Potential Molecular Markers for Estuarine Crocodiles (*Crocodylus porosus*) from Sarawak

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ABSTRACT

Crocodylus porosus, estuarine crocodiles, is the most common crocodile that can be found in Sarawak. They inhabit wetlands near river mouths and migrate to upper side of the rivers during breeding season. Crocodiles are important economically as hides fetch high price when they are transferred into fine leathers. For many generations, the Chinese consider crocodiles have traditional medicinal value, besides their aphrodisiac properties. Conflicts between humans and crocodiles exist worldwide, and debates continue on how to sustainably manage the crocodiles. This study is designed to explore the possibility of establishing molecular markers for future rapid identification of Crocodylus porosus from Sarawak. Molecular biological techniques employed during this project were total genomic DNA extraction, Polymerase Chain Reaction (PCR) and PCR-Restriction Fragments Length Polymorphism (PCR-RFLP). Modified CTAB protocol had successfully extracted the total genomic DNA from tissue sample of C. porosus and PCR had successfully amplified the putative Cytochrome b (Cyt b) gene of approximately between 600-700 bp. PCR-RFLP showed that four restriction enzymes (REs) namely AluI, Csp6I, RsaI and MspI gave restriction profiles, suggesting that they could become molecular markers for C. porosus from Sarawak.

Keywords: *Crocodile, PCR, PCR-RFLP, Sarawak*

Introduction

Crocodiles (Order Crocodilia), comprises 24 species (Martin, 2007), and widely distributed in the tropical and subtropical regions throughout the world. According to Das (2006), there are three species of Crocodilian occur in Borneo, one estuarine species (*Crocodylus porosus* Schneider 1801) and the two freshwater species (*Tomistoma schlegelii* and *Crocodylus rinisus* Müller & Schelegel). Both *C. porosus* and *Tomistoma* are listed in Appendix I CITES, hence trade of these animals is very restricted. In contrast, IUCN Red List had listed *C. porosus* in the LOWER RISK (LR)/Least Concern (Ic) category. Perhaps IUCN information in this matter is out-dated as the data referred to 1994.

Sarawak, the largest state in Malaysia is famous for its enormous amount of natural resources. The Sarawakians take great pride of their natural heritage, however, where estuarine crocodiles are concerned, sentiment among the natives is relatively high. For generations, the natives had learned to co-exist with crocodiles (Ritchie & Jong, 2002). Unfortunately, increasing crocodile attacks have created sense of insecurity among those living near the rivers. Many residents who have the choices to avoid the rivers will choose to join the urbanization or agriculture activities (*e.g.* oil palm plantations) and completely leave the rivers behind. Without activities in the rivers, over along period of time, it is possible that population of crocodiles increase. The fast growing oil palm plantations and other types of grand-scale agriculture coupled with relatively extensive logging in the inland areas had somehow influence the water quality in the rivers. Water quality influence the health of aquatic ecosystem (Wetzel, 2001), either directly or indirectly. Changes of any components in the aquatic food web will create unbalance situation, over some time, will be translated to events happening in the areas. As the population of crocodile grows, and their food supply become limited, it is possible the attacks on humans increased. Therefore, it is crucial to find ways to sustainably manage the crocodiles in these rivers.

Molecular biology techniques have potentials to enhance conservation effort (Avise & Hamrick, 1997). Molecular data play important roles in supporting the traditional data in terms of clarifying species status, addressing phylogeograpy as well as determining genetic pools of each population. With combination of traditional and molecular approaches, management of the natural resources could stay relevant with current trend of living styles and aspirations of the younger generations.

This study is about our preliminary attempt to establish molecular markers for *Crocodylus porosus*, the most abundant crocodile found in Sarawak. The next sections of this paper will describe the materials and methods; results and discussions and finally the conclusions and recommendations.

