and *Alligator sinensis* (Oliveira, Farias, & Hrbek, 2010).

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 (Hekkala *et al*., 2015; Isberg *et al*., 2004; Lapbenjakul *et al*., 2017; Lewis *et al*., 2013; Mauger *et al*., 2017). At the same time, a sufficiently substantial collection of microsatellite primers for different species of crocodiles has been produced for research purposes and it has 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). A preliminary study by Kasim (2011) had used a single set of microsatellite markers on a small number of crocodile samples to investigate the relationship between crocodiles in Sarawak. 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 for findings that 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, concluding that microsatellite approach can be used to determine relationships among crocodiles in Sarawak. The above-mentioned studies used a small number of primers and limited samples.

Physical features such as mountains, large bodies of water, and deserts can prevent the movement of individuals and limit gene flow (Goudarzi *et al*., 2019). In Sarawak, the placement of dams, waterfalls, or rapids along a river can create barriers to dispersal. Microsatellite markers can be used to analyse the genetic profiles of crocodile populations that live upstream and downstream of the dam using DNA samples collected from those populations (Vashistha *et al*., 2020). Changes to the landscape caused by human activities such as deforestation, urbanization, or agriculture can fragment habitats and reduce gene flow (Zemanova *et al*., 2017). In Sarawak, the destruction of wetlands or the construction of roads or buildings can create isolated populations. Some crocodilians, like the saltwater crocodile (*Crocodylus porosus*), are known to migrate over considerable distances during certain seasons between freshwater and marine habitats. The gene flow between various groups could be improved by these migrations. Microsatellite data can show lower genetic divergence and a genetic connection across populations that are located along the same migration route (Fukuda & Saalfeld, 2014).

The study on the genetic relationship of crocodiles in Sarawak is needed for understanding their ecology, behavior, and population dynamics (Abdul Gani, 2019). Secondly, the study can help to identify the genetic diversity of crocodiles in Sarawak, which is crucial for developing conservation strategies and helps to identify patterns of gene flow and population structure, which can inform the management of crocodile populations in the region. Genetic diversity is important for the long-term survival of a species, as it allows adapting to changing environmental conditions, resisting diseases, and maintaining resilience in the face of various threats. In addition, the study can help to identify the different populations of crocodiles in the region, and their levels of gene flow (van Asch *et al*., 2019).

In this study 13 microsatellite markers were applied to assess genetic relationships and structure of crocodile populations from 13 river basins in Sarawak, hoping to shed

light on relationships among wild crocodile populations for future sustainable utilization of the reptile. The findings from this study also partly clarifies 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.

2. Materials and Methods

In total, 62 crocodiles were sampled from thirteen river basins in Sarawak (Table 1). Large adult crocodiles were captured by skilled and experienced individuals, properly restrained, and samples of scute or tissue were collected. As for hatchlings, they were captured using a scoop net and samples were obtained, preserved in 70% EtOH and brought back to laboratory for DNA analysis (Abdul Gani, 2014). All crocodiles were returned to their habitats after sample collection was completed. Total genomic DNA was extracted from crocodile samples (tissues and scutes) using the 2% CTAB protocol (Doyle & Doyle, 1987) and QIAamp DNA Investigator Kit (QIAGEN, Germany) following manufacturer's protocol, then subjected to 1% Agarose Gel Electrophoresis (AGE). Standard Polymerase Chain Reaction involved the 13 sets of microsatellite loci following Isberg *et al*. (2004), using a thermocycler (MyCyclerTM) with one negative control for every batch. The thermal profile used was as follows: initial denaturation at 94°C for 15 seconds, followed by 35 cycles of annealing at 54.6°C for 30 seconds, extension at 72°C for 50 seconds, and then a final 4-minute extension step at 72°C.

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. All positive bands were observed, their sizes estimated, and scored using binary system accordingly. A dendrogram was created based on Unweighted Pair Group with Arithmetic Averages (UPGMA) method after statistical analysis of 13 sets microsatellite marker data using the NTSYSpc.2.02e program (Jamshidi, 2011).

