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Research paper

Molecular genetic variation and structure of Southeast Asian crocodile (*Tomistoma schlegelii*): Comparative potentials of SSRs versus ISSRs

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ABSTRACT

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 Southeast Asia. Indeed, lack of economic value for its skin has led to neglect the biodiversity of the species. The current study aimed to investigate the mentioned case using 40 simple sequence repeat (SSR) primer pairs and 45 inter-simple sequence repeat (ISSR) primers. DNA analysis of 17 *T. schlegelii* samples using the SSR and ISSR markers resulted in producing a total of 49 and 108 polymorphic bands, respectively. Furthermore, the SSR- and ISSR-based cluster analyses both generated two main clusters. However, the SSR based results were found to be more in line with the geographical distributions of the crocodile samples collected across the country as compared with the ISSR-based results. The observed heterozygosity (H_O) and expected heterozygosity (H_E) of the polymorphic SSRs ranged between 0.588–1 and 0.470–0.891, respectively. The present results suggest that the Malaysian *T. schlegelii* populations had originated from a core population of crocodiles. In cooperation with the SSR markers, the ISSRs showed high potential for studying the genetic variation of *T. schlegelii*, and these markers are suitable to be employed in conservation genetic programs of this endangered species. Both SSR- and ISSR-based STRUCTURE analyses suggested that all the individuals of *T. schlegelii* are genetically similar with each other.

1. Introduction

Tomistoma schlegelii is a freshwater crocodilian species native to Southeast Asia. *T. schlegelii* or false gharial is one of the most unusual and little known crocodilian species that can be 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 (Stuebing et al., 2006). The long narrow snout of the beast makes it uniquely distinct than its near taxa (Fig. 1). Presently, there are 23 extant crocodilian species in eight genera of three families coming from Crocodyliforms that had existed on earth since 200 million years ago. Given their old ancestral lineage with little morphological changes (Salisbury et al., 2006) and important functions in the ecosystem balance, more attention should be given to this crocodilian species. In addition, this species is regarded as a keystone species that ensures the balance and function of the habitat through its activities (Rodriguez, 2007).

T. schlegelii is subjected to a population reduction threat due to illegal hunting and environmental changes. Accordingly, it is estimated that a total of 2500–3000 individuals of the species remains in Southeast Asia, which is its main habitat. *T. schlegelii* inhabits peat swamps, and riverine forest habitat loss and illegal hunting are believed to contribute to the decline in the species number (Rödder et al., 2010). Of these individuals, 77 can be found at farms and zoos in Malaysia (Stuebing et al., 2004), while 88 individuals are available in Singapore, Indonesia and Thailand. Reportedly, farms and zoos of the United States of America

Abbreviations: ISSR, inter-simple sequence repeats; SSR, simple sequence repeats; PCA, principal component analysis; RAPD, randomly amplified polymorphic DNA; SM, simple matching; J, Jaccard; UPGMA, unweighted pair group method with arithmetic mean; NTSYS-Pc, numerical taxonomy and multivariate analysis system; AMOVA, analysis of molecular variance; SRW, Sarawak; PM, Peninsular Malaysia; H_O, observed heterozygosity; H_E, expected heterozygosity; N_a, number of effective alleles; N_e, number of observed alleles; PIC, polymorphism information content; RBCs, red blood cells; SDS, sodium dodecyl sulfate; PCI, phenol chloroform isoamylalcohol; EDTA, ethylenediaminetetraacetic acid; CI, chloroform isoamylalcohol; ISSR-PCRs, inter-simple sequence repeats-polymerase chain reaction; SSR-PCRs, simple sequence repeats-polymerase chain reaction; T_a, annealing temperature; PAGE, polyacrylamide gel electrophoresis gels; PCR, polymerase chain reaction; APS, ammonium persulfate; AFLP, amplified fragment length polymorphism; SIMQUAL, similarity for qualitative data; NCBI, National Center for Biotechnology Information.

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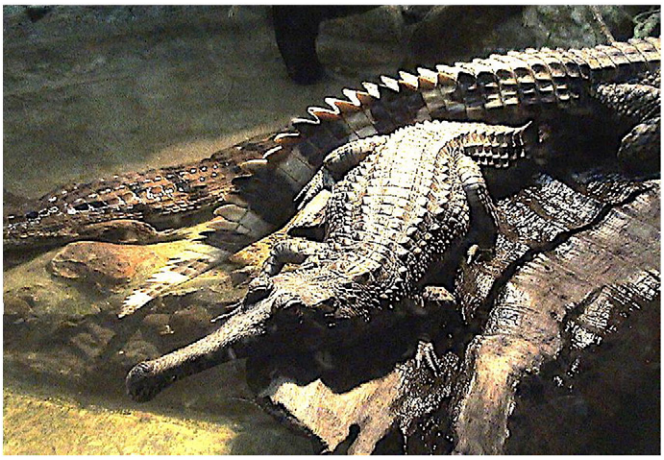

Fig. 1. Morphology of an adult *Tomistoma schlegelii*.

and the European Union possess around 57 individuals of the species. This species was included in Schedule 1 of the Wildlife Protection Act, in 1972, but the conservation of the species needs to be taken into further consideration.

In Malaysia, the Department of Wildlife and National Parks (DWNP) has provided the species with absolutely preserved position in Peninsular Malaysia. Despite all these precautions, not much is known about the species in the wild, and so preventing interspecies breeding is a huge challenge (Mathew et al., 2011).

Several survey studies have been carried out on *T. schlegelii* 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 of these surveys show the distribution, breeding biology, and environmental aspects of the species (Bezuijen et al., 2010).

