

**CHARACTERISATION OF THE GENETIC
DIVERSITY WITHIN AND AMONG
POPULATIONS OF THE ASIAN HORSESHOE
CRAB IN SABAH USING CYTOCHROME
OXIDASE 1 AND MICROSATELLITE MARKERS**



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UMS
UNIVERSITI MALAYSIA SABAH

**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITY MALAYSIA SABAH
2015**

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UMS

**THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE**

**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITY MALAYSIA SABAH
2015**

DECLARATION

I hereby declare that this dissection is of my own work except for the quotations and summaries from which the references are fully acknowledge.

8th August 2015

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To my friendly four-legged companion, I cannot imagine coming home after a stressful day of work and not see you greet me at the door with your wagging tail. Finally to You, Swami for all the love and blessings showered upon me throughout my life.

CERTIFICATION

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OXIDASE 1 AND MICROSATELLITE MARKERS**

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ABSTRACT

Today, only four Horseshoe Crab species exists which includes *Tachypleus tridentatus*, *Tachypleus gigas*, *Carcinoscorpius rotundicauda* (found in Malaysian waters) and *Limulus polyohemus* (found only in American waters). In recent years, a decline in the number of Horseshoe Crabs has occurred due to various phenomena's including habitat degradation which has prompted studies on the genetic architecture and population structure of the Horseshoe crabs. Consequently, this study was carried out using *cytochrome oxidase 1* (C01) gene and microsatellite markers to understand the genetic structure of Horseshoe Crabs in Sabah. A total of 86 Horseshoe Crab samples were collected from five districts in Sabah namely Bongawan (21), Kota Kinabalu (15), Kota Belud (26), Kudat (14) and Tuaran (10). Sequence analysis of the *cytochrome oxidase 1* (C01) gene identified the existence of three species namely *Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda* when tested for similarity using BLAST. Amplification of 10 microsatellite loci (Tt01, PLbp2-2-2, PLbp11-10-2, TTLR1A, TTLR1B2, PLbp11-9-2, HCMS069, TTLR151, TTLR152, and TTLR121) on *T. gigas*, *C. rotundicauda* and *T. tridentatus* was successfully carried out. All loci were observed to be polymorphic except for locus TTLR1A (Kudat) and locus TTLR151 (Tuaran). The number of alleles ranged from 2 to 6 per locus. The mean observed heterozygosities (H_o) and the mean expected heterozygosities (H_e) were between 0.55 and 0.72, and between 0.40 and 0.59, respectively indicating a satisfactory level of genetic diversity of the *T. gigas* population in Sabah. Hardy-Weinberg Equilibrium analysis showed deviation with the highest χ^2 value in locus TTLR1A (93.003) and the lowest in locus TTLR151 (10.108). The dendrogram constructed based on Nei's (1978) genetic distance using UPGMA method showed a distinct cluster of samples from all five locations with samples from Bongawan forming one cluster, samples from Kota Kinabalu and Tuaran forming the second cluster, and Kota Belud and Kudat forming the third cluster, indicating consistency with geographical locations of each population. This study shows moderately high levels of genetic diversity of the Horseshoe Crab population in Sabah. However, population density is constantly declining. This research provides molecular information that could be used to implement different conservation strategies for the Horseshoe Crab population in Sabah.

ABSTRAK

PENCIRIAN KEPELBAGAIAN GENETIK DI ANTARA DAN DI DALAM POPULASI BELANGKAS DI SABAH MENGGUNAKAN LOKUS CYTOCHROM OXIDASE 1 DAN DINUKLEOTIDA MIKROSATELIT

