



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun  
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi

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

**By**

**ADIBAH ABU BAKAR**



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun  
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfilment of the Requirements for the Degree of the Master of Science**

**July 2011**



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun  
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment  
of the requirement for the degree of Master of Science

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

By

**ADIBAH ABU BAKAR**

**July 2011**

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

**Faculty: Biotechnology and Biomolecular Sciences**

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 successfully 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 ( $H_e$ ) and observed heterozygosity ( $H_o$ ) 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

**ADIBAH ABU BAKAR**

**Julai 2011**

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

**Fakulti: Bioteknologi dan Sains Biomolekul**

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. Pulau dari Semenanjung Malaysia. Purata nilai heterozygositi yang dijangka ( $H_e$ ) dan terhasil ( $H_o$ ) 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 di harap dapat membantu dalam pembangunan program pemuliharaan bagi belangkas paya bakau di Asia Tenggara.





## ACKNOWLEDGEMENTS

*In the name of Allah, the most Gracious and the most Merciful.*

First of all, I would like to say *Alhamdulillah*, for giving me the strength and health to complete this research. I am heartily thankful to my supervisor; Prof. Tan Soon Guan, whose encouragement, supervision and support from the preliminary to the concluding level inspired me and enriched my growth as a student, a researcher and a scientist. I would also like to thank my co-supervisors, Dr. Faridah and Dr. Annie for their guidance, support and expertise that were invaluable and will never be forgotten or surpassed. Collective and individual acknowledgments are also owed to my colleagues at the Genetics Lab (UPM) / Tissue Culture Lab (UPM) / Aquaculture Lab (UPM) / Virology Lab (Medic. UPM) and MARDI Animal Breeding Lab whose presence was somehow perpetually refreshing, helpful and memorable. Many thanks go in particular to my dearest friends Pui Ling, Manjeri, Hatta, Amie, Sonia, Haza, Syukri, Chou Min, Syahar and Zamakh for giving me such a pleasant time when working together with them since my first day in UPM.

Special thanks to my family members and husband for supporting and encouraging me to pursue this degree. Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as express my apology that I could not mention them personally one by one.

This study has been financially aided by research grant No. 05/01/07/0220RU (91220) from the Research University Grant Scheme (RUGS), Universiti Putra Malaysia.



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.

Members of the Thesis Examination Committee were as follows:

**Ho Chai Ling, PhD**

Associate Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

**Jothi Malar A/P P.V. Panandam, PhD**

Professor

Faculty of Agriculture

Universiti Putra Malaysia

(Internal Examiner)

**Siti Khalijah binti Daud, PhD**

Associate Professor

Faculty of Agriculture

Universiti Putra Malaysia

(Internal Examiner)

**Badrul Munir Md Zain, PhD**

Associate Professor

Faculty of Science and Technology

Universiti Kebangsaan Malaysia

(External Examiner)



**NORITAH OMAR, PhD**

Associate Professor and Deputy Dean

School of Graduate Studies

Universiti Putra Malaysia

Date: 23 August 2011

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:

**Tan Soon Guan, PhD**

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

**Faridah Qammaruzzaman, PhD**

Associate Professor

Faculty of Sciences

Universiti Putra Malaysia

(Member)

**Annie Christianus, PhD**

Lecturer

Faculty of Agriculture

Universiti Putra Malaysia

(Member)

---

**HASANAH MOHD GHAZALI, PhD**

Professor and Dean

School of Graduate Studies

Universiti Putra Malaysia

Date:





## 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



## TABLE OF CONTENTS

<b>ABSTRACT</b>	<b>Page</b>
<b>ABSTRAK</b>	ii
<b>ACKNOWLEDGEMENTS</b>	iv
<b>APPROVAL</b>	vi
<b>DECLARATION</b>	vii
<b>LIST OF TABLES</b>	ix
<b>LIST OF FIGURES</b>	xii
<b>LIST OF ABBREVIATIONS</b>	xiv
	xvi

