DEVELOPMENT, CHARACTERIZATION AND APPLICATION OF MICROSATELLITE MARKERS OF MANGROVE HORSESHOE CRAB, *Carcinoscorpius rotundicauda* Latreille IN PENINSULAR MALAYSIA

By

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Chairman: **Prof. Tan Soon Guan, PhD**

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Horseshoe crab populations are said to be declining worldwide. However, there is still no published report on the status of horseshoe crabs in Malaysia. In this study, microsatellite markers were developed using 5'anchored ISSR-PCR enrichment procedure to diagnose population genetic structure of the mangrove horseshoe crab, *Carcinoscorpius rotundicauda*. A total of 134 microsatellite regions have been succesfully isolated using this technique and 36 novel microsatellite markers were developed. Out of these, eleven informative microsatellite loci were identified in an analysis of 127 samples representing five *C. rotundicauda* populations namely Kuala Juru, Kg. Permatang, Kg. Chuah, Kg. Sekokoh and Kg. Sg. Pulai from Peninsular Malaysia. The mean expected (He) and observed heterozygosity (Ho) values obtained ranged between 0.299-0.421 and 0.148-0.197 respectively.

Significant departures from Hardy-Weinberg equilibrium were detected in *C. rotundicauda* populations at Kg. Permatang, Kg. Chuah and Kg. Sg. Pulai. These populations also show moderate genetic differentiation with overall F_{ST} values of 0.108. Pairwise genetic distance values analysed between the five populations studied were close to zero which indicate high genetic similarity between populations. Five polymorphic inter-simple-sequence-repeat (ISSR) markers tested also generated comparable results. Thus, samples in each population are presumed to be genetically similar although the populations studied were geographically distant.

Due to the unknown migratory ability of *C. rotundicauda* species, existing habitats must be conserved for the survival of this species especially in Peninsular Malaysia. Findings from this study provide information on the genetic diversity of *C. rotundicauda* in Peninsular Malaysia and are hoped to assist in the development of conservation program for the mangrove horseshoe crabs in Southeast Asia.

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

PEMBANGUNAN, PENCIRIAN DAN APLIKASI PENANDA MIKROSATELIT BELANGKAS PAYA BAKAU, *Carcinoscorpius rotundicauda* Latreille DI SEMENANJUNG MALAYSIA

Oleh

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Populasi belangkas dikatakan sedang merosot di seluruh dunia. Walau bagaimanapun, masih tiada laporan yang diterbitkan berkenaan status belangkas di Malaysia. Dalam kajian ini, penanda mikrosatelit telah dibangunkan dengan menggunakan prosedur '5'anchored ISSR-PCR' untuk mendiagnosis struktur populasi genetik belangkas paya bakau, *Carcinoscorpius rotundicauda*. Sejumlah 134 kawasan mikrosatelit telah berjaya diisolasi menggunakan teknik ini dan 36 penanda mikrosatelit novel telah dibangunkan. Daripada jumlah itu, sebelas lokus mikrosatelit yang informatif telah dikenal pasti melalui analisis 127 sampel yang mewakili lima populasi *C. rotundicauda* iaitu Kuala Juru, Kg. Permatang, Kg. Chuah, Kg. Sekokoh dan Kg. Sg. Pulai dari Semenanjung Malaysia. Purata nilai heterozygositi yang dijangka (He) dan terhasil (Ho) diperolehi adalah masing-masing di antara 0.299-0.421 dan 0.148-0.197.

Penyimpangan dari keseimbangan Hardy-Weinberg yang signifikan didapati pada populasi *C. rotundicauda* di Kg. Permatang, Kg. Chuah dan Kg. Sg. Pulai. Perbezaan genetik di antara populasi adalah sederhana dengan nilai F_{ST} keseluruhan 0.108. Nilai pasangan jarak genetik yang dianalisis antara lima populasi yang dikaji pula didapati hampir sifar iaitu menunjukkan persamaan genetik yang tinggi di antara populasi. Lima penanda inter-simple-sequence-repeat (ISSR) polimorfik yang diuji juga menghasilkan keputusan yang serupa. Oleh itu, sampel dalam setiap populasi dianggap mempunyai persamaan genetik walaupun populasi yang dikaji terletak pada kedudukan geografi yang jauh.