Materials and Methods

Samples of crocodile tissues were collected from Matang Wildlife Centre with some help from staffs of Sarawak Forestry Corporation (SFC). Tissue samples from Miri and Sibu were kindly donated by private crocodile farms. One set of issues samples were preserved in 75% ethanol and another set was kept in -20°C. Extraction of total genomic DNA was carried out with some modifications of the CTAB method (Doyle & Doyle, 1987) followed by standard protocol of Polymerase Chain Reaction (PCR) using CR2H and tPhe-L primers (Allard *et al.*, 1991), and PCR optimization was carried out accordingly based on suggestions by Russello *et al.* (2006). Products of PCR were assessed by 1% agarose gel electrophoresis run in 1% TAE buffer with 80V for one hour. Photograph was captured for record. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was carried out using standard method using Restriction Enzyme (RE) that available in the laboratory namely *Bam HI, Hind III, AluI, Csp6I, RsaI and MspI*. PCR-RFLP products were assessed using 2% agarose gel electrophoresis run in 1% TAE buffer with 60V for one hour and forty-five minutes. Photograph was captured for further analysis.

Results and Discussions

Figure 1 revealed that modified CTAB has successfully isolate total genomic DNA from *C. porosus*. The size of the products was approximately more than 10,000 bp. In general, this protocol had extracted 'good quality' DNA in most cases ('good quality' refer to DNA that could be used as template in PCR and gave PCR product). Absence of bands in lanes 2 and 3 indicated that DNA extraction failed. These two lanes represented product of DNA extraction of scutes, originated from the back of crocodile (from Kuching), perhaps further modification to the current CTAB protocol is needed in order to extract DNA from scutes. Very faint bands were observed in lanes 6 and 7, indicated that very little DNA was extracted. This is perhaps due some technical errors during the experiment. PCR had successfully amplified the *Cyt b* gene, with size of approximately between 600 and 700 bp (Figure 2). This result is coherent with results obtained by Russello *et al.* (2006), who worked with wild samples of *C. porosus* in Palau. Further experiments showed that PCR-RFLP has successfully determined the REs *AluI, Csp6I, RsaI* and *MspI* cut the *Cyt b* gene whereas REs *Bam HI and Hind III* gave no restriction profiles (Figure 3). REs *Bam HI and Hind III* are 6-base cutter RE, thus they may have some difficulties in finding the restriction sites. In addition, it is possible that *Cyt b* gene of Sarawak *C. porosus* does not have DNA sequence of GGATCC and AAGCTT. After treating with RE *AluI,* one fragment of approximately 500 bp was seen in lane marked as 3 & 4, suggesting that this RE cut only once. The other fragment may be small in size thus it may be very faint and appeared at the end of the gel (could not be seen in the gel photograph as agarose gel lack of resolution for small number of bp). Both REs *Cps I* (lane marked as 9 & 10) and *Rsa I* (lane marked as 13 & 14) cut twice and at the same region (restriction site GTAC), producing fragments of approximately 250bp, 200bp and 150bp. The difference between these two REs, with reference to the cutting site is where it cuts because *CspI* cuts at G↑TAC whereas *RsaI* cut at GT↑AC. In addition, agarose gel is not efficient in separating small fragments and could not identify size differences of 1 bp. RE *MspI* had cut the

Cyt b gene once and produced 2 small fragments namely 500bp and 150bp (lane marked as 11 & 12). The total size of these fragments were 650 bp, which matched the original size of the PCR product, therefore one may say that *MspI* is the most suitable RE that could be applied as molecular marker when studying *C. porosus*.

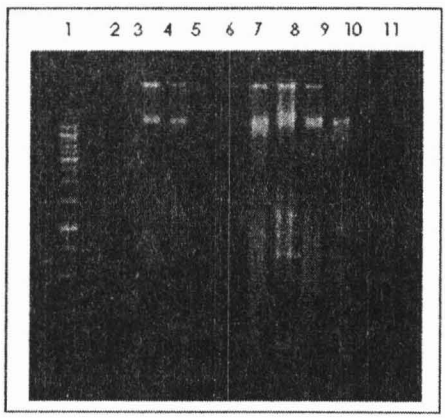

Figure 1: Gel Electrophoresis Photograph showing Extraction Product from the Tissue Samples of Crocodiles. Gel Electrophoresis using 1% Agarose Gel and Run with 1% TAE Buffer.