Pada masa kini, hanya terdapat empat jenis spesis belangkas yang terdiri daripada *Tachypleus tridentatus*, *Tachypleus gigas*, dan *Carcinoscorpius rotundicauda* yang boleh dijumpai di perairan Malaysia dan *Limulus Polyphemus* yang hanya terdapat di perairan America. Sejak kebelakangan ini, kemerosotan bilangan belangkas terjadi disebabkan pelbagai faktor termasuk kemusnahan habitat yang telah mendorong kajian genetik dan struktur populasi belangkas. Kajian ini telah dijalankan menggunakan gen Cytochrome Oxidase 1 (C01) dan petanda-petanda mikrosatelit. Dalam kajian ini, sejumlah 86 sampel belangkas telah diperolehi dari lima daerah di Sabah iaitu Bongawan (21), Kota Kinabalu (15), Tuaran (10), Kota Belud (26) dan Kudat (14). Analisis jujukan gen Cytochrome Oxidase 1 (C01) menunjukkan terdapat tiga spesis belangkas iaitu *Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda* apabila diuji untuk kesamaan menggunakan BLAST. Proses amplifikasi ke atas 10 lokus telah berjaya dilakukan untuk populasi *T. gigas*, *C. rotundicauda* dan *T. tridentatus* di Sabah dan kesemua 10 loki adalah bersifat polimorfik kecuali lokus TTLR1A (Kudat) dan lokus TTLR151 (Tuaran). Bilangan alel yang ditemui adalah di antara dua hingga enam bagi setiap lokus. Nilai min heterozigositi yang dicerap (H_o) dan nilai min heterozigositi jangkaan (H_e) masing-masing adalah di antara 0.55 dan 0.72, dan di antara 0.40 dan 0.59. Ini menunjukkan tahap kepelbagaian genetik belangkas yang memuaskan di Sabah. Analisis keseimbangan Hardy-Weinberg menunjukkan sisihan dengan nilai χ^2 tertinggi dalam lokus TTLR1A iaitu 93.003 dan nilai χ^2 terendah dalam lokus TTLR151 iaitu 10.108. Dendogram yang dibentuk berdasarkan jarak genetik Nei (1978) dengan menggunakan kaedah UPGMA menunjukkan perkelompokan sampel yang berbeza pada umumnya yang agak selari dengan lokasi geografi setiap populasi. Sampel dari Bongawan membentuk kelompok pertama, sampel dari Kota Kinabalu dan Tuaran membentuk kelompok kedua manakala sampel dari Kota Belud dan Kudat membentuk kelompok ketiga. Hasil kajian ini menunjukkan tahap kepelbagaian genetik belangkas yang agak tinggi di belangkas. Walaubagaimanapun, kepadatan populasi belangkas didapati merosot secara berterusan. Kajian ini berjaya menghasilkan maklumat molekular yang dapat digunakan untuk melaksanakan strategi pemuliharaan ke atas populasi belangkas di Sabah.

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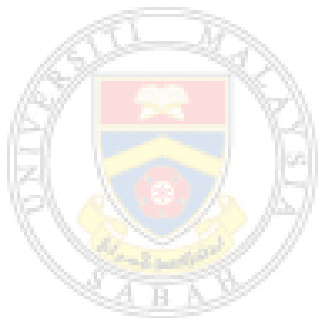


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LIST OF ABBREVIATIONS AND SYMBOLS

-	Negative
%	Percent
+	Positive
°C	Degree Celsius
µg	Microgram
µL	Microlitre
A	Adenine nucleotide
bp	Base pair
C	Cytosine nucleotide
CBOL	Consortium for the Barcode of Life
COI	<i>Cytochrome oxidase I</i>
ddH ₂ O	Deionised Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide-triphosphate
Ed.	Edition
eds.	Editors
EDTA	Ethylenediaminetetraacetic acid
g	Gram
G	Guanine nucleotide
H _E	Expected Heterozygosity
H _O	Observed Heterozygosity
HCl	Hydrochloric Acid
HWE	Hardy-Weinberg Equilibrium
kb	Kilobase pair
KCl	Potassium Chloride
LAL	<i>Limulus</i> Amebocyte Lysate
M	Molar
mg	Milligram
MgCl ₂	Magnesium Chloride
MgSO ₄	Magnesium Sulphate
mL	Millilitre
mM	Millimolar
mtDNA	Mitochondrial DNA
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
PCR	Polymerase Chain Reaction
pg.	Page
pM	Picomolar
RNA	Ribonucleic Acid
rpm	Revolutions per minute
s	Seconds

