DNA Barcoding of Horseshoe Crab From Madura Island

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Abstract. Horseshoe crab or mimi, commonly known as horseshoe crab is an ancient animal that can live until now so it is called a living fossil. Horseschoe crab can be found in Madura, but there is no comprehensive data yet. Accurate data on horseshoe crab species is needed to determine the potential of this ancient animal, especially from the waters of Madura Island. A careful process of collecting, classifying, and identifying species as well as describing new species is still urgently needed to manage the potential of Madura's coastal resources in a sustainable manner. Species identification is the most time-consuming, difficult, and often creates obstacles to data collection and analysis. Identification of horseshoe crab species can be carried out based on horseshoe crab morphology but it is quite complicated to do so a reliable and efficient method is needed. One of the currently developing horseshoe crab identification methods is by using molecular analysis of DNA barcoding. This research is the first attempt to identify species using DNA barcoding molecular analysis to investigate the potential of Madura Island as a source of horseshoe crab genetic diversity and is expected to be useful for the management of horseshoe crab resources. The purpose of this study is to find out the DNA of barcoding horseshoe crab species on Madura Island. Genetic marker Cytochrome Oxidase I of the mitochondrial genome DNA (mtDNA) was used to analyze genetic diversity. Reconstruction of phylogenetic tree and genetic diversity were made by using software MEGA X. Research results showed that sample closely related to *Carcinoscorpius rotundicauda* 99 %.

1 Introduction

Horseshoe crab, commonly known as belangkas or mimi in local name, is an ancient animal that can live until now so it is called a living fossil [1]. Horseshoe crab is a member of the phylum Arthropoda, subphylum Chelicerata, class Merostomata, subclass Xiphosura, order Xiphosurida, and family Limulidae, genus Carcinoscorpius, Limulus, and Tachypleus [2]. There are four species of Horseshoe crab living in the world, namely *Limulus polyphemus* Linnaeus 1758, found only along the Atlantic coast of North America and the Gulf of Mexico (21°-44° N and 68°-90° W), while three other species are only found in Asia (Indo Pacific):

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Tachypleus gigas (Muller, 1785), *T. tridentatus,* and *Carcinoscorpius rotundicauda* (Lattreille 1802), with geographical distribution at 6° S - 31° N and 90°-125° E [3].
Horseshoe crabs live in coastal habitats with low salinity, sandy mud substrates [4] by eating bivalves, crustaceans, and polychaetes [5]. The role of horseshoe crabs is as a sediment modifier through particle/water movement, a protein source for migratory birds, and a pharmaceutical [6].

In general, all three types of Asian horseshoe crab can be found in Madura, but there is no comprehensive data. The three species of horseshoe crab are designated as protected animals based on the Government Regulation of the Republic of Indonesia Number 7 of 1999 concerning the Preservation of Plant and Animal Types and the Regulation of the Minister of Environment and Forestry of the Republic of Indonesia Number P.20 / MENLHK / SETJEN / KUM.1/6/2018, concerning Protected Plant and Animal Species. [7] explain that the presence of all three Asian species in the IUCN category is a lack of data. For this reason, accurate horseshoe crab species data is needed to determine the potential of this ancient animal, especially from the waters of Madura Island.

The diversity of life in the oceans can be analyzed and understood at many levels of organization, including species-level taxonomy. Careful processes in collecting, classifying, and identifying species and describing new species are still urgently needed to sustainably manage Madura's coastal resource potential [8]. Species identification is the most time-consuming, difficult, and often poses obstacles in data collection and analysis. Identification of Horseshoe crab types can be done based on the morphology of Horseshoe crab but it is quite complicated to do so that a reliable and efficient method is needed, one of the Horseshoe crab identification methods that is now developing is to use molecular analysis of DNA barcoding. The advantages of this DNA barcoding method are that the number of samples required is very small, the ability to document taxonomic diversity that is unknown or has never been identified, in addition to being able to discover new diversity in species that were previously classified as just one species [9].

Mitochondrial cytochrome c oxidase I (COI) genes is very effective because it is cost-effective, fast, and accurate for species identification and can generate new insights about genetic diversity and molecular evolution so that it is very beneficial for the preservation of marine resources [10]. Furthermore, DNA barcodes can detect errors in species involvement with very few morphological differences and it is difficult to know complex morphological differences and characteristics, thus adding to genetic data, ecological data, morphological data, geographic data, and behavioral data [11]. Until now, research on Madura Island on Horseshoe crab identification has only been carried out on morphology. The purpose of this study was to determine the DNA barcoding of Horseshoe crab species in Madura. This research is the first attempt to identify species using DNA barcoding molecular analysis and is expected to be useful for Horseshoe crab resource management.

2 Materials and Method

The research was carried out in June 2023 and carried out at the Marine and Fisheries Laboratory, Faculty of Agriculture, Trunojoyo University, Madura. The location of horseshoe crab sampling in the waters of Bangkalan-Madura. Morphological observations are carried out by observing the length, width of all morphological parts of the horseshoe crab as in the following figure 1.

