Molecular Identification and Phylogenetic Reconstruction of Horseshoe Crab (Xiphosura, Limulidae) from the East Java Region

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Abstract

Horseshoe crabs, ancient marine arthropods of the Limulidae family, are currently facing significant threats due to overexploitation driven by high demand for their eggs, which are widely consumed, and their hemolymph, which is critical for biomedical applications. This study aims to evaluate the molecular identification and phylogenetic placement of horseshoe crabs in East Java, a region previously unreported in this context. DNA barcoding was employed to analyze the mitochondrial COI gene region, complemented by detailed morphological identification, to provide a comprehensive understanding of the species in this area. A universal primer LCO/HCO was used for PCR, as described in a previous report, and Sanger sequencing was performed to confirm the approximately 655 bp amplification product. This research identifies horseshoe crabs from six distinct locations in East Java as Tachypleus gigas using molecular methods, with genetic analysis indicating>99% similarity to T. gigas sequences in the GenBank database. Morphological examinations of specimens from seven areas further corroborated the presence of T. gigas, distinguished by characteristic serrations along the telson and a single spine on the posterior region of the opisthosoma. The phylogenetic tree, constructed from our genetic data, shows that Tachypleus gigas populations in East Java are clustered in the same clade as those in Southeast Asia, as supported by GenBank records. This study underscores the importance of integrating genetic and morphological approaches for accurate species identification. Moreover, it provides crucial baseline data for developing conservation strategies to preserve Tachypleus gigas populations in East Java, thereby contributing to broader conservation efforts for this species.

Keywords: *Tachypleus gigas, COI, DNA barcoding, Indonesia, phylogeny, conservation baseline*

Introduction

Horseshoe crabs are remarkable marine arthropods belonging to the Limulidae family that have existed for approximately 485 million years with minimal morphological change. This remarkable evolutionary stability has earned them the title of "living fossils" (Van Roy *et al.,* 2010), although this title is not exclusive to horseshoe crabs. Currently, there are only four extant species of Limulidae: *Limulus polyphemus*, found exclusively in North American waters, and three species, *Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*, which inhabit Asian waters (Shingate *et al.,* 2020). Recently, the wild population of horseshoe crabs has been declining. They are frequently hunted for their eggs, consumed as a delicacy, and for their blood (hemolymph), which is extensively used for biomedical purposes due to its unique properties (John *et al.,* 2018). Additionally, significant changes to their natural habitats and spawning grounds, driven by various anthropogenic activities, have exacerbated population declines (John *et al.,* 2011). Although substantial research has been conducted on the biology, physiology, and ecology of horseshoe crabs, studies focusing on their molecular identification techniques are scarce in Indonesia, particularly in East Java, where such research has been reported. Similar research has recently been reported in Surabaya, Bangkalan, Pasuruan, and Probolinggo, where two species, *T. gigas*, and *C. rotundicauda*, were found (Rahayu *et al.,* 2024). The species *T. tridentatus* has been confirmed to be found in Balikpapan (Wardiatno *et al.,* 2018). However, there are still many limitations to research on this species due to its limited natural habitat.

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Despite their ecological significance, horseshoe crabs are not considered a key fisheries commodity in Indonesia, resulting in limited efforts to cultivate them. This lack of cultivation and conservation has contributed to the exploitation and subsequent population decline of horseshoe crabs in the wild (Carmichael and Brush 2012). Conservation efforts are crucial to maintaining and potentially increasing their population numbers (Tanacredi *et al.,* 2009). Fortunately, horseshoe crabs in Indonesia are protected under Regulation No. P.106/MENLHK /SETJEN /KUM.1/ 12/2018 (John *et al.,* 2018) and Government Regulation Number 7 of 1999. This law (Article 8, paragraph 3) states that the management of protected plant and animal species in their natural habitats includes activities such as identification, inventory, monitoring, habitat and population development, species rescue, and research and development. These regulations provide a framework for implementing effective conservation strategies, including identifying species and providing up-to-date data on their status (Supadminingsih *et al.,* 2018).

Studies on molecular identification in the COI region are important for confirming species identifications, especially along the East Java Coastline, and for adding them to the GenBank database. Understanding the genetic diversity within and between populations of horseshoe crabs is crucial for developing effective conservation strategies. In Indonesia, where molecular genetic studies on horseshoe crabs are limited, there is an urgent need to fill this knowledge gap. East Java, in particular, lacks reported studies on the genetic identification of horseshoe crabs, making this region a priority for research.

A report on horseshoe crab conservation in Asia reveals that Indonesia ranks second-to-last in terms of available genetic databases in the region, on a partial region of COI (John *et al.,* 2018). Studies on genetic diversity are essential for understanding the genetic variation within species. Traditional morphological identification methods alone cannot provide precise and accurate species information; hence, molecular genetic identification is necessary for more reliable results (Hubert and Hanner 2015). One common approach in genetic studies is DNA barcoding, which has gained widespread acceptance in identifying animal species (Andriyono *et al.,* 2022a; 2022b).

