Genetic Analysis on Horseshoe Crab for Phylogenetic Tree Study from Jambi, Bangka Belitung, Central Java, and East Java Province, Indonesia

Nenik Kholilah[1,2], Gabriella Tarida Kurniatami[3], Eka Maya Kurniasih[4], Nining Nursalim[2], Galank Fad'qul Janarkho[2], Ambariyanto Ambariyanto[5], Diah Permata Wijayanti[5], Sapto Purnomo Putro[3], Ni Kadek Dita Cahyani[2,3]*

[1]Marine Science Program Study, Department of Fisheries and Marine Science,
Faculty of Agriculture, University of Mataram
Jl. Pendidikan No. 37, Mataram, West Nusa Tenggara, 83114 Indonesia,
[2]Diponegoro Marine Biodiversity Project Laboratory, Universitas Diponegoro
Jl. Prof. Soedarto, Tembalang, Semarang, Central Java 50275 Indonesia
[3]Biology Department, Faculty of Science and Mathematics, Universitas Diponegoro
Jl. Prof. Jacub Rais, Tembalang, Semarang, Central Java, 50275, Indonesia
[4]Graduate School of Engineering and Science, University of The Ryukyus Nishihara
Okinawa, Japan
[5]Department of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro
Jl. Prof. Jacub Rais, Tembalang Semarang Central Java, 50275, Indonesia
Email: nkdcahyani@gmail.com

Abstract

Order Xiphosura, or horseshoe crabs, are rarely found and classified as macrofossils. In Indonesian waters, there are three species: *Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*. The genetic analysis of their kinship, however, has been extensively unexplored. This study aims to utilize DNA barcoding, focusing on the *cytochrome c oxidase subunit I* (COI) locus from mitochondrial DNA and investigating the phylogenetic relationships of horseshoe crabs from the four sites (Tanjung Jabung Timur Regency in Jambi, Bangka Regency in Bangka Belitung, Demak Regency in Central Java, and Tuban Regency in East Java). Additionally, the study provides insights into the biodiversity and ecological roles of horseshoe crabs within their ecosystems and surrounding habitats. Through molecular methods, this research focuses on biodiversity analysis through Sanger sequencing and MEGA 11 software for constructing phylogenetic trees and calculating genetic distances. With a total of 22 horseshoe crabs, the DNA samples from four different sites were amplified via Sanger sequencing, targeting the COI locus and analyzing with MEGA 11. The phylogenetic tree analysis revealed two distinct species, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*, exhibiting significant genetic variation between them. A clear genetic separation between the two species was observed. Otherwise, within the *C. rotundicauda* species, a minor genetic variation was detected between sampling sites. Notably, the genetic composition displayed greater differences between samples from Java and Sumatra compared to differences within each island. The future research should expand the sampling size and include additional genetic markers to provide a more comprehensive understanding of the genetic diversity and evolutionary history of horseshoe crabs in Indonesian waters. Integrating ecological and environmental data could further elucidate the factors driving genetic differentiation and inform conservation strategies.

Keywords: *horseshoe crab; living fossils; Sumatra; Java.*

INTRODUCTION

Indonesia is renowned for its mega biodiversity, which spans both terrestrial and marine ecosystems, making it one of the most biologically diverse countries on the planet (Rintelen *et al*., 2017). Among its rich biodiversity, the animal kingdom holds particular significance, offering numerous opportunities for exploration and research. Indonesia's marine biodiversity extends from the westernmost point in Sabang to the easternmost region in Merauke, covering an immense range of habitats and species. Among these unique and captivating species is the horseshoe crab, an ancient marine arthropod that has garnered significant scientific interest due to its evolutionary and ecological importance (Carmichael *et al*., 2015; Lamsdell, 2019).