Oleh kerana keupayaan migrasi spesies *C. rotundicauda* tidak diketahui, maka habitat sedia ada perlu dipulihara untuk kemandirian spesies ini terutamanya di Semenanjung Malaysia. Penemuan daripada kajian ini menyediakan maklumat mengenai kepelbagaian genetik *C. rotundicauda* di Semenanjung Malaysia dan diharap dapat membantu dalam pembangunan program pemuliharaan bagi belangkas paya bakau di Asia Tenggara.

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I certify that a Thesis Examination Committee has met on 15 July 2011 to conduct the final examination of Adibah binti Abu Bakar on her thesis entitled "Development, Characterization and Application of Microsatellite Markers of Mangrove Horseshoe Crab, *Carcinoscorpius rotundicauda* Latreille in Peninsular Malaysia" in accordance with the Universities and University College 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 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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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at other institution.

ADIBAH ABU BAKAR
Date: 15 July 2011

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LIST OF ABBREVIATION

1X	one time
□	genetic differentiation among populaions
^{0}C	degree celcius
µg	microgram
µl	microliter
µM	micromolar
A	alleles per locus
Ap	alleles per polymorphic locus
bp	base pair
C	cytosine residue
CI	confidence interval
CO1	cytochrome oxidase 1
CTAB	cetyl trimethyl ammonium bromide
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddH_2O	double distilled water
E	East
EDTA	ethylenediaminetetraacetic acid
F	inbreeding over all populations
f	inbreeding within population
F-coefficient	coefficient of inbreeding
F-statistics	fixation indices
FMP	Fishery Management Plan

G	guanine residue
g	gram
He	expected heterozygosities
Ho	observed heterozygosities
HWE	Hardy-Weinbeg Equilibrium
ISSR	inter simple sequence repeat
IT IS	Integrated Taxonomic Information System
IUCN	International Union for the Conservation of Nature and Natural Resources
KCl	potassium chloride
kb	kilo base
LAL	limulus amoebocyte lysate
LB	Luria Bertani
M	Molar
$MgCl_2$	magnesium chloride
mg	milligram
min	minute
ml	millilitre
mM	milliMolar
mt	mitochondrial
mtDNA	mitochondrial DNA
N	North
No	number
NaCl	natrium chloride
NCBI	National Center for Biotechnology Information
NODC	National Oceanographic Data Center

ng	nanogram
nm	nanometre
P	proportion of polymorphic loci
p-value	probability-value
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	*puissance hydrogen*
RAM	random amplified microsatellites
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	revolution per minute
s	second
SOC	Super Optimal broth with Catabolite repression
SSR	simple sequence repeats
STR	short tandem repeats
Ta	annealing temperature
TBE	tris borate EDTA
TD-PCR	touchdown –polymerase chain reaction
TE	tris EDTA buffer
Tris-HCl	tris-hydrochloride
UPGMA	unweighted pair group method with arithmetic mean
VNTR	variable number tandem repeat
v/v	volume / volume
w/v	weight / volume
X^2	chi-square

CHAPTER 1

INTRODUCTION

Commonly known as the mangrove horsehoe crab, *Carcinoscorpius rotundicauda* is a small-sized horsehoe crab species that has been found living in brackish estuaries. This species lays eggs in sandy-mud layer near river mouth (Sekigutchi *et al.,* 1977) and differs in breeding habit from the other three extant species of horseshoe crabs namely *Limulus polyphemus, Tachypleus tridentatus* and *Tachypleus gigas* which use sandy beach areas to spawn. Morphologically, all of the four species of horseshoe crabs appear to be externally similar but *C. rotundicauda* has a rounded blunt tail which becomes the distinctive character that differentiates it from other horseshoe crab species (Sekigutchi, 1988).