3. Results

In this study, PCR amplification was successful for 60 (out of 62) crocodile samples. PCR amplification of seven markers (Cj127, Cj131, Cj122, Cj101, Cj119, CUD68, and Cj16) showed distinct single bands (except for sample M2), while one marker (Cj35) exhibited double bands. Multiple bands were visible for five markers (Cj105, Cj18, Cj104, Cp10, and Cu4-121).

The unweighted pair group with arithmetic mean (UPGMA) clustering algorithm, with coefficients ranging between 0.51 and 1.00, produced a microsatellite-based unrooted phylogenetic tree (Figure 1). The dendrogram is divided into 6 clades, ranging from A to F.

Clade A includes samples from the following locations: Samarahan RB (SN1; SN2; SN7), Sungai Sarawak RB (SR1; SR2; SR3; SR4; SR5), Saribas RB (SS1, SS2), Tatau RB (TA1), Oya RB (OY1, OY3), Rajang RB (RJ1; RJ2; RJ5; RJ6), Sadong RB (SG1) and Kemena RB (KM5). The samples in clade A had the most representatives from Sungai Sarawak RB with total of 5 samples, followed by Rajang RB (4 samples), Samarahan RB (3 samples), Saribas RB; Oya RB (2 samples) and a sample each from Tatau RB, Sadong RB, and Kemena RB. The samples are closely related based on Sarawak RB map in Figure 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.

Table 1. Location of crocodile samples and voucher number used in this study.

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

Next, clade B includes samples from western, central, and northern regions of Sarawak RB. The samples are from Rajang RB, Samarahan RB, Suai RB, Miri RB, Saribas RB, Sungai Sarawak RB, Niah RB, Tatau RB and Kemena RB. The samples shown in clade B had the largest count from Suai RB with a total of nine samples (SU1; SU2; SU3; SU4; SU5; SU6; SU7; SU8; SU9) followed by Samarahan RB with a total of six samples (SN3; SN4, SN5, SN6, SN8, SN9). A total of four out of five samples from Kemena RB were found in clade B (KM1, KM2, KM3, KM4). 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). Clade C consists of only 2 samples which is from Rajang RB (central region) and Sungai Sarawak RB (western region).

In addition, clade D consists of all seven samples from Miri district, only from the northern region, while Clade E comprises four samples from Sungai Sarawak RB (3 samples) and Samarahan RB (one sample) which are closely

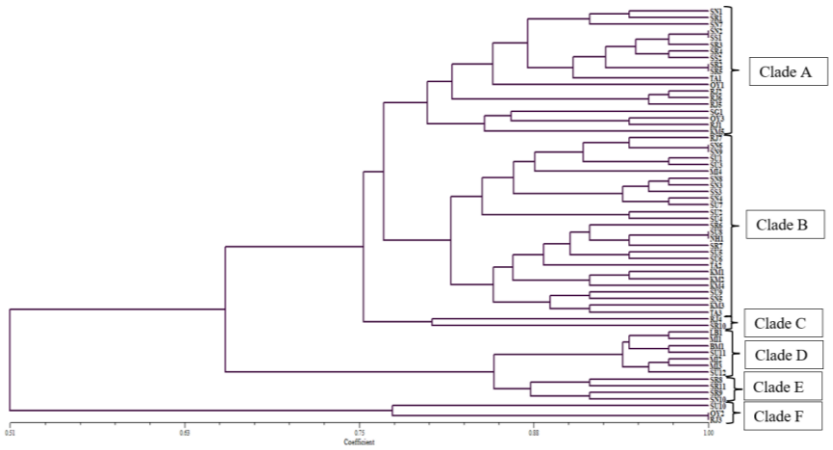

Figure1. Microsatellite-based unrooted phylogenetic tree for 60 Saltwater crocodiles, generated by the unweighted pair group with arithmetic mean (UPGMA) clustering method.

