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Genetic diversity and phylogenetic reconstruction of horseshoe crabs from East Java, Indonesia based on DNA barcode COI

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Abstract. *Rahayu DA, Amarwati R, Faizah U, Nugroho ED, Rusdianto, Ajiningrum PS, Mamat NB. 2024. Genetic diversity and phylogenetic reconstruction of horseshoe crabs from East Java, Indonesia based on DNA barcode COI. Biodiversitas 25: 3789-3802.* Horseshoe crabs or *mimi mintuna* (Indonesian) are an ancient arthropod with 4 species, but there are limited studies on the species from East Java. This indicates the need for the collection of more accurate genetic resources as an important step for its conservation. Therefore, this study aims to examine genetic diversity and phylogenetic reconstruction of horseshoe crabs from East Java using DNA barcode COI. A total of 20 samples were obtained from Beejay Bakau Resort, Probolinggo, Kenjeran Beach, Surabaya, Batah Timur Beach, Madura, and Lekok Beach, Pasuruan. The 621 bp CO1 gene sequence was used for genetic diversity analysis and phylogenetic tree reconstruction. Subsequently, muscle samples were preserved in pure-grade ethanol and subjected to DNA extraction, polymerase chain reaction (PCR), sequencing, and sequence analysis. The sequence data were analyzed using bioinformatics tools, Barcode of Life Data Systems (BOLD) Systems, the Automatic Barcode Gap Discovery (ABGD) web application, and haplotype network. Phenetic taxonomy was further applied using Ntysc to enhance the robustness of the genetic analysis. The results showed that the high haplotype diversity (Hd) of 0.957, 16 haplotypes, and nucleotide diversity (π) of 0.00097 indicated a wide range of genetic variations. The frequency of parsimony informative sites was 26.48%, with 19 polymorphic sites and an overall ts/tv ratio of 2.32. In addition, the phylogenetic trees showed a clear and unambiguous branching pattern for cluster *Carcinoscorpius rotundicauda* Latreille 1802 and *Tachypleus gigas* O.F.Müller 1785. This finding is a matter of concern for managing and conserving horseshoe crab species in East Java waters.

Keywords: Cytochrome c oxidase 1, DNA barcoding, genetic diversity, horseshoe crabs, phylogenetic

INTRODUCTION

Indonesia is an archipelagic and maritime country according to the United Nations Convention on the Law of the Sea. In addition, the Ministry of Maritime Affairs and Fisheries (*Kementrian Kelautan dan Perikanan*/KKP) in the 2011 Marine and Fisheries statistics book stated that Indonesia had an inland territorial sea area of 284.210.900 km^2, an Exclusive Economic Zone (EEZ) of 2.981.211.000 km^2, and a 12-mile sea area of 279.322.000 km^2 (Mangku 2020). Several studies have also shown that the territorial waters of the country are the most affluent marine areas in the Indo-Pacific region. This indicates that the waters have a high diversity of marine life, but some of the species have not been identified (Dhar et al. 2016; Brahmastara 2018; Ravi et al. 2022). Several Indonesian beaches are home to diverse marine life that has not been explored, particularly horseshoe crabs with the local name *mimi mintuna*. At present, a total of 4 species of horseshoe crabs have been recognized around the world. Among these species, the dominant 3 are Asian horseshoe crabs, namely *Tachypleus tridentatus* Leach 1819, *Carcinoscorpius rotundicauda* Latreille 1802, and *Tachypleus gigas* O.F.Müller 1785 (Lee and Morton 2005; Cartwright et al. 2009; Behera et al. 2015; Billy et al. 2017; Haque et al. 2024). The 4th species, *Limulus polyphemus* Linnaeus 1758, inhabits the Atlantic coast of North America (Walls et al. 2002). Horseshoe crabs in Indonesia have also been documented across several major islands, including Sumatra (Anggraini et al. 2017; Fatimah et al. 2023; Dwigothammy et al. 2024), Java (Meilana et al. 2016; Meilana and Fang 2020; Mauludiyah et al. 2022), Kalimantan (Erwyansyah et al. 2018), and Sulawesi (Meilana and Fang 2020).

Previous studies on horseshoe crab in Indonesia have explored a diverse range of topics, including dietary preferences (Nuraisah et al. 2020) and bioactive properties of biomaterials (Asih et al. 2018; Romadhon et al. 2018). Morphometric analyses have also been conducted (Anggraini et al. 2017; Fauziyah et al. 2019; Dwigothammy et al. 2024), along with examinations of environmental (Aini et al. 2020). In addition, several studies have focused on mapping this ancient arthropod's biodiversity and distribution

patterns across the Indonesian archipelago (Mishra 2009; Mashar et al. 2017). Horseshoe crab is a marine arthropod, often referred to as a living fossil, which lives in shallow water habitats and has a low species diversity since the Paleogene era (Obst et al. 2012; Vestbo et al. 2018; Lim et al. 2022). This species has various sizes, influenced by population size, food availability, and environmental conditions. Commercial hunting has also decreased the population in the world in the last 15 years (Mishra 2009). Therefore, further studies are necessary to identify genetic variability and phylogenetic reconstruction of this grouped species. In addition, molecular characterization can be used to strengthen morphological data for species identification. DNA barcoding with molecular marker is often used to strengthen identification based on morphological data (Hubert and Hanner 2016; Trivedi et al. 2016; Abdullah et al. 2019; Alcudia-Catalma et al. 2020; Ambarwati et al. 2021; Basith and Kusrini 2021; Juniar et al. 2021; Vieira et al. 2021; Radulovici et al. 2021; Sari et al. 2021; Nugroho et al. 2023; Rahayu et al. 2023)

Several studies have shown the efficacy of DNA barcoding in identifying horseshoe crab species within Indonesian populations (Meilana et al. 2016; Fatimah et al. 2023). Meilana et al. (2016) also successfully employed COI gene sequences to differentiate between *T. gigas* and *C. rotundicauda* from Java's north coast. In addition, Anggraini et al. (2017) used similar techniques to confirm the presence species in Bengkulu waters, Sumatra. A more comprehensive study by Fauziyah et al. (2019) applied DNA barcoding to samples from various Indonesian locations, revealing distinct genetic. Mashar et al. (2017) used molecular methods to assess the genetic diversity of *T. gigas* populations.

DNA barcoding facilitates rapid and accurate species differentiation, often proving superior to traditional morphological identification methods (Khedkar et al. 2016; Erwyansyah et al. 2018; Li et al. 2022; Meilana and Fang 2020). These gene guarantee the consistency and reliability of genetic data derived from COI sequences for species identification purposes. The stability enhances the utility of COI sequences in molecular identification studies, allowing for quick and reliable differentiation between species, which is particularly useful in large-scale biodiversity assessments and conservation efforts. The knowledge of the population genetic diversity of these species is essential to develop new insight into the management and conservation of endangered marine animals.

MATERIALS AND METHODS

Study area

Specimens of horseshoe crabs were obtained from Beejay Bakau Resort Probolinggo, Kenjeran Beach Surabaya, Batah Timur Beach Madura, and Lekok Beach Pasuruan, East Java, Indonesia (Figure 1). At each location, sampling was conducted randomly, taking into account the lowest tide. Preparation includes sterilizing the biopsy needle and carefully handling the animals. The biopsy process involves carefully inserting the needle into the muscle and extracting a small amount of muscle tissue. The sample is then gently withdrawn, confirming that the muscle tissue has been successfully collected. The horseshoe crab is then conditioned by releasing it back into the sea. Before the genetic sampling, a thorough yet quick morphological observation is conducted carefully.