In DNA barcoding, the cytochrome oxidase subunit I (COI) gene region from mitochondrial DNA is widely recognized as the standard gene region for animal identification (Sachithanandam *et al.,* 2015). Sequencing the COI gene region of horseshoe crabs in East Java can provide valuable genetic information complemented by morphological data. This research aims to identify horseshoe crabs in the East Java region through DNA barcoding, thereby contributing to the body of knowledge necessary for effective conservation efforts. The study's findings are expected to enhance our understanding of the genetic diversity of horseshoe crabs in this region and support conservation initiatives aimed at maintaining their populations in the wild.

Through DNA barcoding, researchers can obtain accurate and reliable genetic data, which is essential for the conservation and management of horseshoe crab populations. The COI gene region is a robust marker for species identification of the horseshoe crab (Dhar *et al.,* 2016), enabling researchers to distinguish between different species and understand their genetic relationships through >2.5 million COI sequences available (Porter and Hajibabaei 2018). This information is vital for implementing targeted conservation measures, such as protecting critical habitats, managing breeding programs, and monitoring population dynamics. This method was successfully documented in several reports on horseshoe crab DNA Barcoding (Dhar *et al.,* 2016, Fairuz-Fozi *et al.,* 2021, Insafitri and Nugraha 2024).

Materials and Methods

Morphometric data collection and sample collection for molecular identification were conducted across six districts or cities in the East Java Province (Figure 1), specifically Tuban (5 samples), Lamongan (4 samples), Surabaya (3 samples), Pasuruan (2 samples), Probolinggo (2 samples), and Bangkalan (3 samples) (Table 1). The sampling period extended from February to April 2024. Horseshoe crab samples were obtained from the bycatch of local fishermen, and this research cannot collect a specific number of samples at each sampling site. The total number of samples used in this study is 23 horseshoe crabs. The number of horseshoe crabs found in each sampling site depends on the local fishermen's bycatch. For each location, horseshoe crabs with intact bodies were selected for morphological observations and morphometric measurements. Tissue from six samples was collected for DNA extraction and further analysis according to the Lobster protocol (Andriyono *et al.,* 2019, Insafitri and Nugraha 2024) or the DNA extraction in Fish. The samples for molecular identification consisted of 0.1 g of walking leg muscle tissue collected from one horseshoe crab at each location (Andriyono *et al.,* 2019). The horseshoe crab was collected from local fishermen. The morphological method involves taking a photo and collecting several standard morphological characteristics after samples are collected, while molecular identification is conducted in the laboratory after tissue preservation. All the samples are dead; therefore, this research did not require an ethics permit. The ethics approval process is not required for projects involving invertebrates other than cephalopods (Andrews 2024). These tissue samples were used for subsequent molecular studies conducted in April and May 2024 at the laboratory of the Professor Nidom Foundation in Surabaya.

Morphological-based Identification

Morphological identification involves in situ observations and measurements of various morphometric parameters as described in the manual identification of horseshoe crabs (Dolejš and Vaňousová 2015). Each intact horseshoe crab specimen was measured for body weight (BW), total length (TL), carapace length (CL), prosomal width (PW), and tail length (TEL) (Figure 2), and the average morphometric measurement data. Length parameters were measured with digital calipers (0.01 mm precision), and body weight was measured with a digital scale (0.01 g). The morphological data were documented for comparison with molecular identification results, providing a comprehensive assessment of horseshoe crab specimens collected from different locations in East Java. Given its protected conservation status in Indonesia, we did not set a specific number of horseshoe crabs to be caught at each location. As a result, the research was unable to ensure that both sexes of horseshoe crabs were collected at each location and could not make sex comparisons across sites.

Despite their ecological significance, horseshoe crabs are not considered a key fisheries commodity in Indonesia, resulting in limited efforts to cultivate them. This lack of cultivation and conservation has contributed to the exploitation and subsequent population decline of horseshoe crabs in the wild (Carmichael and Brush 2012). Conservation efforts are crucial to maintaining and potentially increasing their population numbers (Tanacredi *et al.,* 2009). Fortunately, horseshoe crabs in Indonesia are protected under Regulation No. P.106/MENLHK /SETJEN /KUM.1/ 12/2018 (John *et al.,* 2018) and Government Regulation Number 7 of 1999. This law (Article 8, paragraph 3) states that the management of protected plant and animal species in their natural habitats includes activities such as identification, inventory, monitoring, habitat and population development, species rescue, and research and development. These regulations provide a framework for implementing effective conservation strategies, including identifying species and providing up-to-date data on their status (Supadminingsih *et al.,* 2018).