## CHAPTER

<b>1</b>	<b>INTRODUCTION</b>	<b>1</b>
<b>2</b>	<b>LITERATURE REVIEW</b>	<b>5</b>
2.1	<i>Carcinoscorpius rotundicauda</i>	5
2.2	Distribution	6
2.3	Breeding pattern	7
2.4	Current status of horseshoe crabs	8
2.5	Molecular markers for population genetic	10
2.6	Population genetic study of horseshoe crab	11
2.7	Microsatellites (simple sequence repeat-SSR)	13
2.7.1	Isolation of microsatellite	16
2.7.2	Inter-simple-sequence-repeat (ISSR)	17
2.7.3	Libraries enriched microsatellites loci using 5' anchored ISSR-PCR	19
<b>3</b>	<b>ISOLATION OF MICROSATELLITES FOR DEVELOPMENT OF SINGLE- LOCUS MICROSATELLITE MARKERS</b>	<b>21</b>
3.1	Introduction	21
3.2	Methodology	22
3.2.1	Samples and DNA isolation	22
3.2.2	Constructions of libraries enriched for microsatellites	23
3.2.3	Plasmid DNA extraction	27
3.2.4	Sequencing of plasmid DNA	29
3.2.5	Removing vector sequences	29
3.2.6	Microsatellite sequences submission to Genebank	30
3.2.7	Designing primers	30
3.3	Results	31
3.3.1	DNA quantification	31
3.3.2	Cloning	31
3.3.3	Microsatellite loci isolated	32
3.3.4	Primers designed	40
3.4	Discussion	46
3.4.1	CTAB method for genomic DNA extraction of <i>C. rotundicauda</i>	46

3.4.2	Isolation of microsatellite regions and designing primers	47
3.5	Conclusion	49
4	<b>CHARACTERIZATION AND APPLICATION OF SELECTED SINGLE-LOCUS MICROSATELLITE MARKERS FOR POPULATION STUDY OF <i>C. rotundicauda</i></b>	50
4.1	Introduction	50
4.2	Methodology	51
4.2.1	Samples and DNA isolation	51
4.2.2	Microsatellite markers and amplification	53
4.2.3	Statistical analysis	54
4.3	Results	55
4.3.1	Characterization of selected microsatellite markers	55
4.3.2	Statistical analysis	60
4.4	Discussion	69
4.4.1	Patterns of genetic variation at microsatellite loci and null allele	69
4.4.2	Inbreeding	69
4.4.3	Population differentiation	70
4.5	Conclusion	71
5	<b>ASSESSMENT OF GENETIC VARIATION FOR <i>C. rotundicauda</i> IN PENINSULAR MALAYSIA USING INTER-SIMPLE SEQUENCE-REPEAT (ISSR) MARKERS</b>	72
5.1	Introduction	72
5.2	Methodology	73
5.2.1	ISSR markers and amplification	73
5.2.2	ISSR analysis	74
5.3	Results	75
5.3.1	ISSR markers	75
5.3.2	Pairwise genetic relationship between populations	79
5.4	Discussion	82
5.4.1	ISSR markers	82
5.4.2	Fingerprinting of <i>C. rotundicauda</i>	83
5.5	Conclusion	85
6	<b>GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH</b>	86
	<b>REFERENCES / BIBLIOGRAPHY</b>	89
	<b>APPENDICES</b>	99
	<b>BIODATA OF STUDENT</b>	128
	<b>LIST OF PUBLICATIONS</b>	129

## LIST OF TABLES

Table		Page
3.1	Five anchored ISSR primers screened in this study	24
3.2	Selected ISSR primers with their respective annealing temperature ( $T_a$ )	25
3.3	Clones submitted to the GenBank and the accession numbers	36
3.4	Types of identified microsatellite core units in sequenced clones	37
3.5	Locus-specific microsatellite primers synthesized for <i>Carcinoscorpius rotundicauda</i> species	42
4.1	Latitude and longitude of the sampling locations of <i>C. rotundicauda</i> in Peninsular Malaysia	51
4.2	List of optimized microsatellite primers with the $MgCl_2$ concentration and annealing temperature ( $T_a$ ) for each primer	54
4.3	Banding patterns observed for each optimized primers screened over five <i>C. rotundicauda</i> populations in Peninsular Malaysia	56
4.4	Characteristics of 19 polymorphic microsatellite markers in <i>C. rotundicauda</i>	59
4.5	Population genetic parameters for populations of <i>C. rotundicauda</i> in Peninsular Malaysia (means are over all loci)	60
4.6	Observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities obtained for each populations from Peninsular Malaysia	62
4.7	Estimated null allele frequencies using four algorithms	64
4.8	$F_{IS}$ values per population	65
4.9	$F_{ST}$ values averaged over loci in <i>C. rotundicauda</i> populations	67