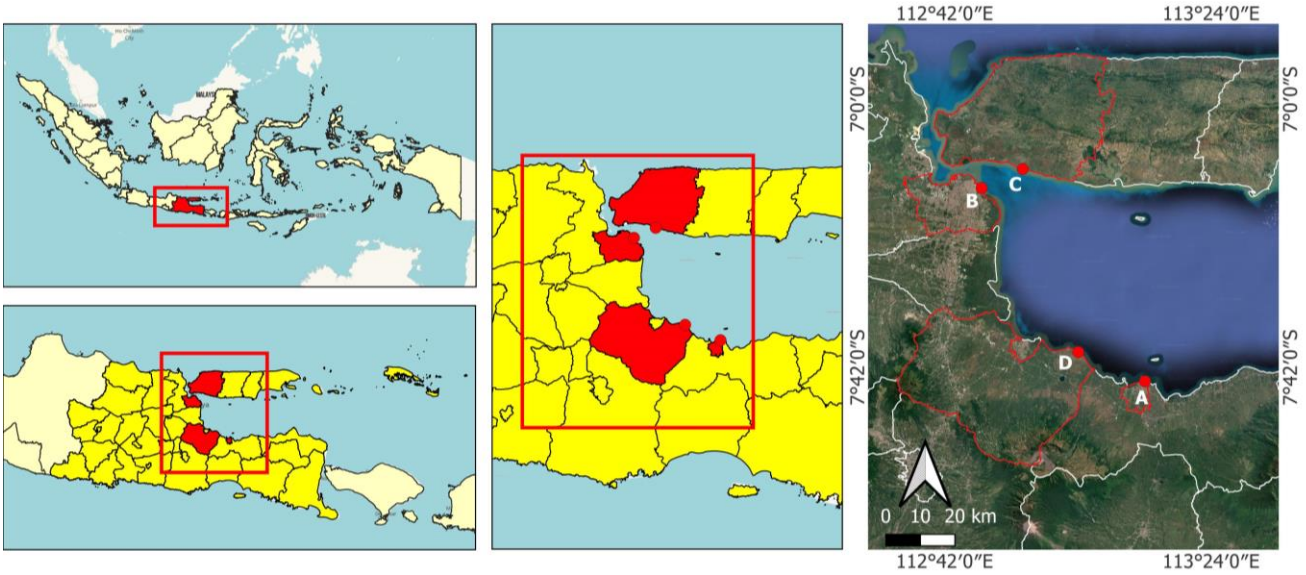

Figure 1. The map of: A. Beejay Bakau Resort, Probolinggo City; B. Kenjeran Beach, Surabaya; C. Batah Timur Beach, Bangkalan District; D. Lekok Beach, Pasuruan, East Java, Indonesa

Samples were abundant at some of the farthest low tides from each beach. Five individuals of each type were identified for further analysis. Subsequently, 15 to 20 mg of muscle were placed into bottles filled with 96% absolute ethanol. Cartwright et al. (2009; 2012) were applied as a reference to analyze the morphological characteristics of horseshoe crab in this study. Based on the morphological characteristics, these samples were grouped into *C. rotundicauda* and *T. gigas*. The samples were undertaken with muscle of 15 to 20 mg for preservation in absolute ethanol (96%), and further prepared for molecular analysis. Molecular analysis was conducted at the Study and Genetics Laboratory, at the Universitas Islam Negeri Malang, Indonesia.

Procedures

Morphological work

In the field, all horseshoe crabs were sorted and washed, to be prepared for morphological observation and identification. Measurements of morphological characteristics were conducted on 8 characters (Telson Shape; Telson Cross-section Shape; Frontal Side; Shape of Third; Appendage on Prosoma; Shape of Second Appendage on Prosoma; Marginal Spine; Genital Pore; and Color) as depicted in Figure 2, using a digital caliper with 0.01 mm precision, following a modified protocol according to Shin et al. (2009) as well as Sekiguchi (2009). Identification procedures were provided by Cartwright et al. (2009; 2012); Tanacredi et al. (2009); Dolejš and Vaňousová (2015). Subsequently, all specimens were preserved in 70% alcohol and deposited at the Zoological Taxonomy, Universitas Negeri Surabaya, Indonesia. Before molecular analysis, the specimens were frozen at -20°C for DNA extraction.

DNA extraction

The isolation of total DNA (whole genome) from muscle samples was conducted using the NextPrep Kit with several modifications. A total of 15 to 20 mg horseshoe crab muscle samples were ground and then placed into a 1.5 mL tube. 200 µL of Buffer GT1 was added to the 1.5 mL tube and mixed using a vortex (Pre-lysis Stage). Subsequently, 200 µL of buffer GT2 and 20 µL of Proteinase K were mixed and vortexed (Lysis phase). The mixed samples were then incubated at 56°C for 10 minutes, during which the tube was inverted every 5 minutes. After this step, the mixture was combined with 200 µL of pure ethanol and mixed using a vortex. The sample was moved to a spin column and spun in a centrifuge at 13,000 rpm for 1 minute. The liquid that passed through was removed, and 500 µL of W1 buffer was introduced to the spin column. This was followed by another centrifugation process at 13,000 rpm for 1 minute. After discarding the flow-through, 700 µl of W2 buffer (which contained ethanol) was added to the Spin Column and centrifuged at 13,000 rpm for 1 minute. The flow-through was removed again, and the spin column underwent an additional 2-minute centrifugation at 13,000 rpm. The DNA captured in the spin column was transferred to a fresh 1.5 ml tube. Lastly, 50 to 100 µL of elution buffer was added, left to settle at room temperature for 1 minute, and then centrifuged at 13,000 rpm for 1 minute.

DNA extraction and sequencing

A segment of approximately 621 base pairs, corresponding to the COI gene region of mtDNA, was amplified through polymerase chain reaction (PCR). This process used universal primers LCO1490 (5' `GGT CAA CAA ATC ATA AAG ATA TTG G` 3') and HCO2198 (5' `TAA ACT TCA GGG TGA CCA AAA AAT CA` 3'), as described by Folmer et al. in 1994. The PCR method used a hot start approach, through Kapa master mix and 2 Taq master mixes. The amplification included 35 cycles, each comprising several steps, namely initial denaturation at 95°C for 3 minutes, followed by denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 2 minutes. A final elongation step at 72°C for 10 minutes concluded the process. These were run on a 1% agarose gel (made with 0.5 g agarose and 50 mL TAE buffer) containing 4 µL Ethidium Bromide as a staining agent to verify the PCR products. 3 µL of PCR product was mixed with 1 µL of loading dye before being loaded into the gel wells. The electrophoresis was conducted at 220 V and 400 mA for 25 minutes. Subsequently, the PCR products were purified using a Qiagen purification kit, following the manufacturer's protocol. The purified samples were sent to First Base, Malaysia, for sequencing.

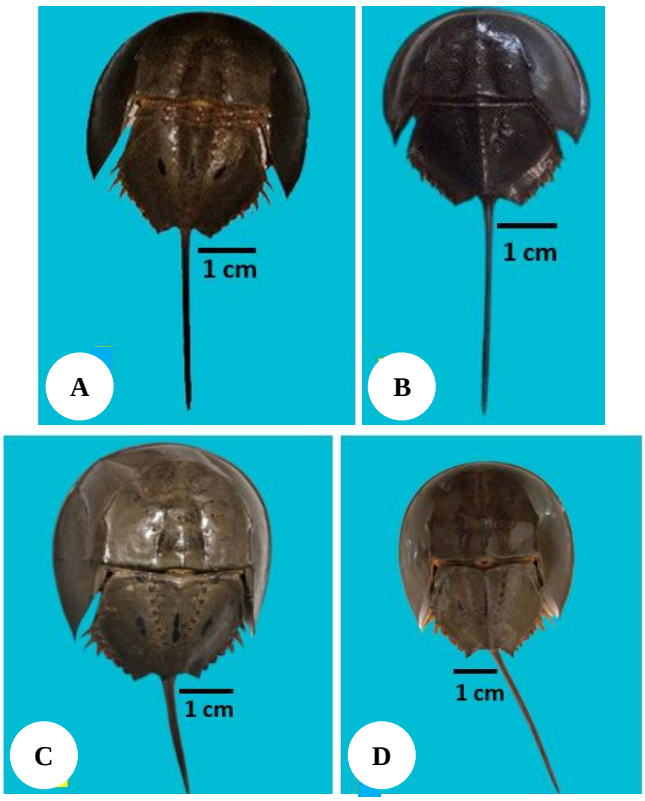

Figure 2. The characteristics morphology of horseshoe crabs from East Java. A. *Carcinoscorpius rotundicauda* (Beejay Bakau Resort, Probolinggo; B. *Carcinoscorpius rotundicauda* (Batah Timur, Madura); C. *Tachypleus gigas* (Lekok Beach, Pasuruan); D. *Tachypleus gigas* (Kenjeran Beach, Surabaya)

Data analysis

Morphology

This study was conducted by characterizing diagnostic data of the horseshoe crab species found, along with several other reference species used to support and complement the diagnosis, and compared with out-group species. Based on this diagnosis, key characters were determined, which were useful for assigning trait scores to each species. However, each species was scored according to its characters using a binary index (present or absent), with scores of 1 and 0 respectively. Phenetic analysis of the tested samples was performed numerically to determine clusters according to the similarity values of each tested sample, using the characters that had been identified, with the aid of Ntysc 2.01 software.