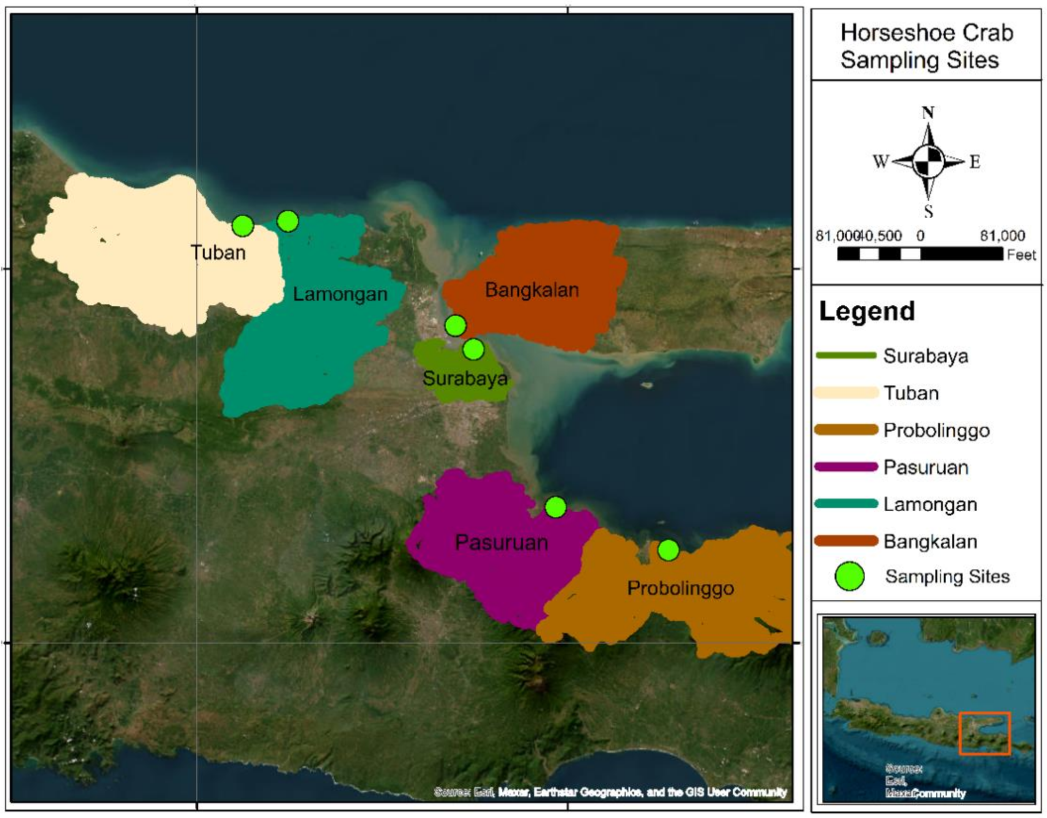

Figure 1. Sampling site for horseshoe crab collection along the north of the East Java coast

Table 1. Geographical coordinate of the sampling site

Location	Samples Number
Palang subdistrict, Tasik Madu village, Tuban.	5
Blimbing, Paciran subdistrict, Lamongan.	4
Kenjeran subdistrict, Bulak Banteng Village, Surabaya.	3
Pengaletan, Jatirejo subdistrict, Pasuruan.	2
Randuputih Village, Tringu subdistrict, Probolinggo.	2
Kamal subdistrict, Gilih Baru Village, Bangkalan	3

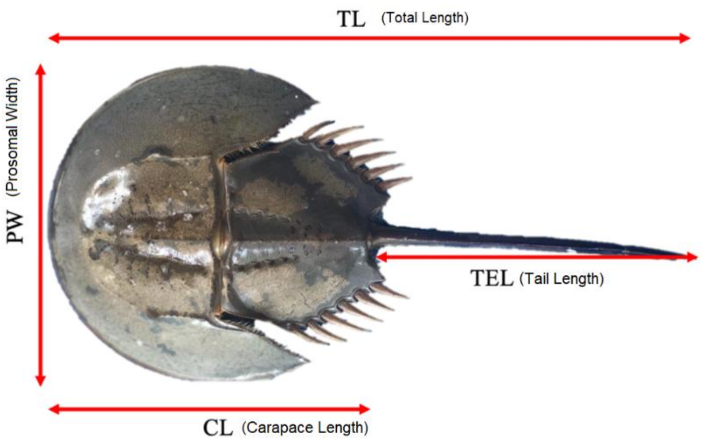

Figure 2. Morphometric measurement of the horseshoe crab

DNA Extraction

A total of seven samples were used for molecular identification. One horseshoe crab was chosen from samples collected in each site, and 0.1 g of leg muscle was taken. Molecular identification was performed using DNA barcoding techniques (Fatimah *et al.,* 2023). The muscle tissue samples collected for this purpose were initially stored in PBS (Phosphate Buffered Saline) solution and kept at -20°C until further processing. DNA extraction was performed using the ZymoBIOMICS™ DNA Miniprep Kit (Irvine, CA) according to the manufacturer's protocol to ensure high-quality DNA yield.

PCR Amplification

The extracted DNA was the template for Polymerase Chain Reaction (PCR) amplification. The target gene region for this study was the cytochrome oxidase subunit I (COI) gene, which is widely used for DNA barcoding in animals (Sachithanandam *et al.,* 2015). Universal primers designed by Hebert *et al*. (2003) were employed: LCO1490F (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO2198R (5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3') (Hebert *et al.,* 2003). The PCR reaction mixture consisted of 50 µL volumes (Vrijenhoek 1994) containing 12 µL PCR-grade pure water, 25 µL of KOD OneTM PCR Master Mix (Osaka, Japan), 1.5 µL of 10 mol forward and reverse primers, and 10 µL of DNA sample to make up the final volume. The PCR cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension at 72 °C for 5 minutes (Vrijenhoek 1994).