Horseshoe crabs, belonging to the order Xiphosura, are often referred to as "living fossils" because of their remarkable evolutionary stasis (Renwick, 1968; Barthel, 1974; Fisher, 1984; 1990,

*) Corresponding author
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Chatterji & Abidi 1993; Kin & Błażejowski, 2014). These organisms have existed since the Paleozoic era, with fossil records dating back more than 450 million years (Dunlop, 2010; Bicknell *et al*., 2020). Despite their primitive classification, horseshoe crabs share a closer genetic relationship with scorpions, falling under the subphylum Chelicerata (Avise *et al*., 1994). This intriguing evolutionary link makes them an essential subject for phylogenetic studies. Notably, their structural changes over time have been minimal, as evidenced by the fossil records of the genus Limulus, further solidifying their status as stabilomorphs (Kin & Błażejowski, 2014).

Research on horseshoe crabs in Indonesia covers several biological aspects, including reproductive biology (Mulya, 2004; Muslihah, 2004), Local knowledge-based study (Meilana and Fang, 2020) morphometrics (Suparta, 1992), and population studies (Rubiyanto, 2012). Known locally as 'Mimi lan Mintuno' on Java, 'Belangkas' in Kalimantan and Sumatra, and 'Bungkak' in South Sumatra, horseshoe crabs are distributed along the northern coast of Java, in areas like Subang, Indramayu, Semarang, and Surabaya, as well as the coastal zones of Jambi on Sumatra (Purwiyanto *et al*., 2019; Meilana and Fang, 2020). This extensive distribution highlights their ecological adaptability and the important role they play in Indonesia's coastal ecosystems (Mashar *et al*., 2017).

While previous studies on horseshoe crabs have concentrated on morphology and physiology, these methods have limitations in uncovering the species' deeper evolutionary relationships (Lamsdell, 2019). Horseshoe crabs are considered cryptic species, making them particularly difficult to identify based on morphological characteristics alone. Advances in genetic techniques, particularly DNA barcoding, now provide a more effective means of exploring genetic diversity and phylogenetic connections among horseshoe crab populations (Citation). This study aims to use DNA barcoding to gather foundational genetic data on horseshoe crabs (Xiphosura) from various regions in Indonesia. Researchers can establish preliminary phylogenetic relationships and gain insights into the evolutionary history and regional divergence of horseshoe crabs.

MATERIAL AND METHODS

The sampling was conducted in four sites: Tanjung Jabung Timur Regency in Jambi, Bangka Regency in Bangka Belitung, Demak Regency in Central Java, and Tuban Regency in East Java (Figure 1). These sites were chosen owing to certain regions having been sampled but had not been molecularly analyzed prior to this study. For the rest reasons, this study background was intended to be a further study. The data on this study provides a starting point for records of Indonesia horseshoe crab biodiversity study due to the lack of data.

Samples were collected from fishermen at the designated destination site. In the field, DNA sampling followed a structured process. First, a photograph of each sample was taken for documentation. Then, specific body parts were carefully separated from the whole organism for DNA analysis. Tissue samples were selected, with a preference for the legs, gills, and, when present, eggs. Approximately 10 grams of tissue were collected from each sample and preserved in 96% ethanol for storage.

DNA extraction was performed using the Chelex 10% method (Walsh *et al*., 1991). Following extraction, DNA was amplified using PCR with the primer pairs JgHCO and JgLCO, where JgHCO (5'-TAIACYTCIGGRTGICCRAARAAYCA-3') and JgLCO (5'-TITCIACI AAYCAYAARGAYATTGG-3') served as the forward and reverse primers, respectively (Geller *et al*., 2013), targeting the Cytochrome Oxidase I (COI) gene. The PCR reactions were conducted in a total volume of 25 µL, incorporating 1.25 µL of template DNA. Each reaction mixture contained 12.5 µL of MyTeq™ Red Mix (Bioline), 1 µL of each primer, and 9.25 µL of double-distilled water (ddH_2O).