DNA barcode

The study obtained partial sequences of the COI gene based on 621 base pairs from 20 horseshoe crabs. Specimens of horseshoe crab were collected from Beejay Bakau Resort Probolinggo, Kenjeran Beach Surabaya, Batah Timur Beach Madura, and Lekok Beach Pasuruan, which constituted the final dataset. Initially, each sequence was translated into an amino acid sequence to eliminate any pseudogenes, following protocols highlighted by Song et al. 2008, Šlechtová et al. 2021, and Muhala et al. 2024. Subsequently, chromatogram analysis was performed using Finch TV software. The translated amino acid sequences were then verified through the ExPASy website (https://web.expasy.org/translate/) (Duvaud et al. 2021). All sequences were compared with close relatives of the samples through Basic Local Alignment Search Tool (BLAST) (Boratyn et al. 2013) and the Barcode of Life Data Systems (BOLD) System (Bejarano et al. 2023). A total of 6 accessions from GenBank (NCBI) were selected as in-group and out-group for phylogenetic tree construction. Multiple sequence alignment was performed using Clustal X (Ferrari and Patrizio 2021) followed by manual verification with BioEdit software. The partial COI gene sequences from horseshoe crabs in this study were submitted to GenBank with corresponding accession numbers as seen in Table 1.

Phylogenetic reconstruction

Phylogenetic reconstruction based on partial sequences of the COI gene included a total of 26 sequences, both in-group and out-group accessions, which were obtained from GenBank. The analysis aimed to construct the grouping of different species with related species. Phylogenetic tree reconstruction was conducted using MEGA X version 10.2.6, with the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods. For both NJ and ML tree reconstructions, the Kimura 2-Parameter (K2P) substitution model was used (Nishimaki and Sato 2019). In addition, the rate variation among sites was modeled with a Gamma distribution, and a bootstrap consensus tree was inferred from 1000 replicates (Russo and Selvatti 2018).

Table 1. DNA Sequences from NCBI GenBank used as comparisons

Species	Location	ACC number
Carcinoscorpius rotundicauda voucher CR_1	Batah Timur, Madura	MW454802
Carcinoscorpius rotundicauda voucher CR_2	Batah Timur, Madura	MW454812
Carcinoscorpius rotundicauda voucher CR_3	Batah Timur, Madura	MW454803
Carcinoscorpius rotundicauda voucher CR_4	Batah Timur, Madura	MW454811
Carcinoscorpius rotundicauda voucher CR_5	Batah Timur, Madura	MW454801
Tachypleus gigas voucher PSR_1	Lekok, Pasuruan	PP939944
Tachypleus gigas voucher PSR_2	Lekok, Pasuruan	PP939943
Tachypleus gigas voucher PSR_3	Lekok, Pasuruan	PP939946
Tachypleus gigas voucher PSR_4	Lekok, Pasuruan	PP939948
Tachypleus gigas voucher PSR_5	Lekok, Pasuruan	PP939947
Tachypleus gigas voucher SBY1	Kenjeran, Surabaya	OP585108
Tachypleus gigas voucher SBY2	Kenjeran, Surabaya	PP940000
Tachypleus gigas voucher SBY3	Kenjeran, Surabaya	OP585110
Tachypleus gigas voucher SBY4	Kenjeran, Surabaya	PP940001
Tachypleus gigas voucher SBY5	Kenjeran, Surabaya	PP940005
*Carcinoscorpius rotundicauda*_PRB1	Beejay Bakau Resort, Probolinggo	PP939073
*Carcinoscorpius rotundicauda*_PRB2	Beejay Bakau Resort, Probolinggo	PP939732
*Carcinoscorpius rotundicauda*_PRB3	Beejay Bakau Resort, Probolinggo	PP939735
*Carcinoscorpius rotundicauda*_PRB4	Beejay Bakau Resort, Probolinggo	PP942715
*Carcinoscorpius rotundicauda*_PRB5	Beejay Bakau Resort, Probolinggo	PP942716
Limulus polyphemus voucher USNM:IZ:1286833	USA	KT959422
Carcinoscorpius rotundicauda isolate Johor_b1	Johor, Malaysia	MF469062.1
Carcinoscorpius rotundicauda isolate Pahang_b51	Pahang, Malaysia	MF469060.1
Tachypleus gigas isolate GP0143	India	KJ825847.1
Tachypleus gigas isolate GP0143	India	JF896107.1
Limulus polyphemus voucher USNM:IZ:1286833	USA	KT959422.1

Species delimitation using ABGD

The Automatic Barcode Gap Discovery (ABGD) method, as outlined by Mu et al. (2023), was used to differentiate horseshoe crab species using the COI gene. This algorithm identified potential species by detecting significant gaps in the distribution of genetic distances between aligned sequences. ABGD was performed by repeatedly dividing the dataset into potential species groups, to achieve the most refined categorization. The process started with the creation of a pairwise uncorrected p-distance matrix using MEGA software, excluding any ambiguous positions in the sequence pairs. The ABGD analysis was conducted using an online tool accessible at http://wwwabi.snv.jussieu.fr/public/abgd/. This analysis incorporated 2 different models, namely Jukes-Cantor and K2P. These models were used to calculate genetic distances, taking into account different assumptions about nucleotide substitution rates. The method aimed to find the point where the distribution of genetic distances showed a clear separation, indicating a potential species boundary. Consequently, this approach provided a systematic way to propose species delineations based on genetic data.

Haplotype analysis using Network

Haplotype analysis using Network software was a widely employed method for visualizing and interpreting genetic relationships among populations based on DNA sequence data. The software constructed median-joining networks that represented haplotypes as nodes, with the node size typically indicating its frequency, and connecting lines showing mutational steps between haplotypes. This approach allowed infer evolutionary relationships, identify ancestral haplotypes, and visualize patterns of genetic diversity. The Network software offered various parameters for customization, including epsilon values and mutation weighting schemes, enabling study teams to refine their analyses. The resulting graphical network could reveal important insights into population structure, demographic history, and evolutionary processes, making it a valuable tool in population genetics and phylogeography studies (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Phenetic taxonomy

Species identification relied on several morphological features, including frontal margin notches, frontal view arching, chelae appearance on the 2nd and 3rd prosomal appendages, opisthosoma spine counts, telson surface spinnerets, and telson shape and cavity structure. This study validated the identification of 2 species in East Java by examining these distinctive morphological traits using phenetic taxonomy, which complemented and reinforced the genetic analysis findings. *C. rotundicauda* had several unique features, such as spineless telson, blunt triangle telson cross-section, and 2 types of second appendage shapes, which were different from *T. gigas* (Table 2).