4.10	Genetic distance between populations calculated using Nei's (1978) unbiased distance formula	68
5.1	Five-anchored ISSR primers screened over 127 samples of <i>C. rotundicauda</i>	73
5.2	Selected ISSR primers with respective annealing temperature ( <i>Ta</i> )	76
5.3	Number of bands observed and total number of polymorphic bands scored for each population	76
5.4	Number of bands observed and scored for each marker	76
5.5	Genetic distance between <i>C. rotundicauda</i> populations derived using Dice (1945) coefficient	80
5.6	Genetic distance between populations derived using unbiased Nei's (1978) genetic coefficient	81

## LIST OF FIGURES

Figure		Page
2.1	Morphology of <i>Carcinoscorpius rotundicauda</i>	6
2.2	Microsatellites repeats	14
2.3	ISSR-PCR	18
3.1	Electrophoresed PCR product for primer BP10 before and after PCR clean-up on 2% agarose gel	32
3.2	Isolated plasmid DNAs from clones C1-C10 for BP1, BP2, and BP8 primers, electrophoresed on 1% agarose gel	33
3.3	Isolated plasmid DNAs from clones C1-C10 for BP9, BP10, and BP13 primers, electrophoresed on 1% agarose gel	34
3.4	Vecscreen result for clone ADBbp1-1	35
3.5	Mononucleotide (A), dinucleotide (B) and trinucleotide (C) microsatellite core units observed	38
3.6	Tetranucleotide (D), pentanucleotide (E) and hexanucleotide (F) microsatellite core units observed	39
3.7	Clones containing perfect microsatellites (A), compound microsatellites (B) and imperfect/interrupted compound microsatellites (C)	41
4.1	Sampling locations of <i>Carcinoscorpius rotundicauda</i>	52
4.2	Polymorphic banding patterns observed for primer ADB1-10-1 (A), primer ADB9-1-1 (B) and primer ADB1-2-1 (C)	57
4.3	Comparison of banding patterns between PAGE gel (A) and Meta®Phor gel (B) for primer ADB1-3-3	58
4.4	UPGMA dendrogram depicting genetic distances calculated using Nei's unbiased distance formula (1978) between five <i>C. rotundicauda</i> populations based on 19 polymorphic microsatellite loci	68
5.1	ISSR banding patterns	78

5.2	Dendrogram from UPGMA analysis using distance data derived from the Dice (1945) similarity coefficient matrix based on ISSR bands of <i>C. rotundicauda</i> populations	80
5.3	Dendrogram from UPGMA analysis using distance data (Table 5.6) generated from Nei's (1978) genetic distance formula	81

**LIST OF ABBREVIATION**

1X	one time
□	genetic differentiation among populaions
°C	degree celcius
µg	microgram
µl	microliter
µM	micromolar
<i>A</i>	alleles per locus
<i>Ap</i>	alleles per polymorphic locus
bp	base pair
C	cytosine residue
CI	confidence interval
CO1	cytochrome oxidase 1
CTAB	cetyl trimethyl ammonium bromide
ddH <sub>2</sub> O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddH <sub>2</sub> O	double distilled water
E	East
EDTA	ethylenediaminetetraacetic acid
<i>F</i>	inbreeding over all populations
<i>f</i>	inbreeding within population
<i>F</i> -coefficient	coefficient of inbreeding
<i>F</i> -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 <sub>2</sub>	magnesium chloride
mg	milligram
min	minute
ml	millilitre
mM	milliMolar
mt	mitochondrial
mtDNA	mitochondrial DNA
N	North
No	number
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NODC	National Oceanographic Data Center





ng	nanogram
nm	nanometre
<i>P</i>	proportion of polymorphic loci
p-value	probability-value
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	<i>puissance hydrogen</i>
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
T <sub>a</sub>	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
$\chi^2$	chi-square





## CHAPTER 1

### INTRODUCTION

Commonly known as the mangrove horseshoe crab, *Carcinoscorpius rotundicauda* is a small-sized horseshoe 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 "Limulus 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 abundance in Malaysia is yet fragmentary. As there is an increased concern over the declining numbers of horseshoe 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.