The thermocycling conditions included an initial denaturation at 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension

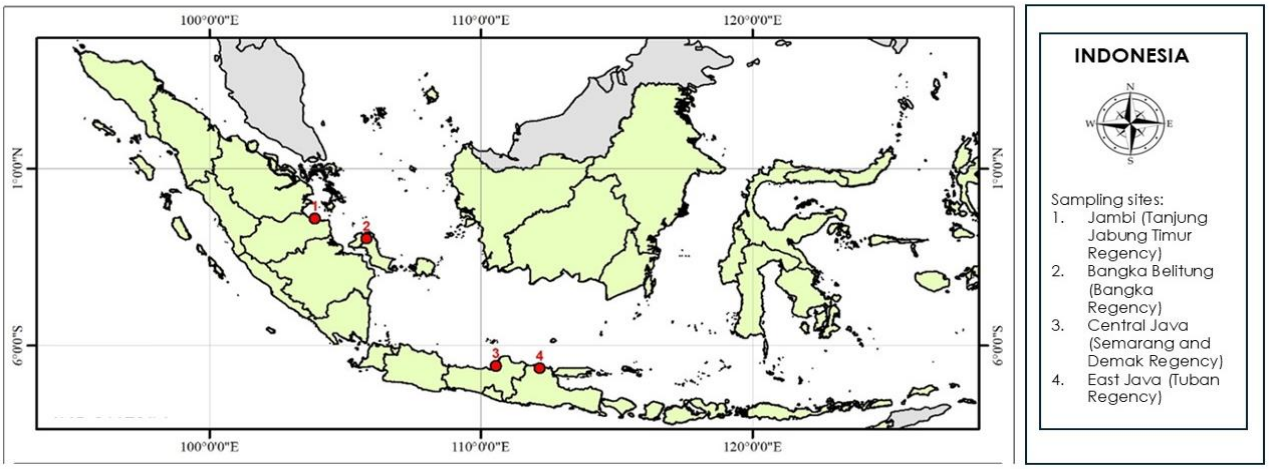

Figure 1. Sampling sites

at 72°C for 1 minute, concluding with a final extension at 72°C for 10 minutes. The PCR products were analyzed using 1% agarose gels stained with FluoroSafe. Finally, the amplified products were sequenced using the Sanger Sequencing method at a dedicated sequencing facility.

The forward and reverse sequences were cleaned and aligned using MEGA version 11 (Tamura *et al*., 2021). The cleaned sequences were then compared against the NCBI database (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov) through the BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) interface (McGinnis & Madden, 2004). This comparison allowed for the identification of sister sequences and differences relative to the data collected in the NCBI database. The information provided by BLAST included species descriptions, scientific names, query coverage, and percent identity.

To construct the phylogenetic tree and assess genetic distances, we utilized MEGA 11, applying the Maximum likelihood method, Hasegawa-Kishino-Yano + G (HKY+G). For the phylogenetic analysis, we downloaded representative sequences of *T. tridentatus* (accession number JN018216.1), *T. gigas* (accession number JF896114.1), *C. rotundicauda* (accession number MW454812.1), and multiple sequences for *L. polyphemus* (accession numbers KT959421.1, KT959422.1, KT959446.1), along with *Harpaphe haydeniana* (accession number KR135994.1) as an outgroup. Genetic distances were calculated by determining the nucleotide differences between sequences (Davidson & del Campo, 2020).

RESULT AND DISCUSSION

Molecular identification of the collected samples revealed two distinct species: *Carcinoscorpius rotundicauda* and *Tachypleus gigas*. A total of 22 sequences were successfully recorded and analyzed, with *C. rotundicauda* identified from 16 samples across two Indonesian islands—four samples from Java (Demak and Tuban) and twelve samples from Sumatra (Jambi and Bangka Belitung). In contrast, *T. gigas* was recorded solely from six samples collected in Java (Demak, Semarang, and Tuban).