The phenogram analysis revealed a complex evolutionary relationship among horseshoe crab species based on similar morphology, with 2 relationship groups identified from closest to farthest, alongside 2 apomorphy and 4 automorphy groups. This relationship showed 2 main subclades, namely the first subclade demonstrated a striking 100% similarity between the sample species and *C. rotundicauda*, with *Tachypleus gigas* as a sister clade also sharing 100% similarity. The unexpectedly high similarity between species from different genera suggested potential taxonomic implications or the need for further genetic studies. The 2nd subclade, Apomorphy B, grouped *L. polyphemus* and *T. tridentatus* with a 75% similarity, an interesting association given their different geographic distributions. This phenogram structure highlighted the complexity of horseshoe crabs taxonomy and evolution, emphasizing the significance of combining morphological and molecular data in future studies to better understand these relationships (Figure 3).

The morphological analysis supported the identification of *C. rotundicauda* and *T. gigas* as distinct species. It also highlighted the value of using multiple morphological characteristics for accurate species identification in horseshoe crabs. The data reinforced the importance of detailed observation in taxonomic studies and provided a solid basis for complementing genetic analyses in species classification. Thus, further investigations and models of the relationship between the species and the areas suggested here should be conducted prior to making decisions regarding conservation.

Table 2. Comparison of samples with other reference species based on the identification book by Sekiguchi and Shuster (2009)

OTU characters	*Carcinoscorpius rotundicauda* (sample)	*Tachypleus tridentatus*	*Tachypleus gigas* (sample)	*Limulus polyphemus*
Telson shape	Without spines	Spines	Spines	Without spines
Telson cross-section shape	Blunt triangle	Sharp triangle	Sharp triangle	Sharp triangle
Frontal side	Curved	With indentation	Curved	Curved
Shape of third appendage on prosoma	Pointed downward	Pointed downward	Pointed downward	Pointed straight
Shape of second appendage on prosoma	Two types	One type	One type	One type
Marginal spine	Clearly visible	Some less visible (reduced)	Some less visible (reduced)	Clearly visible
Genital pore	Lower part fused	Lower part fused	Lower part fused	Lower part not fused
Color	Blackish brown	Greenish brown	Blackish brown	Blackish brown

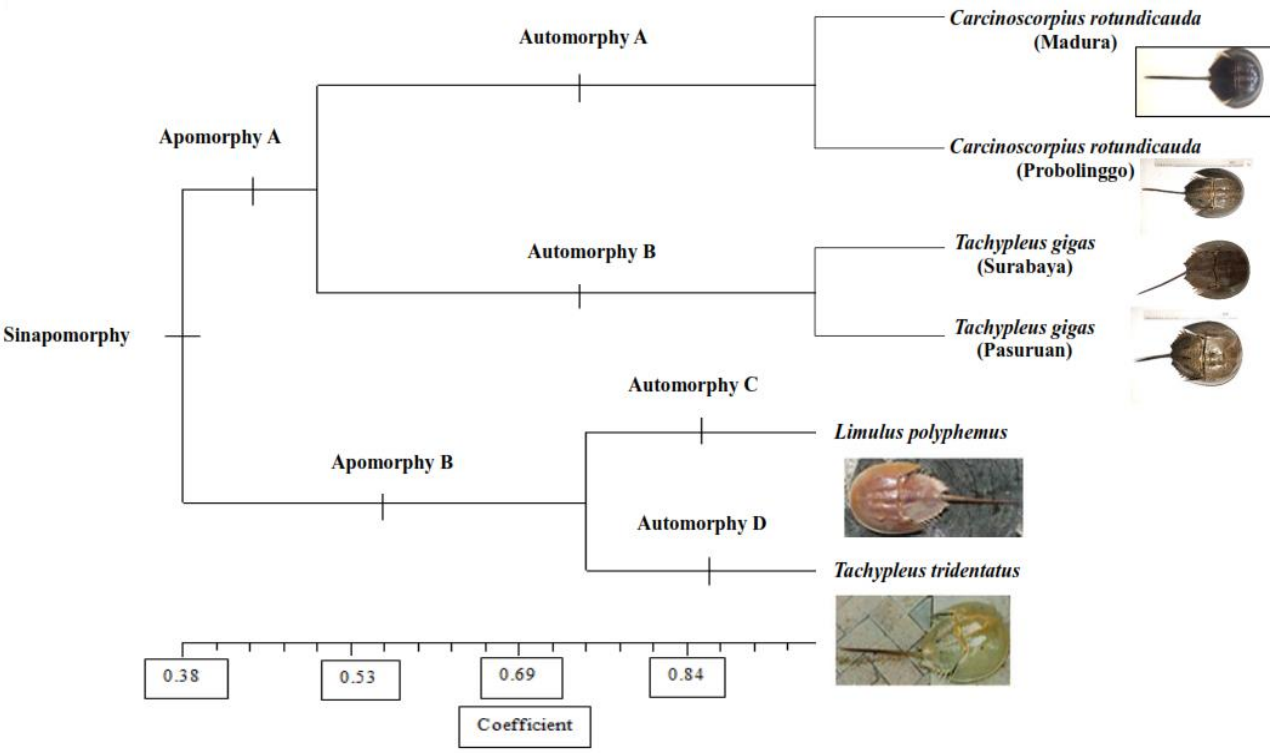

Figure 3. Phenogram of morphological characters of the sample with reference species *Tachypleus tridentatus* and *Limulus polyphemus*

Sequence composition and genetic diversity

The successful amplification of the COI gene was verified by the presence of a clear DNA band at approximately 621 base pairs, with no smearing. This result indicated that the amplification process accurately captured functional mitochondrial COI sequences, as evidenced by the absence of stop codons. Consequently, nuclear DNA sequences derived from mitochondrial DNA (NUMTs), which usually had lengths shorter than 621 base pairs (Ricardo et al. 2020), were not included in the sequencing. The COI gene was widely recognized for its reliability in species identification, as pseudogenes were removed during protein translation. Several studies indicated that COI sequences were effective for distinguishing species across various taxa. For example, a recent study on biodiversity produced new COI sequences for a large proportion of species (Venera et al. 2020).

Sequence validation results conducted through the online facility of BLAST (NCBI) and the BOLD system indicated that the sequence samples matched the available accessions in the database with query cover in the range of 99 to 100% (Table 3). The validation results depicted that the 20 samples were grouped into 2 species, including *C. rotundicauda* and *T. gigas*. According to the results of the sequence validation, the similarity of the sequences reported by Insafitri and Nugraha (2024) was in the range of 99 to 100% sama with *C. rotundicauda*. All species of *C. rotundicauda* and *T. gigas* in this study were species that were commonly found in the waters of the Western Indo-Pacific, especially in Indonesian waters, according to the geographical distribution illustrated by Sekiguchi and Shuster (1993). Insafitri and Nugraha (2024) reported that the findings of *C. rotundicauda* in this study were consistent with the findings in 2 samples of *C. rotundicauda* and *T. gigas* from Madura Island.

DNA barcoding was highly effective in distinguishing horseshoe crab species across various regions, including Java's north coast by Meilina et al. (2016); Bengkulu waters, Sumatra by Anggraini et al. (2017); and Madura waters Insafitri and Nugraha (2024). In this study, the partial sequence profiles of the COI gene were compiled for *Carcinoscorpius rotundicauda* from Batah Timur, Madura, and Beejay Bakau Resort, Probolinggo while *Tachypleus gigas* from Kenjeran Beach and Lekok Beach, Pasuruan District. These findings confirmed the efficacy of DNA barcodes in accurately identifying these species. Importantly, no insertions, deletions, or codon stops were observed upon translation of the nucleotide sequences. These results used the reliability and utility of DNA barcoding as a powerful tool for species identification and biodiversity assessment in freshwater fish populations.