Gel Electrophoresis and DNA Sequencing

The PCR products were verified by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light (Vrijenhoek 1994). Successful amplifications were purified and sent to The1st BASE laboratory in Singapore for Sanger sequencing. DNA sequencing was carried out in three steps; the first step was labeling, where the purified DNA was labeled with the Big Dye™ Terminator kit version 3.1 (Waltham MA). After that, the final step is sequencing using the ABI 310 xL Genetic Analyzer sequencer machine (Applied Biosystems, Inc.). The sequencing services provided high-quality reads of the amplified COI gene region.

Data Analysis

This research presents both morphological and molecular data. The morphology of the horseshoe crab was identified through phenotypic observation and comparison with the identification key provided by Dolejš and Vaňousová (Dolejš and Vaňousová 2015). Molecular identification was performed by extracting DNA, followed by PCR amplification and sequencing of the PCR products. The sequences were initially analyzed using Chromas 2.6.6 (https://technelysium.com.au/wp/chromas/) to trim and verify their accuracy. For species identification, the trimmed sequences were compared against the GenBank database using the Basic Local Alignment Search Tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi on the National Center for Biotechnology Information (NCBI) website. The sequences were matched with known entries to identify species. Further phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGAX) software (Kumar *et al.,* 2018) with the NJ (neighbor-joining) Kimura's 2 parameters as the method used. After morphological identification was completed, accuracy was confirmed by molecular identification. Molecular identification was performed by comparing the DNA sequence obtained with sequences in the GenBank database. Further molecular identification is performed by constructing a phylogenetic tree to determine the position within a clade relative to the same species or other species within the same genus. A phylogenetic tree was constructed to visualize the genetic relationships among the horseshoe crab specimens from different locations in East Java. In addition to the DNA sequences obtained in this study, we also added several DNA sequences obtained from the NCBI GenBank database including *Tachypleus gigas* (PP448062, OP585108, and NC052702) *Tachypleus tridentatus* (MT803341 and NC012574) and *Carcinoscorpius rotundicauda* (NC019623), as well as *Parathelphusa convexa* (OR516568) as an outgroup. This comprehensive approach combined morphological and molecular data to provide a detailed understanding of the region's genetic diversity and the distribution of horseshoe crab species.

Result and Discussion

Based on morphological observations of horseshoe crabs collected from seven locations in East Java (Tuban, Lamongan, Surabaya, Pasuruan, Probolinggo, Situbondo, and Bangkalan), all specimens were identified as belonging to a single species, *Tachypleus gigas*. The primary phenotypic characteristics that differentiate the three Asian horseshoe crab species are the shape of the telson (tail) and the number of spines on the posterior part of the ophistoma (abdomen) (Meilana *et al.,* 2016). The horseshoe crabs from all seven locations exhibited consistent phenotypic characteristics, specifically the presence of serrations along the telson and the absence of three spines on the posterior part of their opisthosoma (Figure 2). These features are distinctive to *T. gigas*, as the other two Asian horseshoe crab species do not share these characteristics (Wang *et al.,* 2020).

Tachypleus tridentatus, while possessing a serrated telson, is distinguished by the presence of three prominent spines on the posterior part of the opisthosoma. Conversely, *Carcinoscorpius rotundicauda* differs significantly from the other two species in having a smooth, non-serrated telson. The consistent shape and color of the horseshoe crabs found in East Java further confirmed their identification as *Tachypleus gigas*. The prosoma of the observed specimens was semi-circular, resembling a horseshoe, and their coloration was generally a dark brownish-green. Telson Shape with spines; Telson cross-section shapes a sharp triangle; frontal side curved; shape of third appendage on prosoma pointed downward; shape of second appendage on prosoma one type; marginal spine reduced; genital pore lower part fused; and color blackish brown.

The morphological homogeneity across all sampled locations in East Java suggests a stable and possibly isolated population of *T. gigas* in this region. The observed characteristics align with the known descriptions of *T. gigas*, reinforcing the accuracy of the morphological identification (Figure 3 and 4).

Horseshoe crabs found in East Java have an average total length range of 25.6 to 44.4 cm (Table 2), with the smallest average total length found in Bangkalan and the largest in Situbondo. Previous research has reported that the horseshoe crabs studied have an average total length range of 36.5 to 36.9 cm (Anzani *et al.,* 2024); however, another report indicates a carapace length range of 25-26 cm (Mashar *et al.,* 2017). The prosomal width of horseshoe crabs in East Java ranges from 11.6 to 22.1 cm, whereas a previous study reports a range of 17.10 to 21.39 cm. The carapace length of horseshoe crabs from East Java ranges from 12.3 to 24.1 cm, whereas a previous study reports a range of 17.92 to 23.90 cm. The body weight of horseshoe crabs in East Java ranges from 159.0 to 762.6 cm; a previous study shows a range of 282.93 to 662.69 g. In this study, we found *T. gigas* to be smaller and bigger than the range size in the previous study. Differences in the size and weight of horseshoe crabs are likely due to differences in food availability and habitat. The food available in mangrove areas with muddy substrates allows for high organic content (Muhammad *et al.,* 2017). In addition, size is closely related to sexual parameters, including the species *T. gigas* (Faurby *et al.,* 2011). Meanwhile, different habitats, such as depth, can affect lighting, including shading from substrates like mangrove areas, seagrass beds, and coral reefs, resulting in varying