SDS	Sodium dodecyl sulphate
SSRs	Simple Sequence Repeats
T	Thymine nucleotide
TBE	Tris/Borate/EDTA buffer solution
Tm	Melting temperature
Tris	Trishydroxymethylaminomethane
Tris-HCl	Tris-hydrochloric acid
UPGMA	Unweighted Pair Group Method with Arithmetic mean
V	Volts
w/v	Weight over volume
X	Times
<i>h</i>	Haplotype diversity
<i>n</i>	Nucleotide diversity
F_{IS}	F statistics
IUCN	the International Union for the Conservation of Nature
ERDG	Ecological Research and Development Group



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CHAPTER 1

INTRODUCTION

1.1 Introduction to Research

Conservation biology is a multidisciplinary science that emphasises biodiversity and its maintenance that has developed as a result of growing awareness of biodiversity loss (Soule, 1985). Conservation genetics in particular, incorporates genetic management of small populations, resolution of taxonomic uncertainties and management units and the application of genetic analysis to preserve and understand species' biology (Frankham, 2003). This research falls in the scope of conservation genetics. The present study aims to understand the population structure of the horseshoe crabs in Sabah using microsatellite markers.

1.2 Issue of Extinction, Conservation Genetics and the Horseshoe crab

Extinction is a natural part of the evolutionary process. For example, the mass extinction at the end of Cretaceous 65 million years ago eliminated much of the previous flora and fauna including the dinosaurs (Cracraft, 2001). Nevertheless, this made way for propagation of the mammals and flowering plants which contributed to the world's biodiversity. In contrast to this, currently the world's biological diversity is rapidly depleting as both direct and indirect consequences of human action and this causes loss of species at rates that far outruns the creation of new species. An unknown but large number of species are already extinct, while many others have reduced population sizes that put them at risk (WCMC, 1992). As a result of this, human intervention is crucial to improve the management and to ensure the survival of many species today.

Conservation genetics is motivated by the need to reduce current rates of extinction and to preserve biodiversity. It is the application of genetics as a tool to preserve species as dynamic entities which are capable of coping with environmental changes. It includes genetic management of populations, resolution of taxonomic uncertainties, defining management units within species, and the use of molecular genetic analysis in forensics and understanding species biology. It also aims to minimize the risk of extinction due to genetic factors.

Horseshoe crabs, also known as living fossils are marine arthropods that are in need of conservation genetic measures. They are facing a rapid decline in the number of populations around the world due to factors such as water pollution, loss of living and spawning habitats and human exploitation (Li *et al.* 2009). Today, only four species of the horseshoe crab exists. Often used as main examples of organisms that survived long periods without any significant changes in their anatomy, horseshoe crabs have puzzled evolutionary biologists for centuries, earning them the name 'living fossils' and 'phylogenetic relicts' (Obst *et al.* 2012). The oldest Horseshoe crab fossil was found in Manitoba, Canada and is 445 million years old (Brut, 2014).

For most of its history, the horseshoe crab was regarded as junk from the sea. Bounties of horseshoe crabs were placed upside down to avoid them from feasting on clams. According to the Atlantic States Marine Fisheries Commission, approximately two million horseshoe crabs were caught yearly to be consumed as fertilizers and livestock feed in the mid 80's. In 1956, Frederik Bang, a John Hopkins biologist discovered that Limulus Amoebocyte Lysate (LAL), refined from the copper-based blood of horseshoe crabs, could be used to detect trace amounts of endotoxin contaminants which is what makes it useful for testing sterility of medical equipment and drugs for human use (Odell et al. 2005).

Aside from the medical benefits, horseshoe crabs also remain as a valuable part of the ecosystem. Many shorebirds, migratory birds, turtles and fish use horseshoe crab eggs as an important part of their diet. In Malaysia, horseshoe crabs are a delicacy in restaurants and command a high price. All this has led to

poaching and over-harvesting to feed the market. This, along with onshore development and coastal disturbance is threatening the horseshoe crab population around the world. Since then, this has led to extensive studies on the horseshoe crab.

While there have been several studies done concerning the general biology and life history of the horseshoe crab, information concerning its status and population dynamics is not adequately known (Walls *et al.* 2002). However, there has been indication of a decline in the number of horseshoe crabs in Asia. As of now, India and Japan have listed the horseshoe crab as an endangered species (The Hindu Business Line, 2005). In addition to this, in Asia, horseshoe crabs are only listed under the data deficiency category (IUCN). In Malaysia, there is still a lack of reliable report on the status of the horseshoe crabs (Tan *et al.* 2010). Thus, in order to prevent further decline in the horseshoe crab population, studies on their population genetics is crucial. It will aid in the knowledge of their variations among different populations and later promote the establishment of conservation strategies for the horseshoe crab species in Malaysia.