The high haplotype diversity (Hd) of 0.957 and nucleotide diversity (π) of 0.1 (Table 4) indicated a wide range of genetic variations within the studied horsehouse crabs in several regions. The frequency of parsimony informative sites at 26.48% suggested the presence of informative genetic variations that could aid in species differentiation. The 19 polymorphic sites and the overall ts/tv ratio of 2.32 further highlighted the genetic variability and evolutionary dynamics within the studied horsehouse crabs in East Java Province. The mean evolutionary rate ranging from 0.00 to 2.69 substitutions per site indicated the rate of genetic changes over time in the COI gene sequences, comparable to Obst et al. (2021) observations in marine arthropods. The assessment of genetic distance between species was crucial for understanding the

relationships and similarities among different horseshoe crabs. A greater genetic distance, such as the value of 0.11 or 11% between *C. rotundicauda* and *T. gigas* indicated a different genetic relationship in the partial COI gene sequences between these species. The highest distance observed between *C. rotundicauda* and *T. gigas* was 12%, while the smallest distance was between *C. rotundicauda* and *T. gigas* at 0.092 or 9.2% (Table 4). The findings of this composition could provide insights into the evolutionary history and divergence patterns within the species from Beejay Bakau Resort, Probolinggo; Kenjeran Beach, Surabaya; Batah Timur Beach, Madura and Lekok Beach, Pasuruan.

Genetic diversity results were obtained from ecological, behavioral, and physical isolation, including limited population size and the selection of specific traits (Mignon-Grasteau et al. 2005; Frankham et al. 2010). Populations exhibiting high genetic diversity were more likely to survive, as this indicated a healthy population with a greater capacity to adapt to environmental challenges, such as overfishing (Hughes et al. 2008). While this study consisted of multiple species, it highlighted the need for future study to focus on examining the genetic diversity of individual horseshoe crab species (Faurby et al. 2010; Smith et al. 2017).

Table 3. Validation of partial sequence of CO1 gene of horseshoe crabs obtained from East Java Province, Indonesia through the BLAST (GenBank/NCBI) and the BOLD system online facilities

Species	Highest BOLD identification	GenBank NCBI			Bold Systems	
		Query cover (%)	E-Value	% Similarity	Similarities (%)	Status
PP942715 *C. rotundicauda* PRB4	*C. rotundicauda*	99	0	99.5	99.84	Published
PP942716 *C. rotundicauda* PRB5	*C. rotundicauda*	99	0	99.4	99.68	Published
PP939735 *C. rotundicauda* PRB3	*C. rotundicauda*	99	0	99.6	99.62	Published
PP939732 *C. rotundicauda* PRB2	*C. rotundicauda*	99	0	99	100	Published
PP939073 *C. rotundicauda* PRB1	*C. rotundicauda*	99	0	99.4	99.72	Published
MW454811 *C. rotundicauda* voucher CR_4	*C. rotundicauda*	99	0	99.2	99.82	Published
MW454812 *C. rotundicauda* voucher CR 2	*C. rotundicauda*	99	0	99.1	100	Published
MW454803 *C. rotundicauda* voucher CR 3	*C. rotundicauda*	99	0	99.8	99.78	Published
MW454802 *C. rotundicauda* voucher CR 1	*C. rotundicauda*	99	0	99.6	100	Published
MW454801 *Carcinoscorpius rotundicauda* voucher CR 5	*C. rotundicauda*	99	0	99.6	99.68	Published
PP939073 *T. gigas* voucher PSR1	*T. gigas*	99	0	99.3	99.81	Published
PP939732 *T. gigas* voucher PSR2	*T. gigas*	99	0	99.2	99.63	Published
OP585108 *T. gigas* voucher SBY1	*T. gigas*	99	0	99.2	99.25	Published
PP940001 *T. gigas* voucher SBY4	*T. gigas*	99	0	99.6	99.63	Published
PP940005 *T. gigas* voucher SBY5	*T. gigas*	99	0	99.4	99.44	Published
OP585110 *T. gigas* voucher SBY3	*T. gigas*	99	0	99.4	99.54	Published
PP940000 *T. gigas* voucher SBY2	*T. gigas*	99	0	99.2	99.68	Published
PP942715 *T. gigas* voucher PSR4	*T. gigas*	99	0	99.4	99.84	Published
PP942716 *T. gigas* voucher PSR5	*T. gigas*	99	0	99.2	99.42	Published
PP939946 *T. gigas* voucher PSR3	*T. gigas*	99	0	99.3	99.44	Published

Table 4. Characteristics of partial sequence of COI gene used for phylogenetic trees reconstruction and genetic distance analysis include sequences from the study sample and the GenBank/BOLD system (in group and out group)

Parameters	Position at codon			
	1st	2nd	3rd	Total
Tyrosine frequency	26.74%	40%	23%	621 bp
Cytosine frequency	29.20%	30.1%	34.1%	621 bp
Adenine frequency	22.65%	14.5%	29.3%	621 bp
Guanine frequency	20.41%	15.0%	13.8%	621 bp
Frequency of invariable sites	59.35%			
Frequency of parsimony informative sites	26.48%			
Nucleotide diversity (Pi)	0.09762			
Haplotype diversity	0.957			
Number of haplotypes	12			
Polymorphic sites	166			
Variance of haplotype diversity	0.00097			
ts/tv ratio (R)	2.32			
Gamma discrete distribution	0,62			
Standard deviation of haplotype diversity	0.031			
Mean of evolutionary rate	0.00, 0.04, 0.05, 0.09, 0.22, 0.26, 0.31, 0.42, 0.56, 0.72, 0.93, 1.20, 2.01, and 2.69 substitutions per site			

Note: The COI gene sequence characteristics were based on the 621bp sequence length

Genetic diversity in horseshoe crabs was influenced by their distribution and habitat characteristics. According to Fozi et al. (2021), the widely distributed marine species, such as horseshoe crabs, often exhibited high genetic variation. The habitat features of horseshoe crabs played a crucial role in determining their genetic diversity. For instance, Faurby et al. (2010) observed that horseshoe crabs living in diverse coastal habitats tended to have higher genetic diversity compared to those in more uniform environments. Complementing this, King et al. (2005) systematically revealed that habitat complexity propelled genetic diversity in horseshoe crabs, with factors such as coastal geomorphology and tidal patterns playing significant roles. This explained the variations in genetic diversity observed between different horseshoe crabs populations along the coast. In addition, Sekiguchi and Shuster (2009) noted that the genetic diversity of horseshoe crabs was closely tied to their specific breeding habitats, with populations that had access to a variety of spawning beaches showing higher genetic variability.

Identification using BOLD systems

The highest identification accuracy achieved for horseshoe crabs from East Java Province using the Barcode of Life Data Systems (BOLD) online platform, ranging from 99.25% to 99.84%, was an impressive indication of the effectiveness of DNA barcoding in species identification. This level of accuracy was particularly notable given the complexity and diversity of horseshoe crab species, which often exhibited subtle morphological differences that could be challenging to discern using traditional taxonomy alone. This study supported the use of the BOLD system as a superior tool for fish identification compared to other databases, aligning appropriately to achieve high identification accuracy, the same findings as the study by Modeel et al. (2023). This result indicated that there were no differences in BOLD identification between the variants observed in *C. rotundicauda* and *T. gigas* (Table 3). A genetic distance value of more than 2% indicated that there were species that were different from other group members. Meanwhile, a genetic distance value of less than 3% suggested that the group or cluster came from the same species or a species (Wong et al. 2009). This genetic distance showed the association with the GenBank data for each sample and related species. Differences in genetic distance showed that it was caused by the presence of genetic diversity within groups. When the genetic distance value was less than 2% in percentage terms, indicating no similar species, the value was called very low. High genetic distances could indicate identification at the family and genus level (Ricardo et al. 2020). According to Chiu et al. (2013), distance genetic diversity could be influenced by environmental factors. Differences in species' geographic location and environmental conditions led to changes in phylogenetic morphology and populations (Twindiko et al. 2013).