Lane 1: 1 kb DNA ladder
Lane 2 & 3: DNA extraction product from scutes
Lane 4 & 5: DNA extraction product from tissue preserved in 75% ethanol
Lane 6-11: DNA extraction product from tissue sample preserved in -20°C

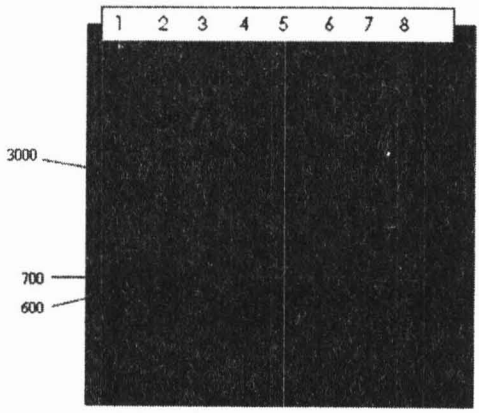

Figure 2: Gel Electrophoresis Photograph Showing PCR Product of *Ctyb* Gene from *C. Porosus*. Gel Electrophoresis used 1% Agarose Gel and Run with 1% TAE Buffer. Clear and Bright Bands Appear Between 600bp and 700bp.

Lane 1: 100bp DNA Ladder (Vivantis)
Lane 2-9: PCR products from *C. porosus*
Lane 10: Negative Control

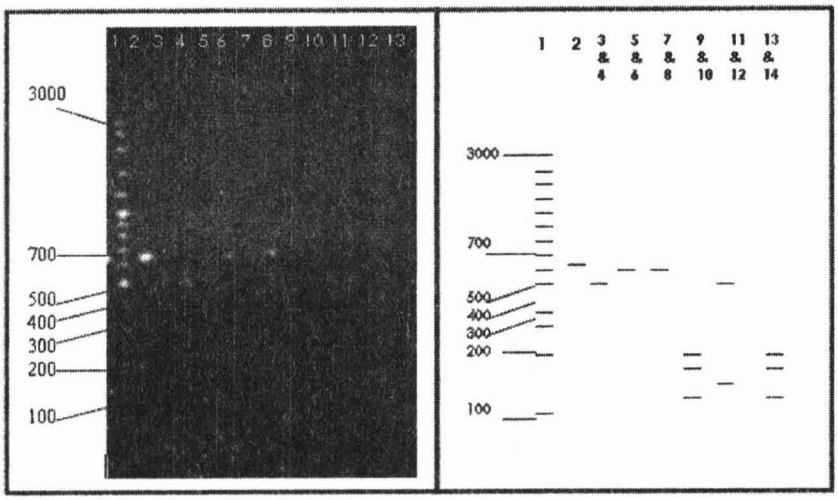

Figure 3 **Figure 3a**

Figure 3 & 3a: Gel Electrophoresis Photograph Showing PCR-RFLPs Patterns using 6 Restriction Enzymes (*BamHI, MspI, AluI, Csp6I, HindIII* and *RsaI*). Figure 3a is the Graphic Presentation of Figure 3. The fragments were Assessed using 2% Agarose Gel and Run with 1% TAE Buffer.

Lane 1: 100 bp ladder (Vivantis)
Lane 2: PCR product (control)
Lane 3&4: Restriction Profile *AluI*
Lane 5&6: Restriction Profile *BamHI*
Lane 7&8: Restriction Profile *HindIII*
Lane 9&10: Restriction Profile *Csp6I*
Lane 11&12: Restriction Profile *MspI*
Lane 13&14: Restriction Profile *RsaI*

Conclusions

Modified CTAB protocols had successfully isolate the total genomic DNA from *C. porosus* tissue samples, with high molecular weight product of more than 10,000 bp. PCR had revealed that putative fragment of *Cyt b* gene from this species was approximately between 600 to 700 bp. Four restriction enzymes namely *AluI, Csp6I, RsaI* and *MspI* gave restriction profiles, suggesting that they could become molecular markers for *C. porosus* from Sarawak. In future, work will focus on sequencing *C. porosus Cyt b* gene from wild and captive populations as data can be useful in developing DNA bar cording for *C. porosus* from Sarawak. It is hoped that these molecular data will help Sarawak State Government to address the issue of Sarawak crocodiles in a more sustainable manner.

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