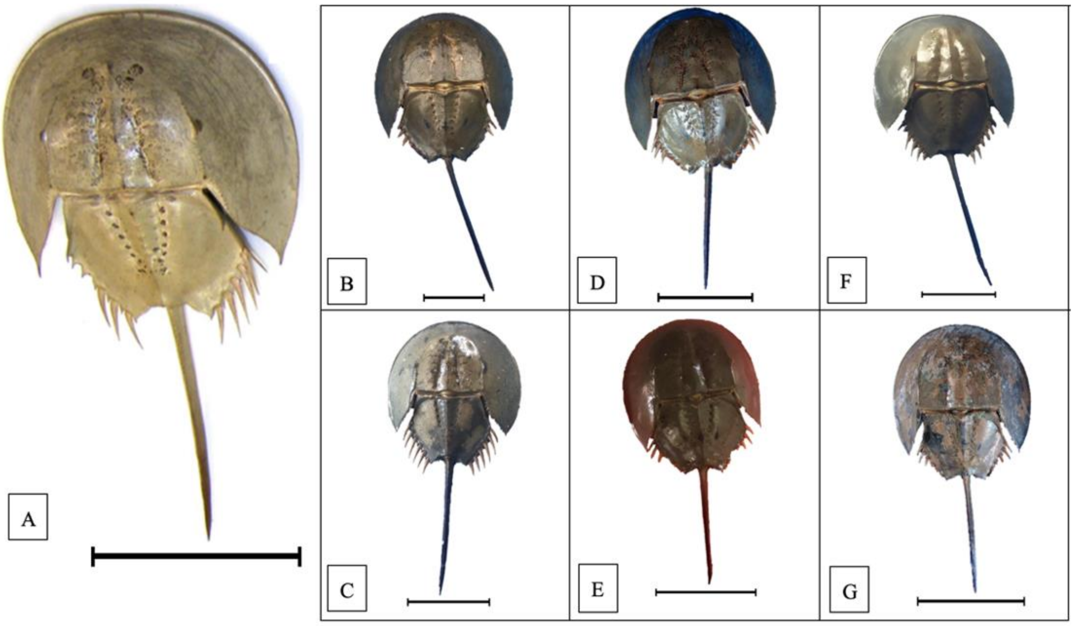

Figure 3. *Tachypleus gigas* is found in East Java. A) morphological features based on Dolejš and Vaňousová (2015); B) Tuban; C) Lamongan; D) Surabaya; E) Pasuruan; F) Probolinggo; G) Bangkalan. Black line: Scale bar of 10 cm.

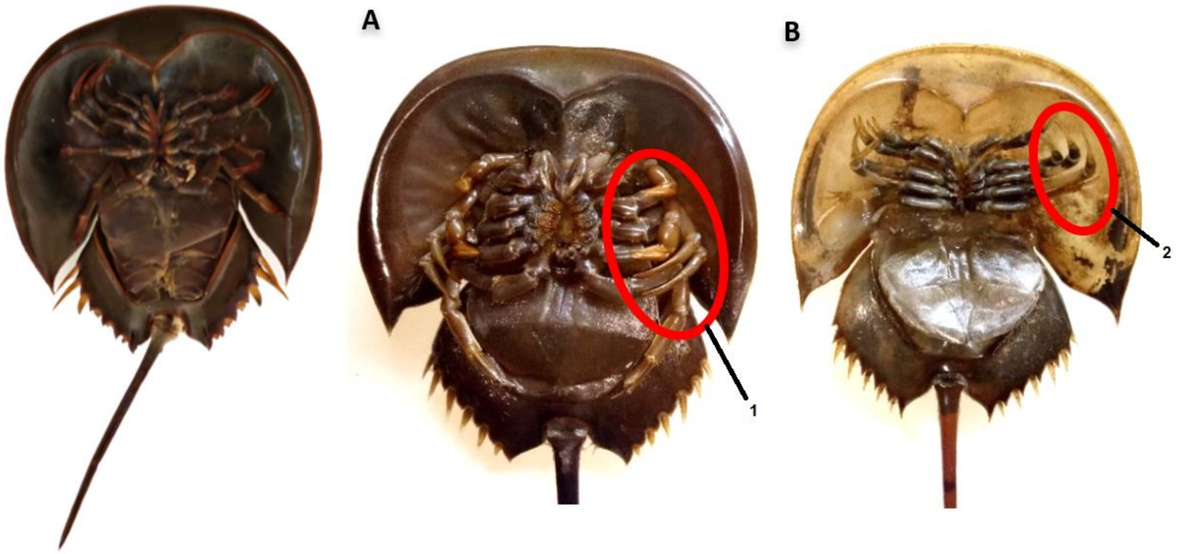

Figure 4. Morphological differences in *Tachypleus gigas*. A. Females have scissor-like chelate pincers (red circle, 1)), while B. males possess hemichelate claspers on walking legs I and II (red circle, 2)

light intensities. It has been reported that *Carcinoscopius rotundicauda* larvae are very sensitive to light conditions, which are thought to influence avoidance of predation, prey selection, and preference for water quality in their natural habitat (Srijaya *et al.,* 2014).