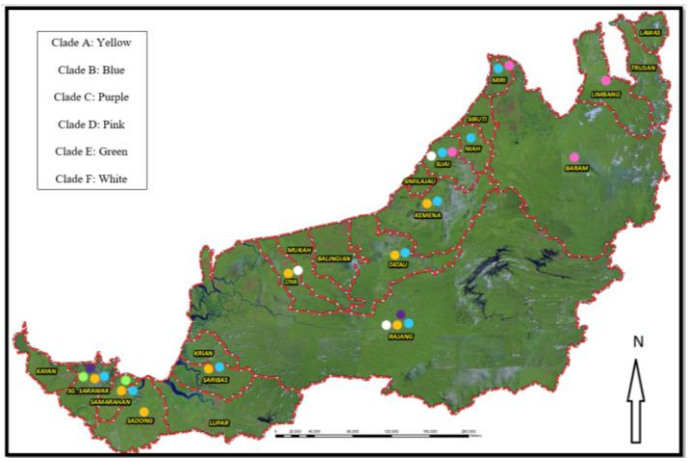

Figure 2. Map of Sarawak River Basins (SRBs) showing the clades at each site (Map adapted from: Sarawak Drainage)

related and located in the western region. The samples in clade D are from Limbang RB (one sample), Miri RB (3 samples), Baram RB (one sample), and Suai RB (2 samples). Furthermore, clade F consists of 3 samples from Suai RB (SU10), Oya RB (OY2), and Rajang RB (RJ3).

4. Discussion

The CTAB procedure (Doyle & Doyle, 1987) and QIAamp DNA Investigator Kit (QIAGEN, Germany) were successful in extraction of whole genomic DNA from crocodiles. In other investigations researchers have 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), or the DNeasy blood and tissues kit (Rossi *et al*., 2020; Russello, Brazaitis, Gratten, Watkins-Colwell, & Caccone, 2007). Techniques to extract DNA have evolved over the years, which allows researchers to choose a much safer and faster as well as low cost method for each project.

The clade F samples (SU10, OY2 and RJ3) were extracted using QIAamp DNA investigator kit (Qiagen, Germany) 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, Rahman, Mirza, & Zafar (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.

Besides that, the crocodile samples in clade A from the western and central region of Sarawak RB came from eight different RB 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 exceed 800 km. Campbell, Dwyer, Irwin, & Franklin (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,

Table 2. List of band scoring for each sample across 13 sets of microsatellite loci

	List of primers																																		
	A-1	B-1	C-1	D-1	E-1	F-1	G-1	G-2	G-3	H-1	I-1	I-2	I-3	J-1	J-2	J-3	J-4	K-1	K-2	K-3	K-4	K-5	K-6	K-7	K-8	K-9	K-10	K-11	L-1	L-2	M-1	M-2	M-3	M-4	M-5	M-6
SN1	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	1	0	0	1	0	1	1	0	0	1
SN2	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	1	0	0	1
SN3	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	1	0	0	1	0	0	0	0	0	1
SN4	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
SN5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	1
SN6	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
SN7	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	1	1	0	1	1	1	0	1
SN8	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	1
SN9	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
SN10	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
SR1	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	1	0	0	1
SR2	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
SR3	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	0	1	0	0	1
SR4	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
SR5	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
SR6	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1
SR7	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	1	0	0	1	0	0	0	0	0	1
SR8	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	0	0	1	0	0	1
SR9	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	0	0	1	0	0	1
SR10	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	1
SR11	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	0	0	1	0	0	1
SS1	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	1	0	0	1
SS2	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
SS3	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	0	0	0	0	0	1
SG1	1	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	1	0	0	1	0	1	1	1	1	1
OY1	1	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
OY2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OY3	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	1	0	0	1	1	1	1	1	1	1
RJ1	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	0	0	0	0	1	1	1	1	1	1	1
RJ2	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1
RJ3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RJ4	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	1
RJ5	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1
RJ6	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1
RJ7	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	1	0	0	0	1	0	0	1	0	0	1
KM1	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
KM2	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1
KM3	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1
KM4	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	1
KM5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1
TA1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1
TA2	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
TA3	1	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
SU1	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
SU2	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	1	0	1	0	1	1	1	1	1	0	0	0	0	0	0	1
SU3	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1
SU4	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0	1
SU5	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1
SU6	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	1	1	1	1	0	0	0	0	0	0	1
SU7	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1
SU8	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
SU9	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	1
SU10	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
SU11	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	0	1	1	1	0	0	0	0	0	1
SU12	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1
NH1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1
LB1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	1
BM1	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	0	1	1	1	0	0	0	0	0	1
MI1	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	0	0	0	0	0	1
MI2	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	0	0	0	0	0	1
MI3	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	0	0	0	0	0	1
MI4	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1

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

suggesting that regular migrations occur among the *C. porosus* in Sarawak. Similarly, clade B contains crocodiles from 9 different river basins that were from five districts in Sarawak which are geographically connected.