Morphologically, *C. rotundicauda* exhibits a rounded protosoma with a bulbous surface, while the edges of its opisthosoma align with this rounded shape, and its telson lacks a serrated surface. This species is adapted to muddy sediment environments, as noted in previous studies (Shin *et al*., 2009; Rubiyanto and Patria, 2018). Conversely, *T. gigas* displays a longer, oval body shape with a protosoma that resembles bunny ears. Its opisthosoma edges are more pronounced and sharper, and its telson features a serrated surface. This species typically inhabits shallow brackish

waters, estuaries, and mangrove areas (Directorate General of Natural Resources and Ecosystem Conservation, 2020).

The analysis targeted the COI gene, with sequence lengths ranging from 634 to 666 base pairs (bp). All sequences were submitted to the NCBI database, and comparisons using BLAST confirmed the presence of two species, *C. rotundicauda* and *T. gigas*, with identity similarity values ranging from 98.75% to 99.85% (Table 1). According to Leray *et al*. (2013), an identity percentage above 98% indicates strong species similarity. All examined samples exceeded this threshold.

The phylogenetic tree constructed in this study confirms the identification of two species within the Asian horseshoe crab group, specifically *C. rotundicauda* and *T. gigas*. Notably, *C. rotundicauda* is found in both Sumatra and Java, while *T. gigas* is exclusive to Java. The tree illustrates three major clades that separate the species. The first clade encompasses *C. rotundicauda*, which further divides into three groups based on geographic samples and NCBI data, including samples from Java (Demak and Tuban) and Madura, followed by those from Sumatra (Pangkal Niur, Bangka Belitung, and Kampung Laut, Jambi) and Malaysia. A significant genetic distance was observed between the samples and those from Bangladesh.

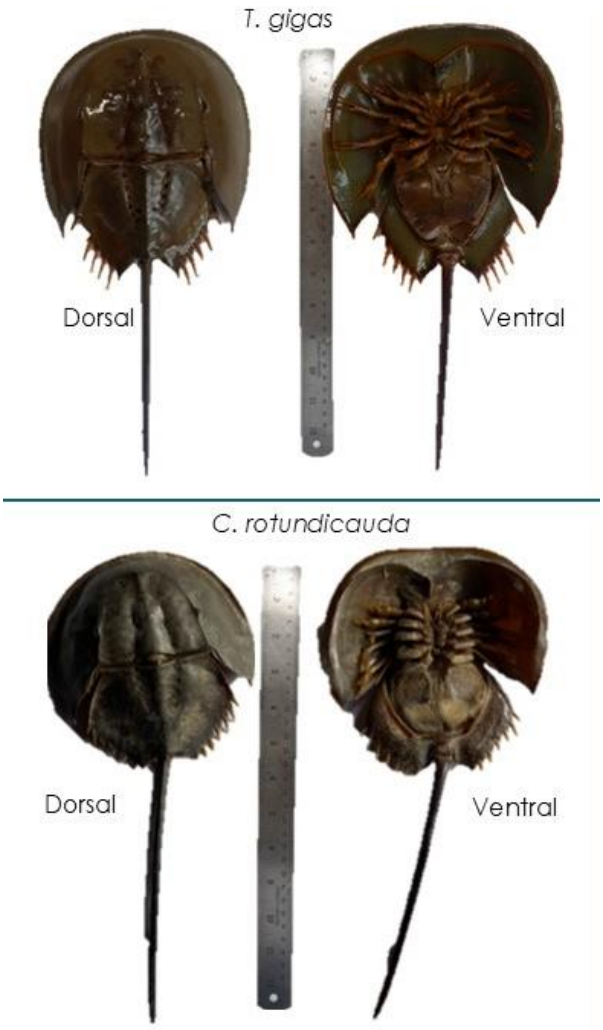

Figure 2. Piture of the sample ollected from the fisherman. Note: *T. gigas;* b. *C. rotundicauda*