Horseshoe Crab Molecular Identification

This research represents the first report of molecular identification of horseshoe crabs, specifically focusing on the East Java region. Through molecular techniques, horseshoe crabs from four districts in East Java were successfully identified. The genetic sequences from horseshoe crabs collected in Situbondo were of low quality, indicating insufficient sequence quality for reliable molecular identification. Therefore, these sequences were excluded from further analysis to maintain accuracy. The six remaining sequences obtained from the samples in Tuban, Lamongan, Bangkalan, Surabaya, Pasuruan, and Probolinggo were of higher quality and subsequently compared with sequences in GenBank for species confirmation.

The BLAST analysis results for the samples are summarized in Table 3. The sequences exhibited over 98-99% similarity with the GenBank sequences, indicating that the horseshoe crab samples from East Java are genetically identical to the species documented in GenBank. Bhattacharjee *et al*. (2012) stated that a sequence identity percentage of 98%-100% denotes homology of the same species (Bhattacharjee *et al.,* 2012). Additionally, the East Java horseshoe crab samples showed an e-value of 0.0, which signifies a highly reliable and identical comparison (Maulid *et al.,* 2016). An e-value closer to one indicates lower identity, but the zero e-value underscores the accuracy of the species match.

The phylogenetic analysis revealed that the four horseshoe crab samples from East Java are closely related to *Tachypleus gigas* specimens from Southeast Asia, as documented in GenBank. The phylogenetic tree also depicts another *T. gigas* clade that is slightly more distantly related to the East Java samples (Aini *et al.,* 2020a, Maulana *et al.,* 2023). The tree shows that position *T. gigas* comprises specimens from India and Bangladesh. Previous reports have highlighted that geographic factors can significantly influence the genetic diversity of horseshoe crabs, with geographic barriers and limited dispersal abilities leading to genetic differences among populations (Aini *et al.,* 2020a, 2021b).

The phylogenetic tree further illustrates the separation of clades for *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*, the two other species of Asian horseshoe crabs (Aini *et al.,* 2021b, Maulana *et al.,* 2023). In contrast, *Carcinoscorpius rotundicauda* appears more distantly related, consistent with its classification in a different genus. Despite these differences, all three species belong to the same family, Limulidae. Additionally, the phylogenetic tree includes a final clade representing *Parathelphusa convexa*, which serves as an outgroup (Winarni *et al.,* 2024). This inclusion helps root the tree and provides context for the relationships among horseshoe crab species.

The high-quality sequences obtained and analyzed in this study have provided definitive molecular evidence for the identification of horseshoe crabs in East Java. The close genetic relationship to *T. gigas* specimens from Southeast Asia supports the morphological identification conducted in the field. Furthermore, the findings underscore the importance of integrating molecular techniques with traditional morphological methods to achieve accurate species identification (Aini *et al.,* 2021a; 2021b).

The genetic homogeneity observed among the East Java samples suggests a stable population of *T. gigas* within this region (Mashar *et al.,* 2017). As previous studies on horseshoe crab populations suggested, this stability might be influenced by geographical isolation or limited dispersal. The consistent morphological and genetic data reinforce the reliability of the identification and underscore the importance of preserving these genetically distinct populations (Aini *et al.,* 2020, 2021b).

Table 2. The average of the morphometrics measurements at each location on the horseshoe crab parameters

Location	Parameters (±SD)				
	BW	CL	TEL	TL	PW
Tuban	433.1±132.2	20.0±1.5	17.5±3.4	37.7±3.5	19.5±1.1
Lamongan	223.0±97.5	15,4±0.9	14.5±1.3	28.5±6.8	14.9±0.9
Surabaya	244.0±42.4	17.0±0.1	16.7±1.1	33.0±1.3	16.5±0.8
Bangkalan	159.0±23.5	12.3±0.5	13.1±1.7	25.6±2.0	11.6±0.8
Pasuruan	161.0±08.4	13.1±02	13.5±0.7	25.7±0.3	12.2±0.1
Probolinggo	227.5±03.5	16.4±0.1	15.1±1.5	31.7±1.8	17.1±0.4

BW: Body Weight; CL: carapace length; TEL: tail length; TL: total length; PW: prosomal width

Table 3. BLAST Nucleotides comparison results to GenBank on NCBI

Sample Code	Location	Species	Identity (%)	Gene Bank Reference
TBN	Tuban	*Tachypleus gigas*	99.20	OP585108.1
LMG	Lamongan	*Tachypleus gigas*	99.42	NC_052701
BKL	Bangkalan	*Tachypleus gigas*	99.83	OP585108.1
SBY	Surabaya	*Tachypleus gigas*	98.79	KJ825848.1
PSR	Pasuruan	*Tachypleus gigas*	98.99	KJ825848.1
PBL	Probolinggo	*Tachypleus gigas*	99.40	KJ825848.1

