Research Article

Relationships among *Tomistoma schlegelii* in Malaysia Based on Cyt *b*-Control Region Gene Analysis

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Tomistoma schlegelii is a slender snout crocodile, secretive in nature which is currently under Appendix I of Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Limited information is currently available on its wild population, especially in Malaysia. Thus this study aims to describe genetic relationship of *T. schlegelii* populations from Malaysia which was done using partial sequencing of Cytochrome *b*-control region mtDNA gene. The study reveals that the genetic diversity among *T. schlegelii* is high, ranging from 0.16% to 3.34%, suggesting healthy populations. Analysis showed that there is gene flow among populations ($D_a = 1.71\%$ to 2.21%) within Western Sarawak, Peninsular Malaysia, and other geographical regions coherent with Sundaland theory, suggesting that there is ancient river system connecting the two regions of Peninsular Malaysia and West Borneo when the Sunda Shelf was exposed. Unique haplotypes had been observed in Northern Sarawak (SAM01 and SAM02) as well as in Sumatera; thus each *T. schlegelii* deserved its own management strategies to ensure the survival of the species.

1. Introduction

Tomistoma, *Tomistoma schlegelii*, is a vulnerable slender snout crocodile, which is currently under Appendix I of Convention on International Trade in Endangered Species of Wild Flora and Fauna CITES (IUCN 2014) with an estimated population of less than 2500 individuals left in the wild [1]. The population is endemic to Malaysia and Indonesia [1, 2]. Reports on *T. schlegelii* sightings in Kalimantan and Sumatera from the 1990s to 2000s were published by [2–4] while in Malaysia sightings have been rare since the late 1980s [5–8].

The main threat of *T. schlegelii* is habitat loss due to deforestation, illegal logging, palm oil plantation, urbanization, fishing activities, and forest fires at peat swamps [2–4]. Currently, wild specimens could be found in captivity in zoos and farms and by individuals who acquired *T. schlegelii* illegally. Zoos and farms in Malaysia have approximately 77 specimens [9] while Singapore, Thailand, and Indonesia have collectively 88 specimens. Zoos and farms in Europe and the United States of America hold approximately 57 specimens. In Malaysia and Indonesia, study of *T. schlegelii* concentrated

on surveys of *T. schlegelii* natural habitats and related issues involving conservation.

Genetic information on *T. schlegelii* is available; for example, [10–12] had sequencing Cyt *b* from *T. schlegelii* as part of the classification of Family Crocodylia. Besides that, [13] reported based on Cyt *b* gene that *T. schlegelii* haplotype in Peninsular Malaysia is unique. Thus, the aim of this study is to infer genetic relationship among *T. schlegelii* from captive and wild specimens using partial DNA sequencing of the mtDNA control region (Cyt *b*-CR) gene. The findings are useful for further support of conservation of *T. schlegelii* in Malaysia and other geographical regions.

2. Materials and Methods

A total 12 *T. schlegelii* samples are obtained from 4 localities (Figure 1), from Miri Crocodile Farm ($n = 5$), from Crocodile Adventureland Langkawi (CAL) ($n = 5$), Samarahan ($n = 1$), and Serian ($n = 1$). Other data were obtained from GenBank (Table 1).

FIGURE 1: Locations of *T. schlegelii* populations involved in this study.TABLE 1: Details of Cyt *b*-CR gene sequences obtained from GenBank involved in this study.

Samples	GenBank accession number	Locality	Collection site
SAR003–SAR005	HM593977–HM593980	Sarawak	MWC
SAR021–SAR023	HM593996–HM593998	Sarawak	Miri
PM006–PM009	HM593981–HM593984,	Peninsular Malaysia	ZN
PM029–PM030	HM593999–HM594000		
PM010–PM013	HM593985–HM593988		
PM014–PM016	HM593989–HM593991	Peninsular Malaysia	MZ
PM017–PM020	HM593992–HM593995	Peninsular Malaysia	TZ
SZ01–SZ010	HM594005–HM594014	Peninsular Malaysia	MZT
HK1–HK4	HM594001–HM594004	Unknown	SZ
SUM36–SUM38	HM594015–HM594017	Unknown	HKW Park
KAL39–KAL43	HM594018–HM594022	Sumatera	PTE
J045–J054	HM594023–HM594032	Kalimantan	CVS
		Java	CWRC