Table 1. Analysis table of DNA extraction data

No.	Sample code	Blast Result Name	Accession Number Sample	length of sequence (bp)	Identity (%)
	Demak, Central Java				
1	DBP020143	*T. gigas*	OP585108.1	666 Bp	99.37
2	DBP020145	*C. rotundicauda*	MW454812.1	666 Bp	99.54
3	DBP020147	*T. gigas*	OP585108.1	666 Bp	98.91
	Semarang, Central Java				
4	DBP020144	*T. gigas*	JF896114.1	666 Bp	98.97
5	DBP020146	*T. gigas*	OP585108.1	666 Bp	99.06
6	DBP020148	*T. gigas*	OP585108.1	666 Bp	98.75
	Kampung Laut, Jambi				
7	DBP020149	*C. rotundicauda*	JF896106.1	666 Bp	99.53
8	DBP020150	*C. rotundicauda*	AF370828.1	666 Bp	99.70
9	DBP020151	*C. rotundicauda*	AF370828.1	666 Bp	99.70
10	DBP020152	*C. rotundicauda*	AF370828.1	666 Bp	99.70
11	DBP020153	*C. rotundicauda*	AF370828.1	666 Bp	99.70
12	DBP020154	*C. rotundicauda*	AF370828.1	666 Bp	99.70
	Pangkal Niur, Bangka Belitung				
13	DBP020155	*C. rotundicauda*	MF469062.1	666 Bp	99.85
14	DBP020156	*C. rotundicauda*	AF370828.1	666 Bp	99.70
15	DBP020157	*C. rotundicauda*	MF469062.1	666 Bp	99.54
16	DBP020158	*C. rotundicauda*	AF370828.1	666 Bp	99.70
17	DBP020159	*C. rotundicauda*	AF370828.1	666 Bp	99.54
18	DBP020160	*C. rotundicauda*	AF370828.1	666 Bp	99.54
	Tuban, East Java				
19	DBP020161	*T. gigas*	JF896114.1	666 Bp	99.11
20	DBP020162	*C. rotundicauda*	MW454812.1	666 Bp	99.39
21	DBP020165	*C. rotundicauda*	MW454812.1	634 Bp	99.22
22	DBP020166	*C. rotundicauda*	MW454812.1	666 Bp	99.54

In the second clade, the *T. gigas* sequences were compared with NCBI data from Malaysia (Periasamy *et al*., 2017), India, Bangladesh, China, and France, indicating a close genetic relationship with Malaysian samples. The sequences from India and Bangladesh clustered together, while those from China and France appeared distantly related. The third clade consists of *Limulus polyphemus*, a horseshoe crab species from America, supporting the genetic distance findings (Figure 2).

Molecular identification techniques, particularly DNA barcoding, are crucial for accurately identifying cryptic species and preventing the mislabeling of wild-caught organisms. Mislabeling poses risks to ecosystem stability and can contribute to the decline of horseshoe crab populations. For instance, in Sarawak, Malaysia, *T. gigas* faces numerous threats, including overharvesting for food, habitat degradation, and insufficient governmental protections (Jawahir *et al*., 2017; Meilana *et al*., 2020).

Similarly, a documented decline in *T. tridentatus* and a corresponding increase in *C. rotundicauda* populations at Pak Nai and Ha Pak Nai, Hong Kong, has been attributed to anthropogenic factors, particularly the construction of a bridge linking Hong Kong to Shenzhen (Lee and Morton, 2016; Meilana *et al*., 2020). The integration of molecular methods into stock assessment practices is essential for effective fisheries management and conservation efforts

