UNIVERSITI PUTRA MALAYSIA

GENETIC VARIATION OF SOUTHEAST ASIAN CROCODILE, TOMISTOMA SCHLEGELII MULLER, USING MICROSATELLITE AND INTER SIMPLE SEQUENCE REPEAT MARKERS

BEHNAM SHAFIEI ASTANI

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By

BEHNAM SHAFIEI ASTANI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the requirement for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to
My Beloved Parents,
My wife
And
My True Friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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Chairman: Professor Soon Guan Tan, PhD
Faculty: Biotechnology and Biomolecular Sciences

Tomistoma schlegelii, also referred to as the "false gharial", is one of the most exclusive and least known of the world's fresh water crocodilians, limited to Indonesia and Malaysia. The species has been recognized for over a century from a museum specimen, and its distribution has been a mystery for a long time. Its lack of economic value and local cultural significance (the skin is not traded locally) has also contributed to the lack of studies on the false gharial.

Tomistoma schlegelii within the Southeast Asia region has been found to suffer from population reduction. This species has to live in small environments, because of the current habitat destruction, hence leading to a steady reduction of species number and, this can be as a reason for an increase in the rate of inbreeding. Nevertheless, lack of information on the genetic variability and structure of the species in Malaysia remains a matter of concern. Hence, the objectives of this study were to determine the current genetic diversity and structure of this endangered species in Malaysia. To achieve this, two types of molecular markers, namely microsatellites (40 primer pairs) and Inter Simple Sequence Repeats (ISSRs, 45 primers) were used.

The Department of Wildlife and National Parks (DWNP), Malaysia provided the samples. In this regard, 17 *T. schlegelii* specimens were collected by the department from different parts of Peninsular Malaysia and Sarawak, and were then transferred to the farms and zoos across the country. The mentioned crocodiles were later subjected to the blood sampling process. Consequently, DNA was isolated from the blood samples of *T. schlegelii* by using the conventional phenol-chloroform method. The PCR amplification of *T. schlegelii* DNA was optimized to obtain the most effective annealing temperature and Mg^{2+} concentration for each individual marker.

Out of the 40 microsatellite primer pairs tested 10 were detected as amplifying polymorphic bands, while 36 ISSR primers were detected as producing polymorphic banding patterns from the 45 tested. The observed heterozygosity (H_O) and the expected heterozygosity values (H_E) of the polymorphic microsatellite loci ranged from 0.588 to 1.000 and 0.470 to 0.891, respectively. The highest average

heterozygosity was observed for locus Cj109 with a value of 0.945 and the lowest average heterozygosity was observed for locus Cs15 with value of 0.529. Among the 45 ISSR markers, 36 showed polymorphic banding patterns (80%). The highest number (6) of polymorphic bands was produced by primer UBC811 and the lowest (1 each) by primers PT2, UBC820, UBC834 and UBC868.

Interestingly, data from both the microsatellite and ISSR marker systems generated two main clusters. The highest repeat motif similarities to the original species from which the loci were developed for three microsatellite loci, namely Cj16, Amiμ16 and Amiμ15, which originated from *C. johnstoni, A. mississippiensis* and *C. siamensis* respectively. The repeat motifs of the Cj16 locus in *T. schlegelii* were found to be a high match with the repeat motif of the same locus in *C. johnstoni*, and the difference was only limited to one repeat unit of "CA". The observed similarity of the repeat motif type of Amiμ16 and Amiμ15 was "AC" and the sizes of the amplified loci in the present study were shorter than the ones reported for the original species.

Both microsatellite and ISSR marker systems have high potential for studying the genetic variation of *T. schlegelii*, and these markers are suitable for conservation genetic programme of this endangered species. Overall, assessment of the results showed that the Malaysian *T. schlegelii* individuals studied in this research could have originated from a core population of the crocodiles. The lack of opportunity for outbreeding with other populations of crocodiles led to the occurrence of low genetic variation of the species in Malaysia.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

VARIASI GENETIK BUAYA DI ASIA TENGGARA, *Tomistoma schlegelii* MULLER, MENGGUNAKAN PENANDA MIKROSATELIT DAN PENANDA INTER SIMPLE SEQUENCE REPEAT

Oleh

BEHNAM SHAFIEI ASTANI

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Tomistoma schlegelii, juga dikenali sebagai "false gharial", merupakan salah satu spesies buaya yang paling kurang diketahui dan hanya boleh dijumpai di Indonesia dan Malaysia. Kewujudan spesies ini telah lama didokumentasi berdasarkan spesimen yang disimpan di muzium. Walaupun begitu, spesies ini kurang mendapat perhatian dalam kajian kerana ia tidak memberi impak ekonomi yang besar dan kurang kepentingan untuk budaya tempatan.