Clade C is fully resolved, with all samples originated from Miri district, which consists of four river basins that share the same geographical area. Interestingly, clade D also entirely stemmed from Samarahan RB and Sungai Sarawak RB. Clade C comprised samples from Miri and clade D cases are from Kuching and Samarahan (these two areas are adjacent to each other). These results indicate that microsatellite data could be used to identify individual crocodiles from different river basins, similar to findings by Abdul Gani (2019).

Clade E grouped samples that could not give any positive PCR products, thus these samples need further investigation. Palumbi *et al.* (2001) reported that there are many factors that could influence the success of PCR, therefore systematic optimization of PCR conditions should be carried out accordingly.

The genetic linkages between individuals and the population genetic structure across several river basins (RBs) 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 distant from each other may be genetically similar if there is a history of gene flow between the populations (Pacheco-Sierra *et al*., 2016).

5. Conclusions

DNA microsatellite analysis divided the crocodiles into five clades (concordant with their geographical locations): A, B, C, D, E and F; whereas one clade remained unresolved. 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 from different basins or clades to minimize the bias that occurred from inferring the non-specific PCR products as microsatellite products so that more rigorous data analysis can be done in the future. In future 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 Fis (inbreeding coefficient), and inferring the recent migration rate (estimated using BayesAss with microsatellite genotypes and Bayesian clustering).

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References

Abdul Gani, M. I. Z. (2014). Population density, human-crocodile conflict and genetic variation among saltwater crocodile, *Crocodylus porosus* in Sarawak (Master's thesis, Universiti Malaysia Sarawak, Sarawak, Malaysia).

Abdul Gani, M. I. Z. (2019). Historical information, population density and distribution, human crocodile conflict, crocodile habitat, selected water parameters, genetic variations among saltwater crocodile, *Crocodylus porosus* in Sarawak. (Doctoral dissertation, Universiti Malaysia Sarawak, Sarawak, Malaysia).

Abdul Gani, M. I. Z., Hassan, R., Tisen, O. B., & Ahmad, R. (2022). Human-crocodile conflicts in Sarawak, Malaysian Borneo: An analysis of crocodile attacks from 2000 until 2020. *International Journal of Biology and Biomedical Engineering*, *16*. Retrieved from https://doi.org/10.46300/91011.2022.16.25

Asif, M., Rahman, M. U., Mirza, J. I., & Zafar, Y. (2008). High resolution MetaPhor agarose gel electrophoresis for genotyping with microsatellite markers. *Pakistan Journal for Agriculture Science, 45*(1), 75-79

Bashyal, A., Gross, B., Venegas-Anaya, M., Lowrance, F., & Densmore, L. (2014). Assessment of microsatellites in estimating inter-and intraspecific variation among neotropical crocodylus species. *Genetics and Molecular Research*, *13*(3), 5492-5502.

Bennett, P. (2000). Microsatellites. *Journal of Clinical Pathology - Molecular Pathology*. London, England: BMJ Publishing Group.

Campbell, H. A., Dwyer, R. G., Irwin, T. R., & Franklin, C. A. (2013). Home range utilisation and long-range movement of estuarine crocodiles during the breeding and nesting season. *PloS One*, *8*(5), e62127.

Cox, J. H., & Gombek, F. (1985). *A preliminary survey of the crocodile resources in Sarawak, East Malaysia*. Kuala Lumpur: Malaysia: World Wildlife Fund (WWF) Malaysia.

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh tissue. *Phytochemical Bulletin*, *19*, 11–15.

Ellegren, H. (2004). Microsatellites: Simple sequences with complex evolution. *Nature Reviews Genetics, 5*, 435-445.