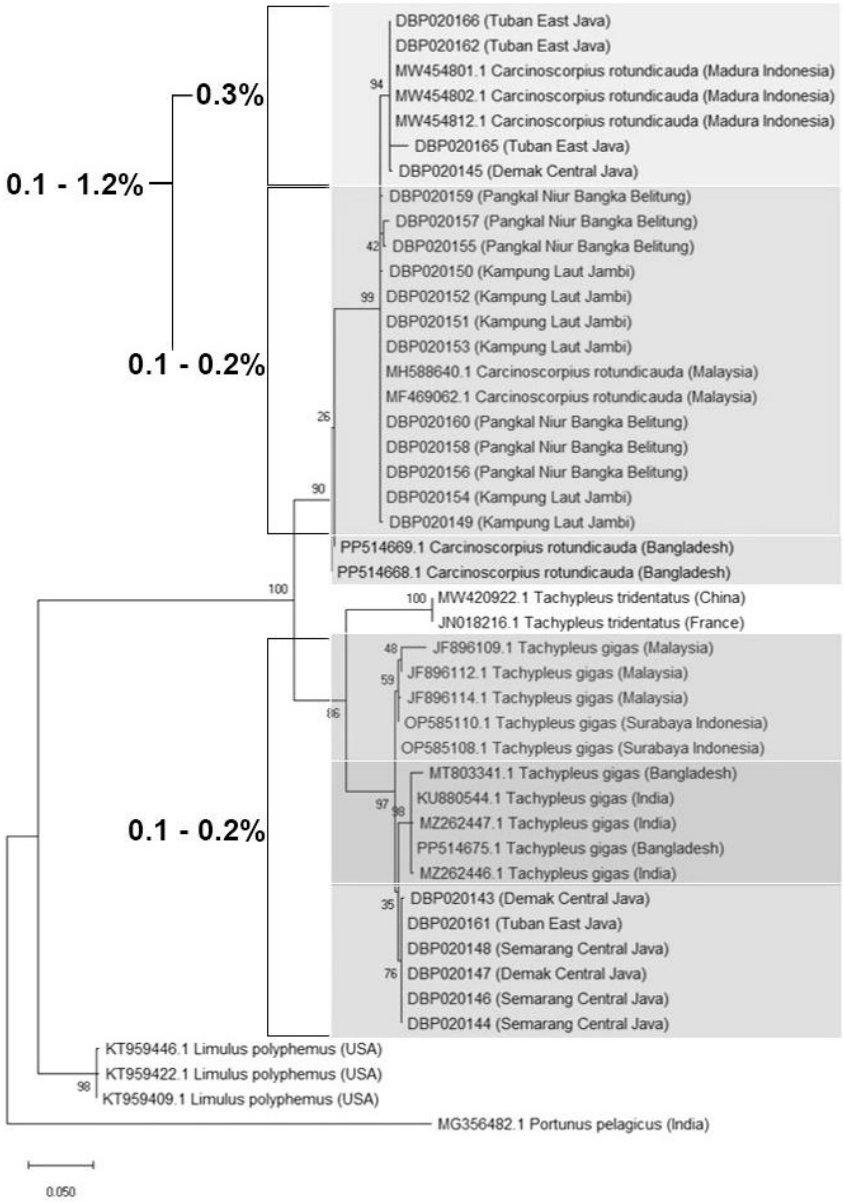

Figure 2. Phylogenetic tree of four different sites of horseshoe crabs. It was performed using the Maximum likelihood method, Hasegawa-Kishino-Yano + G (HKY+G). As an outgroup, one species was added (*Portunus pelagicus*) to strengthen the obtained data.

(Khamnamtong *et al*., 2021; Madduppa *et al*., 2021; Hardianto *et al*., 2022; Hodgdon, 2022; Rumisha and Kochzius, 2023; Joesidawati *et al*., 2023). Enhanced genetic approaches can significantly bolster governmental strategies in sustainable fisheries management.

CONCLUSION

DNA barcoding successfully identified two species from the two islands studied. In Sumatra, samples were collected from two sites, Jambi and Bangka Belitung, revealing *Carcinoscorpius rotundicauda***.** In Java, samples from three sites—Semarang, Demak, and Tuban—identified both *C. rotundicauda* and *Tachypleus gigas*. The phylogenetic tree indicates that genetic closeness varies by site, with *C. rotundicauda* acting as a dividing line between the islands of Sumatra and Java. Notably, *T. gigas* was only found in Java, likely due to a lack of samples and data from

Sumatra, which hindered its detection there. Ultimately, further population studies and ecological research, including habitat assessment, life cycle investigations, and habitat pressure analysis, are essential for a more comprehensive understanding of these species.

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