In Malaysia, horseshoe crabs are well-known in certain local cuisines and are used as bait to catch fish. Unlike *L. polyphemus, T. tridentatus* and *T. gigas*, *C. rotundicauda* species is not eaten as food because it is notably known to be poisonous. Furthermore, outbreaks of food poisoning had been reported in Thailand due to eating toxic eggs of this species (Banner *et al.,* 1966; and Trishnananda *et al.,* 1966). Currently, there is an increased interest from the locals to capture this animal due to the high value of the horseshoe crab blue blood in the biomedical industry. Scientist named Frederick Bang was the first to discover the clotting quality of the horseshoe crab's blood in the 1950s (Bang, 1979). He isolated the chemical in the American horseshoe crab's blood (*Limulus polyphemus*) that caused the clotting and called it "Limilus amoebocyte lysate" or LAL. Research findings showed that LAL

aids to detect human pathogens in patients, injectable drugs and in intravenous devices (Bang, 1979). Due to this valuable endotoxin property of the blood, horseshoe crabs are being over-harvested in certain areas of their habitation.

According to the International Union for the Conservation of Nature and Natural Resources (IUCN) online database, until 2011, *C. rotundicauda* is still listed under the 'data deficiency' category. Moreover, the knowledge of this species abundancy in Malaysia is yet fragmentary. As there is an increased concern over the declining numbers of horsehoe crab, research pertaining to the population status of this species should be carried out to prevent further losses of this 'living fossil' as well as to promote the establishment of conservation strategies for horseshoe crabs in Malaysia.

Molecular genetic markers can be used to diagnose status of a population and provide management information for any organism. The idea of using genetic markers especially after the development of electrophoretic assays such as isozymes has greatly improved global awareness on the potential of genetic markers in biological sciences (Jaccoud *et al.*, 2001). For horseshoe crabs, there were published reports explaining on the intraspecific genetic variations and patterns of population differentiations detected using genetic markers such as allozymes, mitochondrial DNA and microsatellites (Selander *et al.*, 1970; Saunders *et al.*, 1986; Orti *et al.*, 1997).

Microsatellites are a class of repetitive DNA sequences of several nucleotides, usually 2 to 6 base pairs present in all organisms, (Gur-Arie *et al.,* 2000; Morgante *et al.*, 2002). The popularity of microsatellite DNA markers among molecular population biologists is not surprising, considering the special features of these markers and the apparent reliability of data produced from them. This marker system has been employed in different areas ranging from ancient and forensic DNA studies, to population genetics and conservation of biological resources. Though microsatellite markers are initially expensive to develop due to sequencing costs, once developed, microsatellite markers are economical and relatively easy to employ because they require only a small amount of DNA and rather little technical expertise. Due to their high levels of allelic variation, codominant inheritance, and ease of analysis, microsatellite markers are chosen as the markers of choice in this study to evaluate variation and population genetics of *C. rotundicauda* from different locations in Peninsular Malaysia.

There is still no reliable published report on the status of *C. rotundicauda* in Malaysian shores and populations of this species also have never been assessed at molecular level. Thus, to obtain an understanding of *C. rotundicauda* populations in Peninsular Malaysia, the objectives of current study are:

1) To develop single-locus microsatellite markers for *C. rotundicauda* species using the 5'anchored inter-simple-sequence-repeat polymerase chain reaction (ISSR-PCR) enrichment protocol,

2) To infer the population structure of the mangrove horseshoe crab from different areas of Peninsular Malaysia using the newly developed single-locus co-dominant microsatellite markers,

3) To evaluate the potential of using dominant inter-simple-sequence-repeat (ISSR) markers for population genetic study of the mangrove horseshoe crab.