









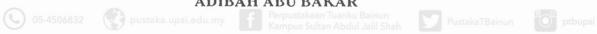
DEVELOPMENT, CHARACTERIZATION AND APPLICATION OF MICROSATELLITE MARKERS OF MANGROVE HORSESHOE CRAB, Carcinoscorpius rotundicauda Latreille IN PENINSULAR MALAYSIA

By

ADIBAH ABU BAKAR











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





















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

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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 (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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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. 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.

menghasilkan keputusan yang serupa. Oleh itu, sampel dalam setiap populasi dianggap mempunyai persamaan genetik walaupun populasi yang dikaji terletak pada kedudukan geografi yang jauh.

Lima penanda inter-simple-sequence-repeat (ISSR) polimorfik yang diuji juga

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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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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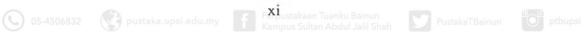






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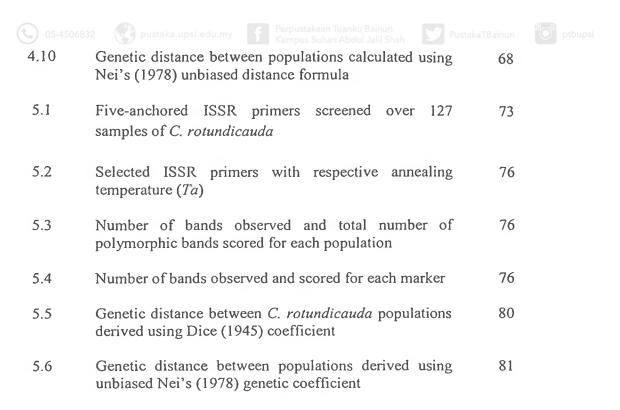




















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LIST OF ABBREVIATION

1X one time

genetic differentiation among populaions

⁰C 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

CTAB cetyl trimethyl ammonium bromide

CO1 05-4506832 pustaka up cytochrome oxidase 1 Abdul Jalil Shah

ddH₂O double distilled water

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

ddH₂O 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

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ptbups

MgCl₂ 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

proportion of polymorphic loci P

p-value probability-value

PAGE polyacrylamide gel electrophoresis

PCR polymerase chain reaction

pН puissance hydrogen

RAM random amplified microsatellites

RAPD random amplified polymorphic DNA

RFLP restriction fragment length polymorphism

rpm revolution per minute

second S

SSR 05-4506832 pustaka.ups

SOC Super Optimal broth with Catabolite repression

simple sequence repeats

STR short tandem repeats

Ta annealing temperature

TBE tris borate EDTA

TD-PCR touchdown -polymerase chain reaction

tris EDTA buffer TE

Tris-HCl tris-hydrochloride

UPGMA unweighted pair group method with arithmetic mean

VNTR variable number tandem repeat

volume / volume v/v

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).









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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:

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 singlelocus 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.

