Conservation plans are predicted to be more efficient with the incorporation of conservation of genetic resources of the species. High genetic variation within a population reflects a healthy population as the species is more adaptive to environmental changes (Ryberg et al., 2002). The use of molecular markers to obtain data on genetic variation is important for the information of conservation strategists. One of the important factors for the achievement of species protection is the conservation of its genetic variation, so that the species can adapt to the changing environment (Frankham, 2005).

Microsatellites (SSRs) and ISSRs are useful molecular markers to study genetic variation. In conservation biology, SSR and ISSR markers are utilized to identify unexpected changes in the inhabitants and the influence of population fragmentation. Besides, these markers are helpful in recognition of new and original inhabitants. SSRs and ISSRs are powerful genetic markers that provide a fine resolution for discriminating populations. Furthermore, SSRs are extremely polymorphic even at the level of small populations of endangered species due to a significant mutation rate (Allendorf and Luikart, 2007; Weber and Wong, 1993).

Loss of habitat and environment owing to legal and illegal means, forest fires, urbanization, and fishing activities is the major factor that threatens this freshwater crocodile species (Auliya et al., 2009). As mentioned before, *T. schlegelii* has been found to suffer from population reduction within the Southeast Asian region. Thus far, studies of *T. schlegelii* have focused on their natural habitats including habitat conservation (Rödder et al., 2010), and classification of these crocodilians (Brochu, 2003; Janke et al., 2005; Li et al., 2007). *T. schlegelii* has to live in small environments, because of the current habitat destruction, hence leading to a steady reduction of the species number. This phenomenon can lead to a lack of genetic diversity as a result of genetic inbreeding.

The main objective of this study was to determine the genetic diversity of the *T. schlegelii* population in Peninsular Malaysia and Sarawak. Therefore, this exploration aimed to evaluate the allelic numbers and their frequencies of SSR and ISSR markers by using cross-amplification of SSR primer pairs developed for other crocodilians species in *T. schlegelii* and the available universal ISSR primers as well as to infer the genetic relationships of the studied samples as its subsidiary purpose.

2. Materials and methods

2.1. Animals

The Department of Wildlife and National Parks (DWNP) provided the samples. In this regard, 17 *T. schlegelii* specimens were collected from different parts of Peninsular Malaysia and Sarawak, and were then transferred to the farms and zoos across the country (Table 1). The mentioned crocodiles were later subjected to the blood sampling process.

2.2. DNA extraction

The DNA extraction of all samples was carried out following Chong et al. (2000) with some modifications explained as below.

Twenty microliters of red blood cells (RBCs) of each sample was used for DNA extraction. The RBCs were mixed with 558 μL of lysis buffer (Tris–HCl (50 mM) and EDTA (100 mM)) adjusted to pH 8. The next step was to mix 30 μL of 20% sodium dodecyl sulfate (SDS) and 12 μL of 20 mg/mL proteinase K into the previous tube containing the RBC mixture. The samples were homogenized by resuspending it, and then the samples were incubated at 37 °C overnight. Next, 600 μL of phenol chloroform isoamylalcohol (PCI) with the ratio of 25:24:1 was added into the mixture followed by inverting the tube for 30 min. Then, the tubes were centrifuged for 10 min at 14,000 rpm. Subsequently, the supernatant was transferred to a new 1.5 mL microcentrifuge tube. The step was repeated by adding 600 μL of chloroform isoamylalcohol (CI) with the ratio of 24:1. This time, the step was done without phenol, unlike the previous step. The next step was DNA precipitation. This step was done by adding 2 volumes of pure chilled ethanol to the samples. Then, the samples were subjected to gentle inversion for 45 min using HP9300/HP9310 mini oven. The samples were centrifuged at 10,000 *g* for 5 min using Sigma 1–14 to remove excess ethanol. The washing step was carried out twice using 70% ethanol. 70% ethanol was added into the samples followed by inversion for a few times. Then, the samples were centrifuged to remove the remaining ethanol for 5 min at 10,000 *g*. Samples were allowed to dry at room temperature. After the samples were completely dried, 20 μL of 10 mM Tris–HCl was added to each sample and left overnight at 4 °C before storage at −20 °C.

Table 1
Sampling location and blood types of *T. schegellii*.

Sample	SSC	Collection site	Origin	Region	Sample type
TK002	1	Matang Wildlife Centre	SWK	Sadong	RBC
TK003	1	Matang Wildlife Centre	SWK	Runjing	RBC
TK021	2	Miri Crocodile Farm	SWK	Kuching	Whole blood
TK022	2	Miri Crocodile Farm	SWK	Miri	Whole blood
TK007	3	Zoo Negara	PM	Selangor	RBC
TK008	3	Zoo Negara	PM	Selangor	RBC
TK010	4	Zoo Melaka	PM	Selangor	RBC
TK011	4	Zoo Melaka	PM	Selangor	RBC
TK012	4	Zoo Melaka	PM	Selangor	RBC
TK013	4	Zoo Melaka	PM	Selangor	RBC
TK014	5	Zoo Taiping	PM	Terengganu	Whole blood
TK015	5	Zoo Taiping	PM	Selangor	Whole blood
TK017	6	Zoo Mini Temerloh	PM	Pahang	Whole blood
TK020	6	Zoo Mini Temerloh	PM	Pahang	Whole blood
TK024	3	Zoo Negara	PM	Zoo Bred	Whole blood
TK025	3	Zoo Negara	PM	Zoo Bred	Whole blood
TK027	3	Zoo Negara	PM	Zoo Bred	Whole blood

SSC: sampling site code, PM: Peninsular Malaysia, SWK: Sarawak, and RBC: red blood cells.