In all biological research, the identification of species is critical. Accurate identifications reveal known information about each organism, its ecological roles, its physiological and biochemical properties, and its societal risks and benefits (Seifert *et al.* 2006). In addition to this, to the untrained eye, the differences in morphology and taxonomic characteristics of the four horseshoe crab species are subtle (Awise *et al.* 1994) and scientists are unable to distinguish one species from another. Besides that, morphological similarities have caused a struggle in understanding phylogenetic relationships among the horseshoe crab lineages (Xia, 2000).

Species identification, such as for the horseshoe crab in this study, requires the usage of a specific gene region. The region should be able to distinguish different species through polymorphism in the DNA sequences and should have a universal conserved primer region that can be used for different species. The

cytochrome oxidase I (COI) gene region satisfies this requirement and it has been proven useful in numerous barcoding research (Kress *et al.*, 2005).

Microsatellites, a highly versatile genetic marker which are also known as Simple Sequence Repeats (SSRs) are stretches of short DNA sequence in which a motif is tandemly repeated. Due to its habitually high variability, microsatellites have been a useful marker to address matters such as discrimination, relationships, structure and classification, not only at the population level, but also at the individual level (Wan *et al.* 2004).

In the past two decades, such genetic markers have been used to determine the status of several endangered species and have provided significant understandings that have critically influenced management decisions and created benefits for several species (Wan *et al.* 2004). In this study, the *cytochrome oxidase 1* (CO1) gene region was used in the identification of the horseshoe crab species, while microsatellite markers were used in the assessment of the population structure of the horseshoe crabs in Sabah.

1.3 Problem Statement

There is significant decrease in the number of horseshoe crabs in Sabah.

1.4 Significance of Study

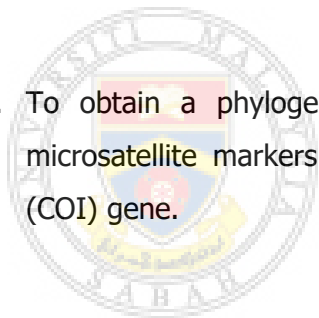
Taking into consideration the various interconnected issues affecting the horseshoe crab population and its possibility of becoming an endangered species in Malaysia, developing a successful horseshoe crab management strategy is important. More so, with its proven biomedical benefits, horseshoe crabs need to be protected through proper understanding of its population status encompassing its breeding pattern and its genetic diversity. In order to have conservation management plans, research and documentation of information is vital. This research is one step towards the documentation of scientific information of the horseshoe crab population in Sabah. This kind of information is also important considering the future prospects for Malaysia to start its own biomedical industry in which horseshoe crab bleeding can be carried out for the production of LAL. In this

context, future cultivation of horseshoe crabs can also benefit economically. As such, scientific information of this species has medical, ecological, and economical importance.

1.4 Objectives

The aim of this study is to identify the genetic structure of the wild populations of the horseshoe crabs in Sabah. Three specific objectives were taken to achieve this goal.

1. To identify the species of the Asian Horseshoe Crabs in Sabah using the primer sequences for *cytochrome oxidase I* (COI) gene of Asian horseshoe crab.
2. To characterize the genetic population structure of the Horseshoe crab populations through DNA profiling of single-locus microsatellite markers.
3. To obtain a phylogenetic tree of the horseshoe crab population through microsatellite markers and sequence homology of the *cytochrome oxidase I* (COI) gene.



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CHAPTER 2

LITERATURE REVIEW

2.1 General Biology, Ecology & Life History of the Horseshoe Crab

2.1.1 Phylogeny, Taxonomy and Distribution

Horseshoe crabs are marine chelicerates closely related to arachnids. Horseshoe crabs are the closest living relatives to the trilobites (Shuster, 1982). They have lived for more than 500 million years since the Palaeozoic Devonian era. The oldest fossil found dates back between 360 and 405 million years ago (Chatterji *et al.*, 1992). The ancestor of the present species of the Horseshoe crab originated from the Mesozoic waters of Europe.