Phylogenetic reconstruction

Phylogenetic reconstruction analysis resulted in the construction of NJ and ML trees depicted in Figures 4 and 5, respectively. Each species was linked to a distinct DNA barcode cluster, facilitating the clear delineation of phylogenetic relationships among them. Both NJ and ML trees exhibited 2 divergent clusters, with bootstrap values exceeding 100%, indicating robust support for the inferred relationships. The phylogenetic trees demonstrated an unambiguous branching pattern for *C. rotundicauda* from Beejay Bakau Resort, Probolinggo, and Batah Timur Madura with *T. gigas* from Lekok Beach Pasuruan and Kenjeran Beach Surabaya forming their monophyletic branches and different location. However, the proximity of these 2 species at the same node suggested a close evolutionary relationship, consistent with the calculated genetic distance of 0.11, which indicated the furthest genetic separation between them. Overall, the NJ, ML, and genetic distance analyses supported the genetic distinctiveness of *C. rotundicauda* and *T. gigas*, highlighting their divergence and evolutionary relationships within the horseshoe crabs for sustainability and conservation.

The phylogenetic reconstruction analysis, using both NJ and ML methods, resulted in robust phylogenetic trees (Figures 4 and 5). These methods were widely used in molecular phylogenetic studies and often provided complementary insights into evolutionary relationships (Yang and Rannala 2012). The clear separation of the species into distinct monophyletic branches supported their taxonomic status as separate species. However, their proximity to the same node suggested a close evolutionary relationship. This was consistent with previous studies showing phylogenetic closeness between these 2 genera (Obst et al. 2012). The different sampling locations for the species suggested potential geographical variation in their distribution, which was crucial to consider in conservation efforts (Vestbo et al. 2018). High bootstrap values (>80%) in both phylogenetic trees indicated strong support for the inferred relationships. These high bootstrap values increased confidence in the resulting tree topologies (Madduppa et al. 2017). Collectively, the NJ, ML, and genetic distance analyses supported the genetic distinctiveness of *Carcinoscorpius rotundicauda* and *Tachypleus gigas* while revealing their evolutionary relationships. This information was valuable for conservation efforts, as understanding genetic diversity and phylogenetic relationships was key to designing effective conservation strategies (Frankham et al. 2010).

These findings contributed to the understanding of horseshoe crabphylogeny and genetic diversity, which was essential for their conservation. Furthermore, as these ancient marine arthropods faced increasing threats from habitat loss and overexploitation, such genetic information became crucial for developing targeted conservation measures (Smith et al. 2017). The result of this study revealed that *C. rotundicauda* and *T. gigas* were resolved sister taxa with a robust bootstrap support of 99%. The genetic diversity observed between these clusters was very low, with values greater than 2%. According to established criteria, a genetic distance value exceeding 2% typically indicated species differentiation, while values below 3% suggested intra-specific or conspecific groupings (Chakraborty and Ghosh 2014; Bektas et al. 2018).

Consequently, the genetic distance analysis supported the distinction between species and provided insight into the genetic diversity between the species in Indonesia, especially East Java, Indonesia.

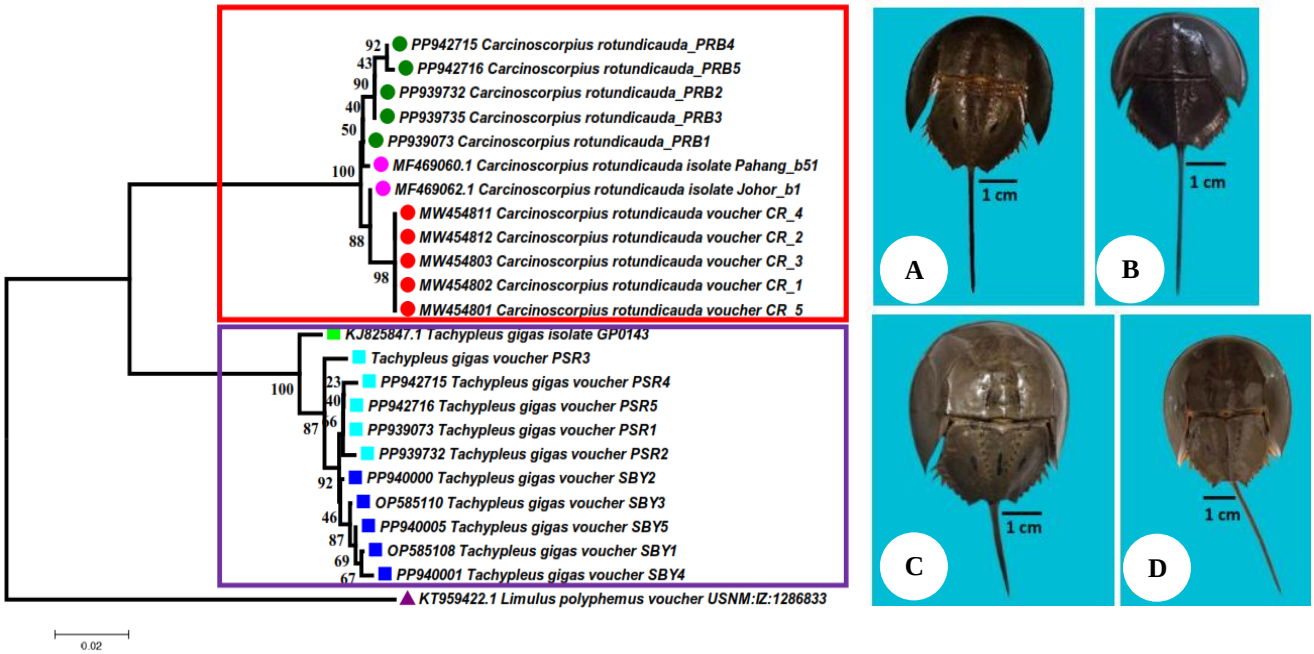

Figure 4. Neighbour Joining (NJ) phylogenetic tree of horseshoe crabs based on partial sequence of COI gene. A. *C. rotundicauda* (Beejay Bakau Resort, Probolinggo; B. *Carcinoscorpius rotundicauda* (Batah Timur, Madura); C. *Tachypleus gigas* (Lekok Beach, Pasuruan); D. *T. gigas* (Kenjeran Beach, Surabaya)

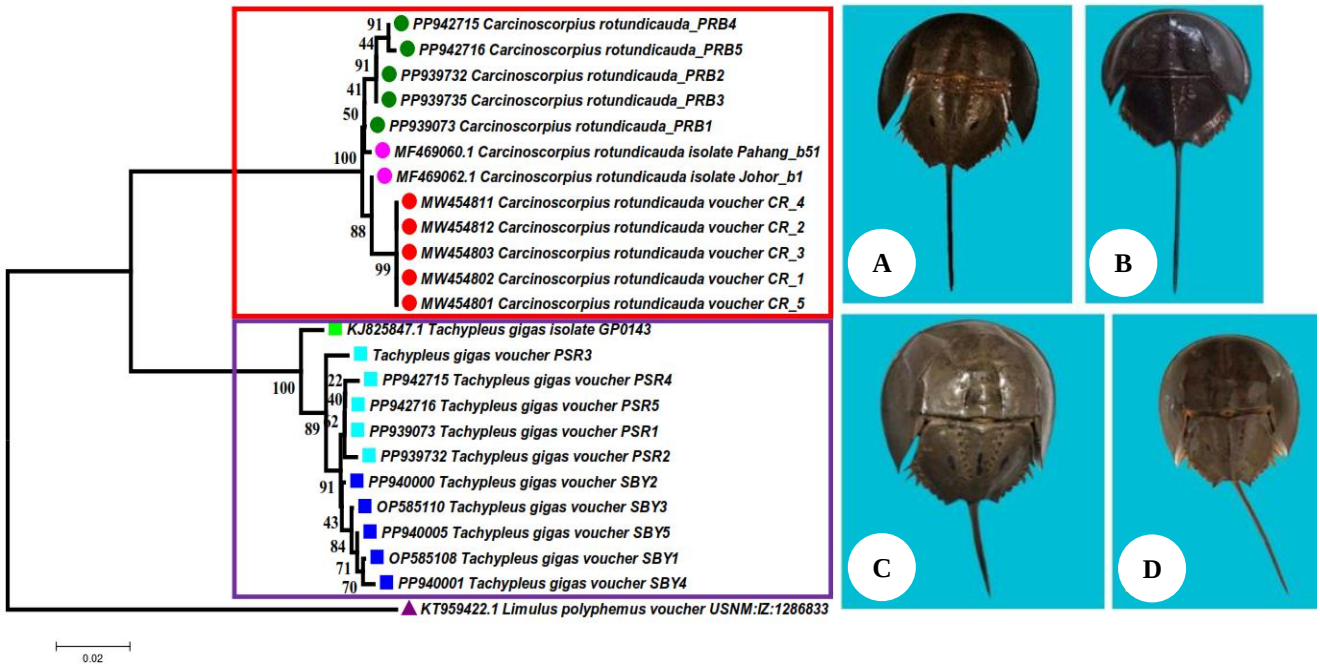

Figure 5. Maximum Likelihood (ML) phylogenetic tree of horseshoe crabs based on partial sequence of COI gene. A. *Carcinoscorpius rotundicauda* (Beejay Bakau Resort, Probolinggo; B. *Carcinoscorpius rotundicauda* (Batah Timur, Madura); C. *Tachypleus gigas* (Lekok Beach, Pasuruan); D. *Tachypleus gigas* (Kenjeran Beach, Surabaya)