Tomistoma schlegelii di rantau Asia Tenggara didapati telah mengalami pengurangan populasi. Disebabkan pemusnahan habitat, spesies ini terpaksa hidup di dalam kawasan yang kecil dan ini akan mengurangkan lagi bilangan spesies ini, justeru meningkatkan kadar 'inbreeding' dalam spesies ini. Kekurangan maklumat mengenai variasi dan struktur genetik spesies ini di Malaysia adalah sesuatu yang membimbangkan. Oleh itu, objektif kajian ini adalah untuk menentukan variasi serta struktur genetik spesis ini di Malaysia. Untuk mencapai matlamat ini, dua jenis penanda molekul, iaitu mikrosatelit (40 pasang 'primer') dan 'Inter Simple Sequence Repeats' (ISSRs, 45 'primers') telah digunakan.

Jabatan Perlindungan Hidupan Liar dan Taman Negara (PERHILITAN) telah membekalkan sampel untuk kajian ini. Sebanyak 17 spesimen buaya *T. schlegelii* telah dikumpulkan oleh PERHILITAN dari peninsula Malaysia dan Sarawak untuk diagihkan ke ladang-ladang penternakan dan zoo-zoo tempatan. Sampel darah kemudiannya diambil daripada spesimen-spesimen buaya ini. DNA telah dikeluarkan dari sampel darah spesies ini dengan menggunakan teknik 'phenol-chloroform'. Amplifikasi PCR dengan DNA *T. schlegelii* telah dijalankan untuk mendapatkan suhu 'annealing' dan konsentrasi Mg^{2+} yang paling optimum.

Daripada 40 pasang 'primer' mikrosatelit, 10 didapati mengamplifasikan band yang polimorfik, manakala 36 dari 45 'primer' ISSR didapati mengamplifikasikan band yang polimorfik. Nilai-nilai 'observed heterozygosity (H_O)' dan 'expected heterozygosity (H_E)' penanda-penanda mikrosatelit masing-masing telah didapati berada dalam jangka 0.588 ke 1.000 dan 0.470 ke 0.891. Nilai 'average heterozygosity' yang paling tinggi telah diperoleh dengan penanda Cj109 dengan nilai

0.945 manakala nilai yang paling rendah telah diperoleh dengan penanda Cs15 dengan niali 0.529. Antara 45 ‘primer’ ISSR, 36 ‘primer’ (80%) telah menunjukkan band yang polimorfik. Bilangan band yang paling banyak telah diperoleh dengan menggunakan ‘primer’ UBC811 (6 band) dan bilangan band yang paling rendah telah diperoleh dengan menggunakan ‘primer’ PT2, UBC820, UBC834 and UBC868 (masing-masing 1 band). Data daripada penanda mikrosatelit dan ISSR telah menunjukkan pembahagian kepada dua kumpulan. ‘Repeat motif’ yang paling bersamaan dengan spesies lain telah diperoleh dengan menggunakan penanda-penanda Cj16, Amiμ16 dan Amiμ15 daripada spesies buaya *C. johnstoni, A. mississippiensis* dan *C. siamensis*. ‘Repeat motif’ untik Cj16 adalah “CA” manakala ‘repeat motif’ untuk Amiμ16 and Amiμ15 adalah “AC”.

Kedua-dua penanda mikrosatelit dan ISSR mempunyai potensi yang tinggi bagi kajian variasi genetik *T. schlegelii*, dan amat sesuai untuk digunakan oleh program perlindungan genetik bagi spesies ini. Hasil kajian ini telah mendapati bahawa *T. schegelii* di Malaysia berkemungkinan berasal daripada 1 populasi spesies buaya ini, dan telah mengakibatkan kekurangan variasi genetik dalam kalangan spesies ini di Malaysia.

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I certify that a Thesis Examination Committee has met on 16/01/2015 to conduct the final examination of Behnam Shafiei Astani on his thesis entitled "Genetic Variation of Southeast Asian Crocodile, *Tomistoma Schlegelii* Muller, Using Microsatellite and Inter Simple Sequence Repeat Markers" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.
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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

1X	One time
A	Adenine
AFLP	Amplified Fragment Length Polymerase
APS	Ammonium Persulfate
bp	Base pairs
C	Cytosine
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylene diamine tera acetic Acid
G	Guanine
H_E	Expected Heterozygosity
H_O	Observed Heterozygosity
ISSR	Inter Simple Sequence Repeat
MCMC	Markov Chain Monte Carlo
$MgCl_2$	Magnesium chloride
mtDNA	Mitochondrial DNA
Na	Observed number of alleles
NaOH	Sodium Hydroxide
Ne	Effective number of alleles
ng	Nanogram
NJ	Neighbor Joining
PAGE	Polyacrylamide Gel
PCR	Polymerase chain reaction
PIC	Polymorphism Information Content
RAPD	Random amplification of polymorphic DNA
SAHN	Sequential, Agglomerative, Hierarchical and Nested clustering
SSR	Simple sequence repeat
T	Thymine
T_a	Annealing temperature
TBE	Tris-borate EDTA
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultraviolet
VNTR	Variable Number Tandem Repeat

CHAPTER 1

INTRODUCTION

1.1 Study Background

Tomistoma schlegelii known as Buaya jenjulong in Malaysia is a freshwater crocodilian species that can only be found in Southeast Asia. *T. schlegelii* or false gharial is one of the most unusual and little known crocodilian species (Bezuijen *et al.*, 2010; Kaur *et al.*, 2013). Presently, there are 23 extant crocodilian species in eight genera of three families coming from Crocodyliforms that had existed on earth 200 million years ago. Given their old ancestral linage with little morphological changes (Salisbury *et al.*, 2006) and important functions in the ecosystem balance, more attention should be given to this crocodilian species. This species is regarded as a keystone species that ensures the balance and function of the habitat through its activities (Rodriguez, 2007).