MWC: Matang Wildlife Centre; ZN: Zoo Negara; MZ: Melaka Zoo; MZT: Mini Zoo Temerloh; TZ: Taiping Zoo; SZ: Singapore Zoo; HKW: Hong Kong Wetland; PTES: PT Eka Nindya, Sumatera; CVS: CV Surya Raya Farm, EK; CWRC: Cikananga WRC, Java.

Total genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method [14] with the presence of the proteinase K. The amplification of mtDNA control region (Cyt *b*-CR) gene fragment was conducted using oligonucleotide primers L14930 (5'-AGC GGG CAA AAT AGA AAA CTGA-3', forward) and H15630 (5'-ATA GAG ATG CCG GGA TTA CGAA -3', reverse) [13]. PCR followed [15] using programmable gradient-enabled thermocycler (Bio-Rad MyCycler™ Thermal Cycler).

Approximately, 50–100 ng of the template DNA was amplified in a 25 μ l reaction mixture containing 50 mM 10x buffer, 2 mM MgCl₂, 0.4 mM of dNTPs (Promega), 0.2 mM of each primer, and 0.5 U of Taq DNA polymerase (Promega). The cycle parameters involved predenaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 60 s with the final extension step at 72°C for 5 min. All PCR products were subjected to gel electrophoresis using 2% agarose gel stained with two drops of ethidium bromide with TAE buffer for 100 to 120 minutes at 80 V with 100 bp DNA ladder

used to estimate the size of the bands. The gel was visualized under the UV light and photographed using UV camera. The PCR products were directly sent to First Base Sdn Bhd to be sequenced.

Multiple alignments of the Cyt *b*-CR gene sequences were constructed using the CLUSTAL X program v2.0 [16]; the stop codon sequence was removed and subsequently aligned by eye. The phylogenetic tree was constructed using the MEGA 4.0 software [17], besides PAUP version 4.0b [18] and Mr. Bayes version 3.1.2 [19]. This software was also used to calculate genetic divergence values based on Kimura 2 parameter approach. The measurement of population genetic parameters such as genetic diversity (the probability that two randomly chosen mtDNA sequences differed in the sample) and nucleotide diversity (per nucleotide site, i.e., the probability that two randomly chosen homologous nucleotides differ in the sample) [20] was estimated from the mtDNA dataset using DNASP 4.0 [21]. To be noted, the samples from GenBank abbreviations followed the voucher numbers stated in [13].

TABLE 2: Summary of genetic distance in percentage (%) for mtDNA Cyt b-CR gene sequences of *T. schlegelii* based on the locality in Malaysia.

	Samarahan	Serian	Miri	Langkawi
Samarahan	—			
Serian	0.31	—		
Miri	2.21–2.53	0.16–1.25	—	
Langkawi	2.05–2.37	2.53–3.34	0.31–0.62	—

TABLE 3: Analyses of mtDNA Cyt b-CR gene sequences among *T. schlegelii*.

Species	Haplotype	Locality	Samples
<i>T. schlegelii</i>	Hap_1	Sarawak, Peninsular Malaysia, unknown, Sumatera	SZG9 SZG2 J052 J053 J049 J054 J048 HK2 HK1 SZG6 SZG3 SAR003 SAR002 SAR022 PM016 PM019 PM020 SUM037 SUM038 SUM036 PM010 SZG4 SAR023 PM015 PM009 SAR021 SAR005 SAR004 SZG1 HK3 SZG10 HK4 SZG5 J045 SZG7 PM008 SZG8 PM007 J047 PM012 PM014 PM017 PM018
<i>T. schlegelii</i>	Hap_2	Langkawi (in this study) and Peninsular Malaysia	LTS02 LTS05 LTS01 PM006 PM011 PM013 LTS03 LTS04
<i>T. schlegelii</i>	Hap_3	Sarawak (in this study)	MTS01 MTS05 MTS04 MTS02 MTS03
<i>T. schlegelii</i>	Hap_4	Sarawak (in this study)	SAM01
<i>T. schlegelii</i>	Hap_5	Kalimantan and unknown	J046 J050 KAL041 J051 KAL040 KAL039 KAL043 KAL044
<i>T. schlegelii</i>	Hap_6	Peninsular Malaysia	PM29 PM30
<i>T. schlegelii</i>	Hap_7	Sarawak (in this study)	SAM02