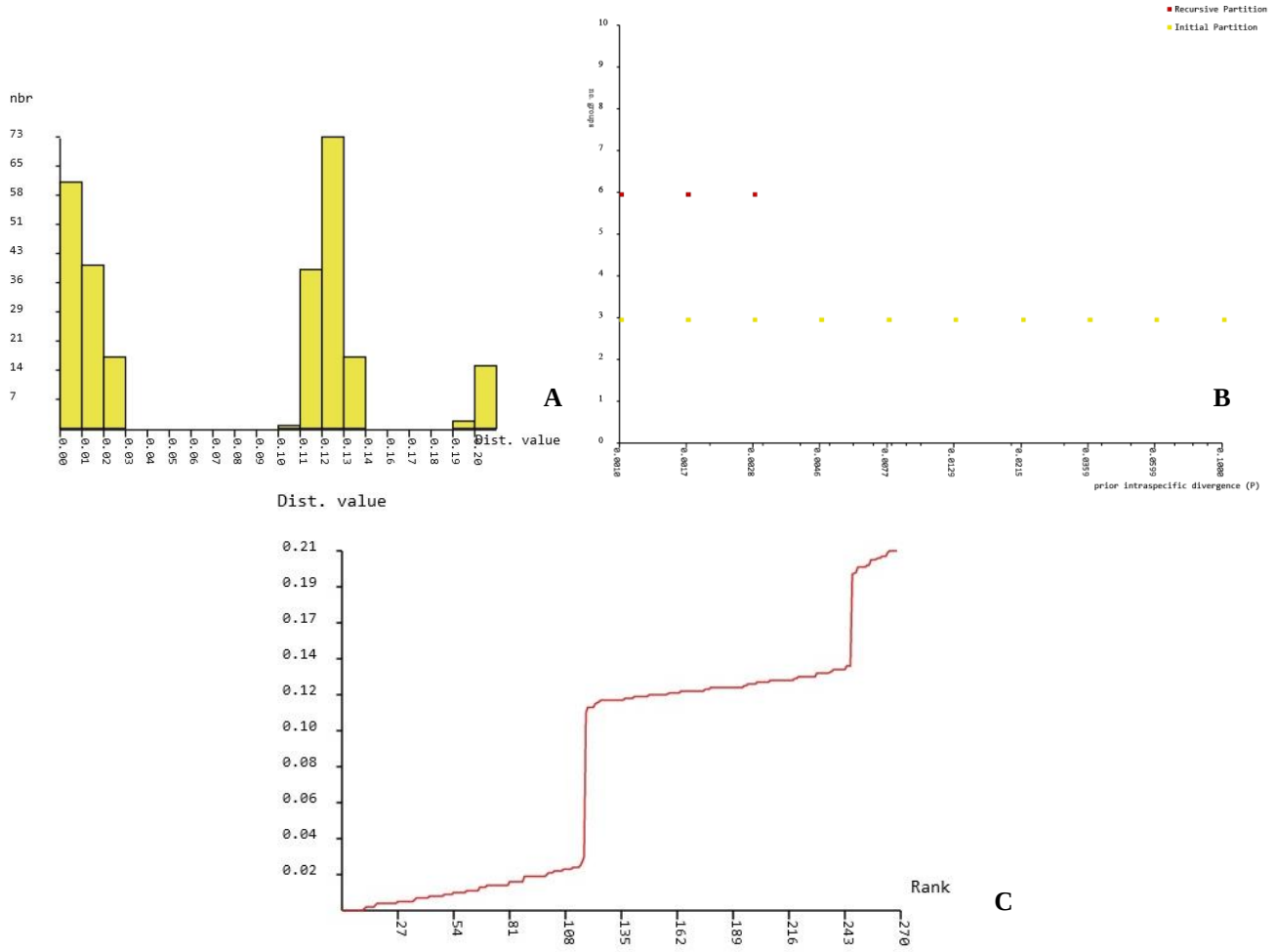

Figure 6. Analysis of Gap Barcodes of horseshoe crab species generated by Automatic Barcode Discovery Gap Discovery (Puillandre et al. 2012). Distribution of K2P distances between each pair of specimens for the COI gene. A: distance histogram; B: rank distance; C: number of PSH obtained for each previous intraspecific divergence

Species delimination using ABGD

ABGD method identified 3 groups for horseshoe crabs specimens with the initial approach and the barcode gap threshold calculated by the analysis of the COI dataset (see Figures 6.A and 6.B). Initial partition with prior maximal distance P=2.15e-02; Barcode gap distance = 0.100, distance K80 Kimura =1.50. This method also identified a barcode gap centered around 1.8% of divergence between the available COI sequences. The analysis defined the existence of 3 to 7 hypothetical species in all recursive partitions with prior intraspecific genetic divergence values between 0.15% and 0.26%, which was considered more likely than 3 or more species with intraspecific divergence values below 0.28% or as a single species with intraspecific divergence values greater than 2.15%. This was consistent with the results of the ABGD grouping, which divided the species into 3 groups (Figure 6.C).

The ABGD method, a widely used approach for species delimitation in DNA barcoding studies (Puillandre et al. 2012), identified 3 distinct groups of horseshoe crabs in the initial approach. This method used the barcode gap, which was the difference between intraspecific and interspecific genetic distances, to delineate species boundaries. The analysis revealed a barcode gap threshold at a distance of 0.100, with an initial partition prior maximal distance of P=2.78e-03. The K2P (K80) distance was calculated as 1.50, which was a commonly used model in molecular evolution studies for estimating genetic distances (Kimura 1980). Importantly, the ABGD method identified a barcode gap centered around 1.5% divergence among the available COI sequences. This finding was consistent with previous studies on arthropods, where COI divergence values between 1 to 2% were suggested as potential thresholds for species delimitation (Hebert et al. 2003). The analysis defined between 3 to 7 hypothetical species across all-recursive partitions, with prior intraspecific genetic divergence values ranging from 0.15% to 0.26%. This result was deemed more plausible than alternatives such as 3 or more species with intraspecific divergence below 0.28%, or a single species with intraspecific divergence exceeding 2.15%. This interpretation was in accordance with the principle of parsimony in species delimitation (Carstens et al. 2013).

The final ABGD grouping, which divided the specimens into 3 groups (Figure 6.C), aligned with the current taxonomic understanding of horseshoe crabs in the region. This result was specifically interesting in the context of Asian horseshoe crabs' diversity, where 3 extant species were generally recognized, namely *L. polyphemus*, *T. gigas*, and *C. rotundicauda* (Obst et al. 2012). These findings contributed to the understanding of horseshoe crabs' diversity and phylogenetics, which was crucial for conservation efforts. As these living fossils faced increasing threats from habitat loss and overexploitation, accurate species delimitation became essential for developing targeted conservation strategies (Vestbo et al. 2018).