Presently, the majority of individuals of the endangered species are found in farms and zoos, and they are obtained illegally. Therefore, with 2,500 estimated remaining individuals, the false gharial native to Southeast Asia illustrates a model of an endangered flagship species resulting from large loss of habitat (Rödder *et al.*, 2010). Almost 77 individuals of the species can be found at the farms and zoos in Malaysia (Stuebing *et al.*, 2004), while 88 other individuals can be found in Singapore, Indonesia, and Thailand. Farms and zoos in the United States of America and the Europe have around 57 individuals (Melino, 2011). *T. schlegelii* is a species found in Peninsular Malaysia, West Borneo, Java, and Sumatra. There is still an argument whether this species belongs to the Gavialidae or the Crocodylidae family (Mathew *et al.*, 2011). This species is an endangered species due to habitat loss. *T. schlegelii* inhabits peat swamps, and riverine forests habitat loss and illegal hunting are believed to contribute to the decline in the species number (Rödder *et al.*, 2010).

Aside from rapid status assessments, little is known about the species ecosystem. However, these rapid assessments have found that, overall, the *T. schlegelii* population in Southeast Asia has declined or fragmented due to habitat degradation, while Tanjung Puting National Park in Central Kalimantan still holds a stable population (Auliya *et al.*, 2009). Southeast Asia's tropical forests are rich with species variety probably because they act as a protection through past climate variations. However, these forests are subjected to modifications by human activities that might affect other living organisms (Gatesy *et al.*, 2003). Several survey studies have been done in Sumatra (Bezuijen *et al.*, 2001), East and Central Kalimantan (Fraser and Bernatchez, 2001), Peninsular Malaysia (Simpson *et al.*, 1998), and Sarawak (Stuebing *et al.*, 2004). The results obtained from these surveys show the distribution, breeding biology, and other aspects of *Tomistoma* environmental science (Bezuijen *et al.*, 2010).

As the genetic variation of this endangered species at the population level is not well studied, this makes conservation efforts difficult to be implemented due to lack of information and sources (Kaur *et al.*, 2013). However, conservation effort still needs to be done to avoid total species extinction. In the early stage, conservation efforts for

Tomistoma are carried out by focusing on the field surveys to record the existing populations and to identify conservation priorities (Bezuijen *et al.*, 2010).

To study the genetic variation, microsatellite and ISSR can be useful molecular markers. In conservation biology, microsatellite and ISSR markers can be utilised to identify unexpected changes in the inhabitants and influence of population fragmentation. Besides that, these markers are helpful in recognition of new and original inhabitants. Microsatellites and ISSR are powerful genetic markers that provide a fine resolution for discriminating populations (Tanya *et al.*, 2011).

These markers, which have become more important in recent times, provide precise evaluation of genetic structures of many organisms. Microsatellite markers are polymorphic and are distributed randomly in the genome to help differentiate populations (Avise, 2000).

1.2 Problem Statement

Tomistoma schlegelii within the Southeast Asia region has been found to suffer from population reduction. Human activities such as illegal fishing, urbanisation, as well as the environmental changes, and consequently habitat destruction as the main threatening factors (Auliya *et al.*, 2009) have led to decrease in the number and density of the endangered freshwater crocodile species "*Tomistoma schlegelii*" (Kaur *et al.*, 2013). Therefore, conservation efforts need to be initiated. In line with this, further extensive surveys to accurately identify the wild population size in Malaysia are required. In another word, conservation plans on habitat protection, population monitoring measurements, and land management should be determined. To achieve these, a clear overview on the genetic variation of the species across the country is unavoidable, while the present knowledge on the genetic diversity of *T. schlegelii* population in Malaysia is not sufficient (Harshman *et al.*, 2003; Janke *et al.*, 2005; Roos *et al.*, 2007). It is hypothesized that molecular markers such as microsatellites and ISSRs will generate useful information on the genetic structure and variation of this species. Such information can be employed for developing any conservation program in the future.

1.3 General Objectives

The main objective of this study was to determine the molecular genetic diversity of *T. schlegelii* samples obtained from Peninsular Malaysia and Sarawak. Currently, few microsatellite and ISSR marker studies have been conducted for *T. schlegelii*. The species was only included in these studies as an additional crocodilian species to test cross taxa amplificability of the developed markers.

1.4 Specific Objectives

The specific objectives of this study are as follows:

1. To determine the genetic diversity of *T. schlegelii* by using microsatellite and ISSR markers.
2. To estimate the allelic numbers and frequencies of the cross species amplified microsatellite markers.
3. To infer the genetic relationships of the *T. schlegelii* samples.

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LIST OF PUBLICATIONS

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