3. Results and Discussion

A total 700 bp of mtDNA control region (Cyt *b*-CR) gene of *T. schlegelii* had been successfully amplified using L14930 primers for all specimens. The sequence matched *T. schlegelii* with accession number HM593977 [13].

The overall frequency distributions of nucleotides at the 1st, 2nd, and 3rd codon position were as follows: T = 29.5%, C = 28.6%, A = 29.9%, and G = 12.0%; T = 26.0, C = 33.6%, A = 29.1%, and G = 11.2%; T = 30.0%, C = 26.4%, A = 32.6%, and G = 11.3, respectively. A compositional nucleotide bias analysis revealed no significant bias ($p = 1.29$) across the *T. schlegelii* sequence data. Among the 700 bp of the partial mtDNA control region (Cyt *b*-CR) gene, only 32 (4.5%) sites were variables with 6 singleton sites, leaving 26 (3.7%) potentially parsimoniously informative characters, indicating that this gene is a reliable marker to infer genetic variations at the population level and 668 (95.5%) sites were conserved. The variable site had also shown that the transition (16 sites) occurred more than transversion (10 sites). Similarly, reports claimed that transition occurs more than transversion in animal mitochondrial genomes [22].

The genetic distance values among individuals were 0.16% to 3.34% (Table 2). The highest genetic distance values had been recorded between sample Langkawi and Serian localities probably because of geographical distance, while samples from Miri and Langkawi have the lowest genetic distance

which is 0.16% perhaps due to the same gene pool. The range value was large due to Cyt *b*-CR region having a high rate of evolution in mtDNA [13].

The analysis also revealed that high genetic distance values were recorded between *T. schlegelii* populations in which the highest variation of 3.34% was recorded in Langkawi specimen (Table 2). Langkawi population also shows the high genetic distances when compared to Samarahan population as 2.05% to 2.37% genetic distances were recorded among *T. schlegelii*. The result was different with the findings of [13] which revealed a low level of inter- and intrapopulation genetic distances of 0.08% to 0.18%, respectively. According to [23], the high genetic distance could be explained by several factors including small population size, pass bottleneck event, and physical barriers among population. The genetic variation and structure also increased due to geographical distance [24]. Hence, the population genetic analysis is conducted to further understand the genetic structure of *T. schlegelii* from Malaysia and other regions.

Among the 68 individuals sequenced (12 from this study and 56 from GenBank) 7 haplotypes were identified in total with 3 haplotypes being shared among them as shown in Table 3, namely, H1, H2, and H5. The sharing of haplotype indicates there is gene flow among them. Moreover, nucleotide divergence analysis revealed that high nucleotide diversity (π) ranges from 0.3% to 0.9% within *T. schlegelii* population (Table 4). Among populations, high nucleotide

TABLE 4: Measures of haplotypes and nucleotide diversity within populations of *T. schlegelii* analyzed by location.

Locality	H	Percent (%) pairwise divergence	Gene diversity	Nucleotide diversity
Samarahan	1	0.0	0.01 ± 0.13	0.003 ± 0.03
Serian	1	0.0	0.03 ± 0.23	0.002 ± 0.03
Miri	1	0.4–0.9	1.00 ± 0.18	0.009 ± 0.01
Langkawi	1	0.4	1.00 ± 0.50	0.004 ± 0.01

TABLE 5: Measures of nucleotide diversity (π) and net nucleotide divergence (Da) among populations of *T. schlegelii* analyzed by location.