Up to date, only one consensus has been reached in which the Atlantic species (*Limulus polyphemus*) is a sister taxon to the three Indo-Pacific species (*Tachypleus gigas*, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*). The consensus is consistent with the current taxonomy in which *Limulus* belongs to the subfamily Limulinae and the three other extant species belonging to Tachypleinae. All four species of Limulidae is the only living representatives of Merostomata (Yamaksai, 1988).

At present, the phylogenetic relationship between the three Indo-Pacific species remains unsolved despite the multitude of analyses conducted. Results were highly differed, leading to some research suggesting that the three species constitute a phylogenetically irresolvable Trichotomy which could have resulted from all three Indo-Pacific species forming within a short geological time (Avisé *et al.*, 1994).

Nevertheless, re-examination on phylogenetic relationships of the horseshoe crab species was conducted by Obst *et al.* (2012) using an intra-specific analysis involving 18S rDNA, 28S rDNA and mitochondrial gene cytochrome *c* oxidase I (COI). The results suggest:

- 1) Strong support for a monophyletic genus *Tachypleus* and a diversification of the three Asian species during the Paleogene period. "Speciation events were temporally well-separated by several million years" (Obst *et al.*, 2012).
- 2) Tree topology suggests that the "three Asian species originated in central South East Asia from a marine stem group that inhabited the shallow coastal waters between the Andaman Sea, Vietnam, and Borneo".
- 3) "*C. rotundicauda* probably separated from the *Tachypleus* stem group by invading estuarine habitats, while *T. tridentatus* most likely migrated northeast along the Southern coast of China and towards Japan" (Obst *et al.*, 2012).

Horseshoe crabs belong to the phylum of Arthropods, consisting of animals that have articulated bodies and limbs. The three major classes of Arthropods are Insects, Arachnids and Crustaceans. The horseshoe crabs belong to its own class called Merostomata. The term merostomata means "legs attached to the mouth". Horseshoe crabs are most closely related to trilobites that existed 544 million years ago (Shuster, 1982). The taxonomic classification of the horseshoe crabs is shown below.

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Meristomata
Order	:	Xhiphosurida
Family	:	Limulidae
Genus	:	<i>Limulus, Tachypleus, Carcinoscorpius</i>
Species	:	<i>Limulus polyphemus, Tachypleus gigas, Tachypleus tridentatus, Carcinoscorpius rotundicauda</i>

The present distribution of Horseshoe crabs suggest they migrated when the shallow seas disappeared while the European land mass was formed. One group moved to the east and one moved to the west (Shuster, 1982). Today, Horseshoe crabs are found in only two regions of the world; the coastal waters of Asia, from India to Japan, and along the Atlantic coastlines of North America (Table 2.1 and Figure 2.1)

Table 2.1 : Distribution of the four remaining horseshoe crab species

Species	Distribution
<i>Limulus polyphemus</i>	Atlantic coast of America and distributed extensively from Maine to the Florida Keys and around the periphery of the Gulf of Mexico up to south of Yucaton Peninsula
<i>Carcinoscorpius rotundicauda</i>	Found on the West coast of Malaysia and Thailand (in the mouth of Mae am River). Also found on the East coast of Orissa, India
<i>Tachypleus gigas</i>	Occurs along both the coasts of Malay Peninsula, around West coast of Singapore and along the coast of Orissa (India) to Indo-China, Borneo, Java (Indonesia), Torres Straits and Celebes
<i>Tachypleus rotundicauda</i>	It is the most common Asian species and found rich in the western and southern coasts of Japan. Also found along the coast of Vietnam, China (South and East coasts), Taiwan, Philippines, North Borneo and in the Indian Ocean side of Sumatra

Source : Biomedical Potentials of the Indian Horseshoe Crab. INFOFISH International. (Lakshmanan & Venkateshvaran, 1999).