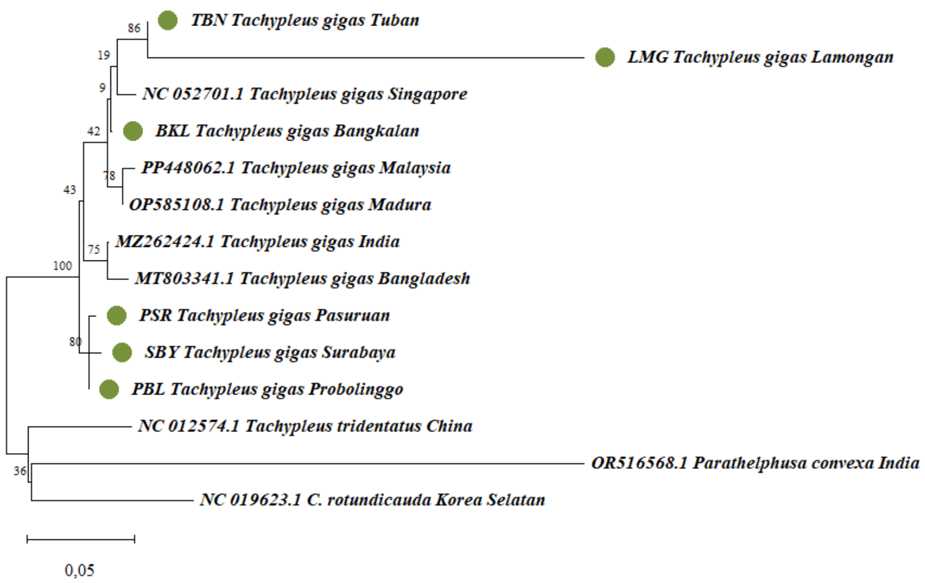

Figure 5. Phylogenetic tree of the horseshoe crab's COI region gene constructed using MEGAX software (Kumar *et al.,* 2018). Note: green dot indicates the samples in this study

This study successfully employed molecular identification techniques to accurately identify horseshoe crabs in East Java. The high sequence similarity with known *T. gigas* specimens from Southeast Asia, combined with detailed phylogenetic analysis, confirms the species' identity and provides insights into the genetic diversity and evolutionary relationships among horseshoe crab populations. This research fills a critical knowledge gap and contributes valuable genetic data for the conservation and management of horseshoe crabs in Indonesia. Integrating molecular and morphological data sets provides a robust foundation for future studies and conservation efforts, ensuring the protection and sustainability of these ancient marine arthropods.

Horseshoe Crab Conservation Status

Indonesia has recently recognized *Tachypleus gigas* as a protected species, Law No. 106/MENLHK/SETJEN/KUM.1/12/2018 (Alfiana 2020). This protection status reflects the growing awareness of the species' ecological importance and the need to conserve its populations. Additionally, according to the above regulation, *T. gigas* is not considered harmful to humans. 19/PERMEN-KP/2020. However, the international conservation status of *T. gigas* remains concerning, as it is categorized as Data Deficient by the IUCN (International Union for Conservation of Nature and Natural Resources) Red List of Threatened Species (Aini *et al.,* 2021a, Pati *et al.,* 2021). This classification indicates that insufficient information is available to assess the extinction risk of this species. Notably, *T. tridentatus* is the only Asian horseshoe crab species well documented by the IUCN, and it is currently listed as Endangered, a status only two levels below Extinct in the Wild. This endangered status highlights the rarity of *T. tridentatus* in natural habitats, underscoring the urgency for conservation efforts (Laurie *et al.,* 2019, Zhu *et al.,* 2020).

Fatimah *et al.* (2023) reported the presence of *T. tridentatus* in Banyuasin, South Sumatra, Indonesia, confirmed through molecular identification. This finding suggests that *T. tridentatus*, although endangered, still exists in certain regions of Indonesia (Fatimah *et al.,* 2023). Conversely, *Carcinoscorpius rotundicauda,* similar to *T. gigas*, is also classified as Data Deficient by the IUCN, indicating a lack of comprehensive data on its population status and distribution.

Nonetheless, exploitation of the horseshoe crab population in Indonesia continues. On the east coast of North Sumatra, this is triggered by the very tempting price of the horseshoe crab, which can reach Rp. 150,000.head^{-1} for a female horseshoe crab that lays eggs. Other factors that threaten the declining horseshoe crab population are reduced food sources, overharvesting, and increased predation; the horseshoe crab is also found to be consumed by the long-tailed monkeys (*Macaca fascicularis*). In East Java, where this study was conducted, most research suggests that the declining population is due to the loss of suitable spawning grounds (Aini *et al.,* 2021). Furthermore, communities around East Java still commonly consume horseshoe crabs, especially eggs.

Moreover, the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) does not list *T. gigas* in any of its appendices. CITES primarily focuses on regulating international trade to ensure it does not threaten the survival of species in the wild. The absence of horseshoe crabs from CITES' appendices suggests that the international trade of these species has not been adequately documented or regulated. This omission is contradictory to the findings of John *et al*. (2018), who reported a significant demand for horseshoe crabs in China (John *et al.,* 2018). *T. gigas*, exported from Vietnam to China, is widely utilized for biomedical purposes, including the extraction of its blood for the *Tachypleus* Amebocyte Lysate (TAL) test (Krisfalusi-Gannon *et al.,* 2018). Following blood extraction, the meat is processed for food, and the exoskeleton carcasses are sun-dried and sold for chitin production (John *et al.,* 2011).