Haplotype network horseshoe crabs

The Median Joining network analysis provided a comprehensive description of the genetic variation within horseshoe crabs, highlighting 16 haplotypes grouped into 3 distinct haplogroups. The network depicted that horseshoe crab species formed an in-group based on their unique haplotypes, with each fish possessing a distinct, non-homologous haplotype and their location. This genetic clustering suggested a close relationship of *C. rotundicauda* with the origin locations of Probolinggo and Madura, as depicted in Figure 7. Similarly, the grouping of *T. gigas* from Kenjeran, Surabaya, and Lekok Beach, Pasuruan also clustered according to their types but were not homologous due to geographical differences. These findings provided valuable insights into the genetic diversity and relationships within horseshoe crabs species in East Java, emphasizing the importance of further studies and conservation efforts for these unique arthropod populations.

The haplotype network of 20 samples from 3 clusters (Figure 7) showed that the number of mutational steps from the Probolinggo and Madura was much smaller towards Surabaya and Pasuruan populations. A genetic break of 19 mutational steps in COI was found between these regions, compared with only one or two steps between most other populations at similar or much larger geographical distances. This suggests a substantial genetic differentiation between these regions, which could be due to geographical barriers or historical separation (Searle 2000). The detailed haplotype network revealed 11 haplotypes in *T. gigas* and 7 haplotypes in *C. rotundicauda*. A star-like network in both places indicates radiation from a common haplotype for just one or two mutational steps (seldom more), and confirms the accumulation and maintenance of diversity in *C. rotundicauda* and *T. gigas* maternal lines locally. The most important observation was that a few haplotypes are shared between distant populations. Even more interesting is that several individuals from distant locations, have a shared haplotype, indicating historical connectivity of a maternal lineage over distances in their locations. The presence of shared haplotypes between distant populations suggests historical connectivity or gene flow between these populations. This is particularly interesting for horseshoe crabs, as it provides insights into their dispersal capabilities and population structure.

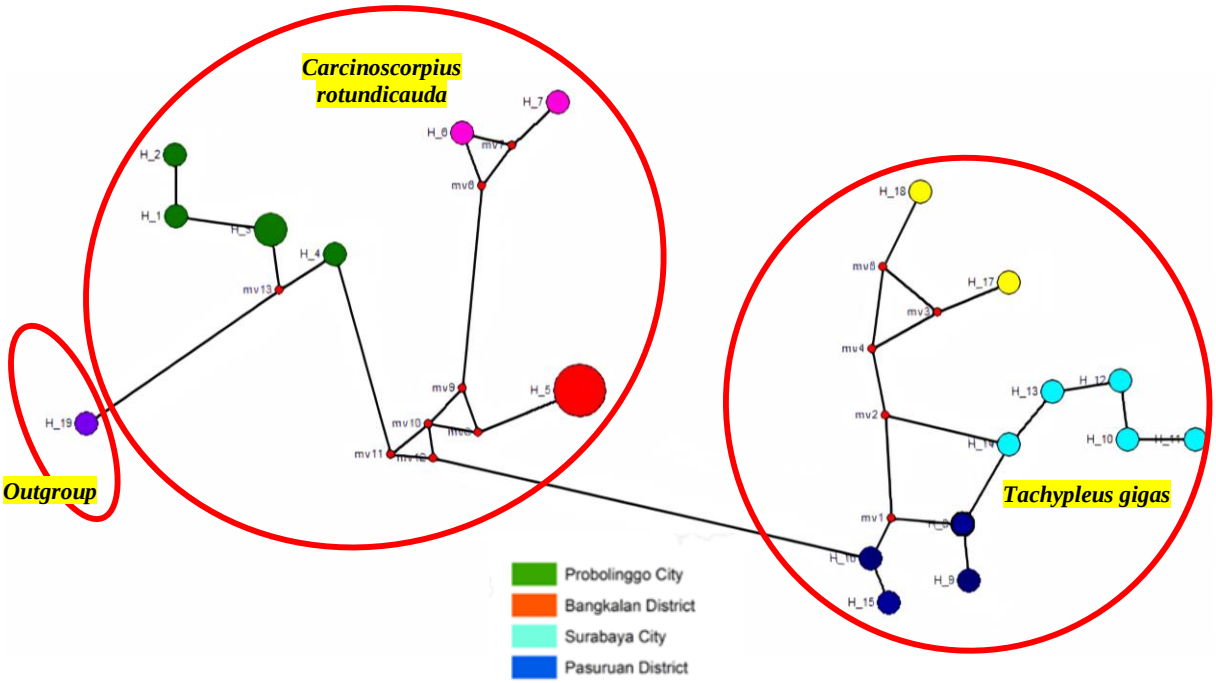

Figure 7. Haplotype network of *Nemacheilus* spp. Haplotype network of 12 haplotypes based on the COI gene sequence. Haplotypes are shown in different circle shapes and patterns, the number of individuals is shown in different circle shapes (large: 3 individuals; small: 1 individual). Branching between haplotypes is shown by substitution based on the alignment position of the COI gene sequence

The Median Joining network analysis revealed a complex pattern of genetic variation within horseshoe crabs, identifying 16 haplotypes clustered into 3 distinct haplogroups. This method, which was particularly useful for intraspecific data analysis, provided a visual representation of the relationships between closely related haplotypes (Bandelt et al. 1999). The network structure indicated that horseshoe crab's species form an in-group based on their unique haplotypes, with each individual possessing a distinct, non-homologous haplotype related to its location. This pattern of genetic clustering was consistent with previous studies on horseshoe crabs' population genetics, which showed significant genetic structuring among populations (King et al. 2005). The close relationship observed between *C. rotundicauda* haplotypes and their origin locations in Probolinggo and Madura (Figure 7) suggested a degree of population isolation and potential local adaptation. This finding was consistent with a study by Sarmiento et al. (2021), who observed significant genetic differentiation among *C. rotundicauda* populations in Malaysia.

Similarly, the grouping of *T. gigas* haplotypes from Kenjeran, Surabaya, and Lekok Beach, Pasuruan clustered according to their types, but indicated non-homology due to geographical differences. This pattern of genetic structuring was consistent with the limited dispersal capabilities of horseshoe crabs, which could lead to genetic differentiation over relatively short geographic distances (Faurby et al. 2010). Furthermore, these findings provided valuable insights into the genetic diversity and relationships within horseshoe crab populations in East Java. The observed genetic structuring emphasized the importance of considering local population dynamics in conservation planning. According to a study by Smith et al. (2017), understanding genetic diversity patterns was crucial for developing effective conservation strategies for these ancient arthropods.

This study validated the effectiveness and reliability of targeted DNA barcoding and phenetic taxonomy for analyzing genetic diversity and species-level relationships. Furthermore, it presented the first comprehensive report on the morphology, genetic identification, and phylogenetic reconstruction of horseshoe crabs from East Java waters using partial sequences of the COI gene. The study facilitated the conservation management of local East Java horseshoe crabs through grouping according to species and genetic composition, while suggesting potential avenues for cryopreservation to support sustainability and domestication efforts. The findings demonstrated that a molecular approach using partial COI gene sequences corroborated genetic diversity data published in the GenBank (NCBI) database. This study provided validated and enhanced identification of the phenetic taxonomy and molecular characteristics of horseshoe crabs. By establishing a reliable DNA barcode reference library for East Java, Indonesia, this study represented a pioneering effort to improve monitoring, conservation, and management of fisheries in these overexploited regions.

This study was the first to report on the genetic diversity and phylogenetic reconstruction of horseshoe crabs from specific locations in East Java, including Kenjeran Surabaya, Batah Timur Madura, Lekok Beach Pasuruan, and Beejay Bakau Resort Probolinggo, based on partial sequences of the COI gene. While the genetic diversity appeared relatively high, the study showed that habitat destruction and overfishing had inevitably led to a decline in diversity. This conclusion synthesized the key findings and implications, emphasizing its novel contributions to horseshoe crabs' study and conservation in East Java, Indonesia.

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