Fitzsimmons, N. N., Tanksley, S., Forstner, M. R. J., Louis, E. E., Daglish, R., Gratten, J., & Davis, S. (2000). Microsatellites markers from crocodylus: New genetic tools from populations genetics, maintain system studies and forensics. *Crocodilian Biology and Evolution*, 51–57.

Fukuda, Y., & Saalfeld, K. (2014). Abundance of saltwater crocodile hatchlings is related to rainfall in the preceding wet season in Northern Australia. *Herpetologica*, *70*(4), 439–448. Retrieved from https://doi.org/10.1655/herpetologica-d-13-00096r3

Gratten, J. (2003). The molecular systematics, phylo geography and population genetics of Indo Pacific crocodylus (Doctoral Dissertation, University of Queensland, Australia).

Goudarzi, F., Hemami, M.-R., Rancilhac, L., Malekian, M., Fakheran, S., Elmer, K. R., & amp; Steinfartz, S. (2019). Geographic separation and genetic differentiation of populations are not coupled with niche differentiation in threatened kaiser's spotted newt (Neurergus Kaiseri). *Scientific Reports*, *9*(1). Retrieved from https://doi.org/10.1038/s41598-019-41886-8

Hassan, R., Ahmad, R., Md Adzhar, M. A. A., Abdul-Gani, M. I. Z., Ayob, A., & Zainudin, R. (2016). Notes on the wild tomistoma populations in western Sarawak, Malaysian Borneo. *International Journal of Ecology, 2016*, 1-7.

Hassan, R., Md Adzhar, M. A. A., Abdul-Gani, M. I. Z., & Ahmad, R. (2018). Assessment of wild saltwater crocodile population in Bako River, western Sarawak, Malaysian Borneo for potential ecotourism industry. *Malaysian Applied Biology, 47*(1), 131-138.

Hassan, R., Mohd Azizi, N. F., Md Adzhar, M. A. A., Abdul Gani, M. I. Z., Ahmad, R., & Moi Ung, C. L. (2018). A taphonomic study of *Crocodylus porosus* (crocodylidae) and *Tomistoma schlegelii* (gavialidae) remains from western Sarawak, Malaysian Borneo: Applications for public education. *Trends in Undergraduate Research*, *1*(1). Retrieved from https://doi.org/10.33736/tur.1139.2018

Hekkala, E. R., Amato, G., DeSalle, R., & Blum, M. J. (2010). Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations. *Conservation Genetics*, *11*(4). Retrieved from https://doi.org/10.1007/s10592-009-9970-5.

Isberg, S. R., Chen, Y., Barker, S. G., & Moran, C. (2004). Analysis of microsatellites and parentage testing in saltwater crocodiles. *Journal of Heredity*, *95*(5). Retrieved from https://doi.org/10.1093/jhered/esh067

Jamshidi, S. S. (2011). NTSYSpc 2.02e implementation in molecular biodata analysis (clustering, screening, and individual selection). *International Conference on Environmental and Computer Science*, *19*.

Kasim, N. Y. (2011). Genetic diversity of saltwater crocodiles (*Crocodylus porosus*) from Sarawak using microsatellite approach (Project Report, Universiti Malaysia Sarawak, Sarawak, Malaysia).

Lapbenjakul, S., Thapana, W., Twilprawat, P., Muangmai, N., Kanchanaketu, T., Temsiripong, Y., & Srikulnath, K. (2017). High genetic diversity and demographic history of captive Siamese and saltwater crocodiles suggest the first step toward the establishment of a breeding and reintroduction program in thailand. *PloS One*, *12*(9), 1-21.

Lewis, J. L., FitzSimmons, N. N., Jamerlan, M. L., Buchan, J. C., & Grigg, G. C. (2013). Mating systems and multiple paternity in the estuarine crocodile (*Crocodylus porosus*). *Journal of Herpetology, 47*(1), 24-33.

Li, Y., Wu, X., Ji, X., Yan, P., & Amato, G. (2007). The complete mitochondrial genome of salt-water crocodile (*Crocodylus porosus*) and phylogeny of crocodilians. *Journal of Genetics and Genomics*, *34*(2). Retrieved from https://doi.org/10.1016/S1673-8527(07)60013-7

Martin, S. (2008). Global diversity of crocodiles (crocodilia, reptilia) in freshwater. *Hydrobiologia, 595*(1), 587-591.