In Malaysia, horseshoe crab eggs have been exported for human consumption since 2012, highlighting the species' economic importance in the region (John *et al.,* 2018). Additionally, there have been instances of illegal transport of horseshoe crabs collected from Indonesian waters, intended for export to Thailand via Malaysia (Botton *et al.,* 2015, John *et al.,* 2018). These reports indicate substantial, often unregulated, international trade in horseshoe crabs, which poses a significant threat to their wild populations. Given these circumstances, there is a compelling need for CITES to begin documenting and regulating the trade of horseshoe crabs to mitigate illegal exports and overexploitation. Establishing regulatory frameworks and international cooperation is crucial to ensuring the sustainable management of horseshoe crab populations. Enhanced data collection and monitoring of the horseshoe crab trade can provide a foundation for developing effective conservation strategies (Chen *et al.,* 2004, Wang *et al.,* 2020). The findings from this study emphasize the critical need for comprehensive conservation measures for *T. gigas* and other horseshoe crab species in Indonesia. While national regulations provide a legal framework for protection, their effectiveness depends on enforcement and the cooperation of local communities and international bodies. Addressing data and regulatory gaps will be essential to the long-term preservation of these ancient marine arthropods.

Horseshoe Crab Conservation Status

Indonesia has recently recognized *Tachypleus gigas* as a protected species, Law No. 106/MENLHK/SETJEN/KUM.1/12/2018 (Alfiana 2020). This protection status reflects the growing awareness of the species' ecological importance and the need to conserve its populations. Additionally, according to the above regulation, *T. gigas* is not considered harmful to humans. 19/PERMEN-KP/2020. However, the international conservation status of *T. gigas* remains concerning, as it is categorized as Data Deficient by the IUCN (International Union for Conservation of Nature and Natural Resources) Red List of Threatened Species (Aini *et al.,* 2021a, Pati *et al.,* 2021). This classification indicates that insufficient information is available to assess the extinction risk of this species. Notably, *T. tridentatus* is the only Asian horseshoe crab species well documented by the IUCN, and it is currently listed as Endangered, a status only two levels below Extinct in the Wild. This endangered status highlights the rarity of *T. tridentatus* in natural habitats, underscoring the urgency for conservation efforts (Laurie *et al.,* 2019, Zhu *et al.,* 2020).

Fatimah *et al.* (2023) reported the presence of *T. tridentatus* in Banyuasin, South Sumatra, Indonesia, confirmed through molecular identification. This finding suggests that *T. tridentatus*, although endangered, still exists in certain regions of Indonesia (Fatimah *et al.,* 2023). Conversely, *Carcinoscorpius rotundicauda,* similar to *T. gigas*, is also classified as Data Deficient by the IUCN, indicating a lack of comprehensive data on its population status and distribution.

Nonetheless, exploitation of the horseshoe crab population in Indonesia continues. On the east coast of North Sumatra, this is triggered by the very tempting price of the horseshoe crab, which can reach Rp. 150,000.head^{-1} for a female horseshoe crab that lays eggs. Other factors that threaten the declining horseshoe crab population are reduced food sources, overharvesting, and increased predation; the horseshoe crab is also found to be consumed by the long-tailed monkeys (*Macaca fascicularis*). In East Java, where this study was conducted, most research suggests that the declining population is due to the loss of suitable spawning grounds (Aini *et al.,* 2021). Furthermore, communities around East Java still commonly consume horseshoe crabs, especially eggs.

Moreover, the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) does not list *T. gigas* in any of its appendices. CITES primarily focuses on regulating international trade to ensure it does not threaten the survival of species in the wild. The absence of horseshoe crabs from CITES' appendices suggests that the international trade of these species has not been adequately documented or regulated. This omission is contradictory to the findings of John *et al*. (2018), who reported a significant demand for horseshoe crabs in China (John *et al.,* 2018). *T. gigas*, exported from Vietnam to China, is widely utilized for

biomedical purposes, including the extraction of its blood for the *Tachypleus* Amebocyte Lysate (TAL) test (Krisfalusi-Gannon *et al.,* 2018). Following blood extraction, the meat is processed for food, and the exoskeleton carcasses are sun-dried and sold for chitin production (John *et al.,* 2011).

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Conclusion

Based on morphological identification, horseshoe crabs found at seven locations in East Java are all the same species, *Tachypleus gigas*. The distinguishing phenotypic characteristics observed included serrations along the telson and the presence of only one spine on the posterior part of the opisthosoma, rather than three. Molecular identification of the COI gene region corroborated these findings, confirming that horseshoe crabs from four locations in East Java are indeed *T. gigas*. According to GenBank data, the phylogenetic analysis further supported this conclusion, showing that the T. gigas specimens from East Java are most closely related to other T. gigas specimens originating from Southeast Asia. These findings highlight the genetic homogeneity of *T. gigas* populations in East Java and underscore the importance of integrating morphological and molecular approaches for accurate species identification. This research provides a foundation for future conservation efforts to protect the *T. gigas* populations in Indonesia.

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