Locality	Distance (km)	Nucleotide diversity (π)	Net nucleotide divergence (Da)
Samarahan/Miri	800	0.0156	0.0178
Samarahan/Langkawi	1200	0.0031	0.0171
Samarahan/Serian	40	0.0141	0.0103
Miri/Langkawi	1400	0.0191	0.0221
Miri/Serian	760	0.0022	0.0212
Langkawi/Serian	1200	0.0032	0.0220

diversity (π) was observed which is from 0.3% to 1.9% and 1.7% to 2.0% net nucleotide divergences (Da) (Table 5). The net nucleotide divergence (Da) (Table 5) values were higher when comparing Samarahan with Miri (1.78%) than Samarahan with Langkawi (1.71%) because Samarahan and Langkawi are separated by more than 1200 km distance. It could be due to more genetic drift in Miri population compared to Langkawi from Samarahan specimen. The highest net nucleotide divergence (Da) (Table 5) can be observed among Miri and Langkawi (2.21%) suggesting high genetic differentiation among the population being separated with about 1400 km from each other.

Phylogenetic analyses of *T. schlegelii* from Sarawak produced same tree topologies for Neighbour-Joining (NJ) (not shown), Maximum Likelihood (ML) (not shown), maximum parsimony (MP) (Figure 2), and Bayesian inference tree (not shown). They revealed a monophyly *T. schlegelii* with respect to outgroups *G. gangeticus*, *C. johnsoni*, and *C. palustris* with the bootstrap value of 98% (MP), 99% (NJ), 100% (ML), and 1.0 Bayesian Posterior Probability. The monophyly status of *T. schlegelii* with respect to outgroup was congruent with [13].

There is only single clade in the phylogenetic tree (Figure 2) with 4 subclades: *T. schlegelii* from wild population in this study, namely, SAM01 and SAM02, in the same subclade with all 5 semiwild *T. schlegelii* from Miri, namely, MTS01, MTS02, MTS03, MTS04, and MTS05 (Subclade 1), with 98% (MP), 99% (NJ), 100% (ML), and 1.0 BPP.

Five *T. schlegelii* from Langkawi (LT01, LT02, LT03, LT04, and LT05) are closely related to three samples from Peninsular Malaysia (PM06 from Zoo Negara and two other samples, namely, PM011 and PM013, from Melaka Zoo whose locality is in Selangor) in Subclade II with bootstrap value of 99% (MP), 99% (NJ), 100% (ML), and 0.9 BPP. The owner of CAL reported that all *T. schlegelii* were bought from Singapore in 1980 with unknown locality. This however cannot determine whether the origin of *T. schlegelii* in Langkawi is Selangor or

other places although all *T. schlegelii* as mentioned are placed in Subclade II. Subclade III comprises specimens from Kalimantan, Peninsular, and Java while Subclade IV comprises all other specimens which form largest subclade. However, with low bootstrap value (50% (MP); 62% (NJ); 54% (ML), and 0.5 BPP) in between Subclade 4 and Subclade 1, the tree is not resolved. More research needs to be done so the information is helpful for the farm in terms of historical information and could be raised during education briefing to the tourists.

4. Conclusion

In conclusion, genetic diversity among *T. schlegelii* is high, ranging from 0.16% to 3.34%, suggesting healthy populations. Analysis showed that there is gene flow among populations (Da = 1.71% to 2.21%) within Western Sarawak, Peninsular Malaysia, and other geographical regions coherent with Sundaland theory, suggesting that there is ancient river system connecting the two regions of Peninsular Malaysia and West Borneo when the Sunda Shelf was exposed. Unique haplotypes had been observed in Northern Sarawak as well as in Sumatera; thus each *T. schlegelii* deserved its own management strategies to ensure the survival of the species.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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FIGURE 2: Maximum parsimony of the 50% majority rule consensus tree of mtDNA Cyt b-CR gene sequence of *T. schlegelii*. Bootstrap values and Bayesian posterior probabilities (BPP) are accordingly indicated below the branch nodes.

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