Mauger, L. A., Velez, E., Cherkiss, M. S., Brien, M. L., Mazzotti, F. J., & Spotila, J. R. (2017). Conservation genetics of American crocodile, *Crocodylus acutus*, populations in Pacific Costa Rica. *Nature Conservation*, *17*. Retrieved from https://doi.org/10.3897/natureconservation.17.9714.

Miles, L. G., Isberg, S. R., Moran, C., Hagen, C., & Glenn, T. C. (2009). 253 Novel polymorphic microsatellites for the saltwater crocodile (Crocodylus porosus). *Conservation Genetics*, *10*(4). Retrieved from https://doi.org/10.1007/s10592-008-9600-7

Oliveira, D.P., Farias, I.P., & Hrbek, T., 2010. Microsatellite markers for mating system and population analyses of the spectacled caiman crocodilus (Linnaeus 1758). *Conservation Genetics Resources*, *2*, 181–184. doi:10.1007/s12686-010-9221-6

Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grabowski, G., University of Hawaii at Manoa, Kewalo Marine Laboratory. (2002). *The simple fool's guide to PCR*. Honolulu, HI: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

Pokhriyal, B, Thorat, K, & Dubey, R., 2012. Microsatellite markers – a novel tool in molecular genetics. *International Journal of Research in Pharmacy and Chemistry, 2*, 397–412.

Rossi, N. A., Menchaca-Rodriguez, A., Antelo, R., Wilson, B., McLaren, K., Mazzotti, F., . . . Amato, G. (2020). High levels of population genetic differentiation in the American crocodile (Crocodylus acutus). *PloS One*, *15*(7). Retrieved from https://doi.org/10.1371/journal.pone.0235288

Russello, M. A., Brazaitis, P., Gratten, J., Watkins-Colwell, G. J., & Caccone, A. (2007). Molecular assessment of the genetic integrity, distinctiveness and phylo geographic context of the Saltwater crocodile (Crocodylus porosus) on Palau. *Conservation Genetics*, *8*(4). Retrieved from https://doi.org/10.1007/s10592-006-9225-7

Shafiei-Astani, B., Ong, A. H. K., Valdiani, A., Tan, S. G., Yien, C. Y. S., Ahmady, F., Kuar, T. (2015). Molecular genetic variation and structure of southeast Asian crocodile (*Tomistoma schlegelii*): Comparative potentials of SSRs versus ISSRs. *Gene, 571*(1), 107-116.

Tisen, O., & Ahmad, R. (2010). *Crocodylus porosus in Sarawak*: *Status and Management*. Paper presented at the IUCN-SSC crocodile specialist group (CSG) Workshop on Human crocodile conflict, Sabah, Malaysia.

van Asch, B., Versfeld, W. F., Hull, K. L., Leslie, A. J., Matheus, T. I., Beytell, P. C., . . . Rhode, C. (2019). Phylogeography, genetic diversity, and population structure of Nile crocodile populations at the fringes of the southern African distribution. *PloS One*, *14*(12). Retrieved from https://doi.org/10.1371/journal.pone.0226505

Vashistha, G., Deepika, S., Dhakate, P. M., Khudsar, F. A., & Kothamasi, D. (2020). The effectiveness of microsatellite DNA as a genetic tool in crocodilian conservation. *Conservation Genetics Resources*, *12*(4), 733–744. Retrieved from https://doi.org/10.1007/s12686-020-01164-6

Webb, G. J. W., Manolis, C., & Brien, M. L. (2010). Saltwater crocodile, *Crocodylus porosus*. In C. Manolis & C. Stevenson (Eds.), *Crocodile*. *status survey and conservation action plan* (3rd ed., pp. 99-113). Darwin: Australia: Crocodile Specialist Group.

Zemanova, M. A., Perotto-Baldivieso, H. L., Dickins, E. L., Gill, A. B., Leonard, J. P., & amp; Wester, D. B. (2017). Impact of deforestation on habitat connectivity thresholds for large carnivores in tropical forests. *Ecological Processes*, *6*(1). Retrieved from https://doi.org/10.1186/s13717-017-0089-1