Tachypleus tridentatus and *Carcinoscorpius rotundicauda* are from the kingdom Metazoa, phylum Arthropoda, subphylum Chelicerata, class Merostomata, order Xiphosura, and family Limulidae. *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* is also known as the Asian horseshoe crab. *Tachypleus tridentatus* is found mostly on the west and south coast of Japan and also found along the coast of Vietnam, south and east coast of China, Taiwan, Philippines and also north of Borneo (Lakshmanan & Venkateshvaran, 1999). *Carcinoscorpius rotundicauda* is found on the west coast of Malaysia, Thailand (in the mouth of Mae Nam river) and also on the east coast of Orissa of India (Lakshmanan & Venkateshvaran, 1999).

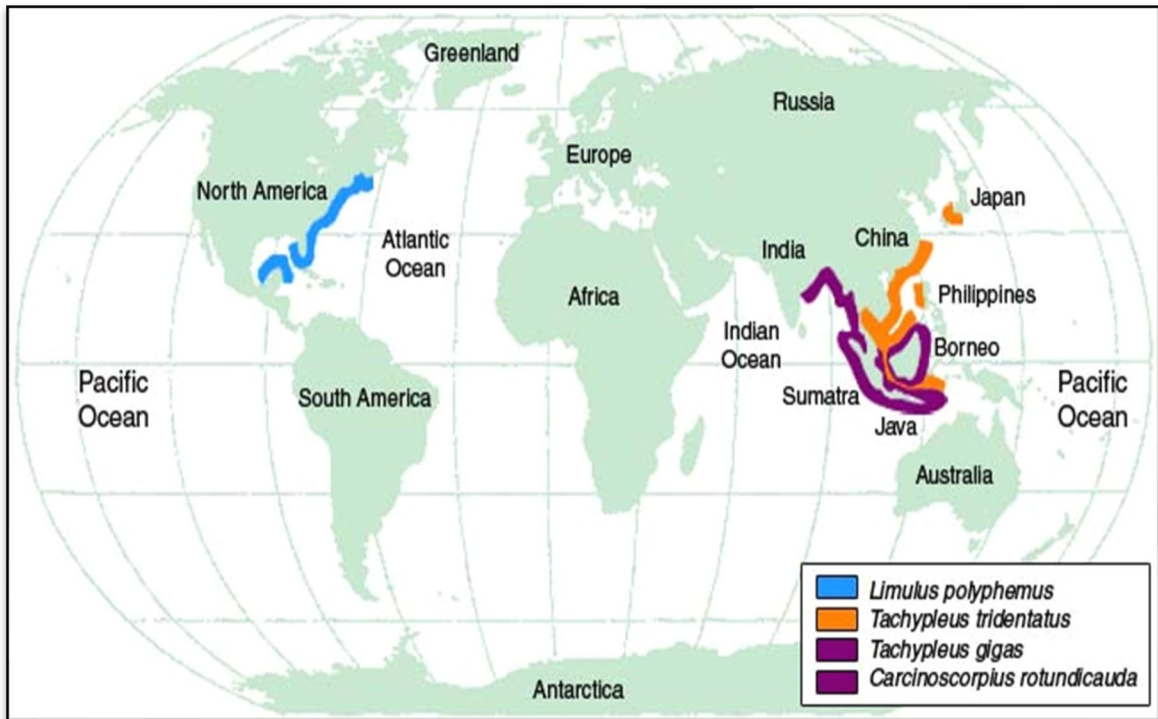


Figure 2.1 : Distribution of the four remaining horseshoe crab species.

Source : University of Delaware Graduate College of Marine Studies and the Sea Grant College Program, Project Galathea, 2008.

2.1.2 Morphology

One of the most distinguished features of the horseshoe crab is its alien-like body. Despite its appearance, the horseshoe crab is harmless. The body of the horseshoe crab is divided into three regions: the prosoma, the opisthosoma and the teson (Rudkin and Young, 2009). These are sometimes referred to as the cephalothorax, the abdomen, and the tail.

The cephalothorax, also referred to as the carapace or shell, is the large anterior segment of the horseshoe crab. The carapace protects the legs and organs of the horseshoe crab and also keeps the animal upright in rough waters. The prosoma contains an intestinal tract with an esophagus and proventriculus which is used by the crab to grind food. It also contains a nervous system concentrated into a brain, a tubular heart, excretory glands and connective tissues and cartilaginous plates. The