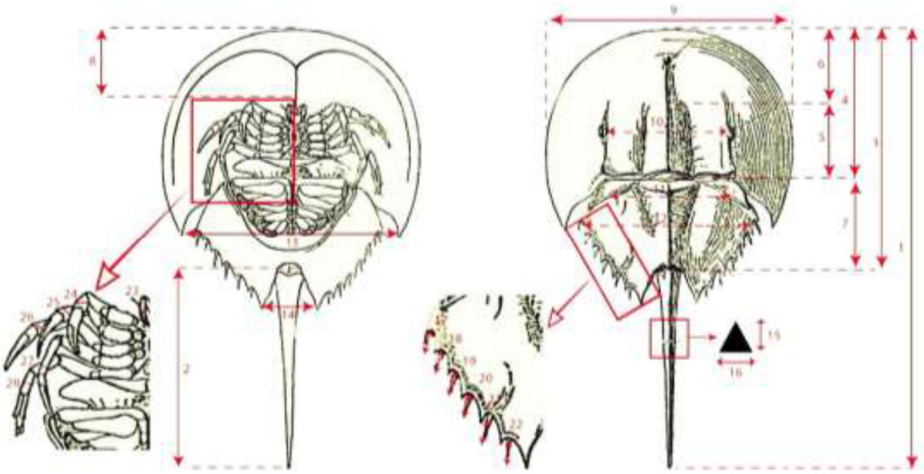

Fig. 1. The body of the horseshoe crab [8]. Description: XX1: character body size (1. Total length (X1), 2.Telson length (X2), 3. Body length (X3), 4. Prosoma length (X4), 5. Median ridge length (X5), 6 Length of front occeli (X6), 7. Length of ophistoma (X7), 8. The thickness of ventral messel (X8), 9. Maximum width of prosoma (X9), 10. Distance between compound eyes (X10), 11. Distance between auricula spine (X11), 12. Distance between marginal processes (X12), 13. Distance between posterior angles (X13), 14. Distance between anal angles (X14), 15. Mid-telson height (X15), 16. Mid-telson width) (X16). XX2 is the character of the size of the limbs, 17. Length of marginal spine 1 (X17), 18. Length of marginal spine II (X18), 19. Length of marginal spine III (X19), 20. Length of marginal spine IV (X20), 21. Length marginal spine V (X21), 22. Length of marginal spine VI (X22), 23. Diameter of cheliceral claw (X23), 24. Pedipalpi claw diameter (X24), 25. Diameter of walking claw I (x25), 26. Diameter of walking claw II (X26), 27. Diameter of walking claw III (X27), Diameter of walking claw IV (X28).

2.1 DNA barcoding procedure

2.1.1 Samples and preparation of Horseshoe crab

Tissue sampling on the gills, cut with scissors and tweezers that have been sterilized previously with 75% ethanol and clean with tissue. Then sterilize the tweezers and scissors again, rinse with ethanol and clean with tissue to be reused to the next individual sample. Each small piece is then put into a 2 mL cryovial labeled. To avoid resampling, each cut sample is tagged using a small plastic number and cable binder. The sample is preserved in 95% ethanol until it is used [12].

2.2 DNA extraction

DNA extraction is performed using a fast and simple DNA extraction method with Chelex 100 resin (BioRad). The 0.2 mm section of tissue is scraped off using a sterile spatula and inserted into a 1.5 mL tube containing 250 uL of 10% Chelex [13]. The sample is then vortex in the Chelex slurry for 10-15 seconds briefly at high speed and centrifuged for 10-15 seconds. Incubated for 45 minutes at 95 °C [14]. The chelex is then centrifuged again for 10-15 seconds to ensure that all contents are at the bottom of the microcentrifuge tube. Direct supernatants are used for Polymerase Chain Reaction (PCR) amplification.

2.3 PCR amplification

The benefits of PCR (Polymerase Chain Reaction) can detect differences in gene expression between cells, tissues and organisms [15]. The PCR reaction was carried out in a volume of 25 µL, using a 1.25 µL template. Universal primer for COI gene, consisting of forward primer JgLCO1490 : 5'-TIT CIA CIA AYC AYA ARG AYA TTG G-3' and reverse primer JgHCO2198 : 5'-TAI ACY TCI GGR TGI CCR AAR AAY CA-3' [16]. The PCR reaction was carried out in a volume of 25 µL, using 1.25 µL of DNA prints. Each reaction included 12.5 µL MyTeqTM Red Mix (Bioline), 1 µL each primer and 9.25 µL ddH2O. The thermocycling profile includes an initial denaturation of 95°C for 4 minutes, 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with a final elongation of 72°C for 10 minutes. The PCR reaction was examined on a 1% agarose gel stained with Florosafe.

2.4 DNA sequence analysis

The forward and backward sequences were corrected using MEGA X and then aligned using ClustalW [17]. Individual sequences were compared with National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) data using the Program's Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi (BLAST). The Neigbor Joining (NJ) method was used to produce phylogenetic reconstructions of NJ analysis based on genetic distance, performed in MEGA X with the 2-Parameter Kimura model using 1000 bootstrap iterations to assess clade support [18].

3 Results and Discussion

3.1 Morphological characteristics of horseshoe crab

Based on the results of observations that have been made and seen from the horseshoe crab characters that have been observed obtained from the research location show the same characteristics as the species *Carcinoscorpius rotundicauda*, the determination of the horseshoe crab species found refers to the identification key according to [19] and [20].

Horseshoe crab *C. rotundicauda* according to [19] and [20] has the following taxonomy:

Phylum : Arthropods
Sub Phylum : Chelicerata
Class : Meristomata
Sub Class : Xiphosura
Order : Xiphosurida
Super Family : Limulacea
Family : Tachypleinae
Genus : Carcinoscorpius
Species: *Carcinoscorpius rotundicauda*

Morphological observations of horseshoe crab samples have flatter and lower groupers, the spines on the prosoma are also small. The telson in the sample is longer than the prosoma and ophistosomes and does not contain any fine or plain spines. Based on measurements, the total length of the sample is 28 cm and this is the same as the study of [21] which states that in male *C. rotundicauda* have a total length range of 26-28.5 cm with an average of 27.38 cm, while in females the total length ranges from 24-28 cm with an average of 25.8 cm. As [22] stated that in comparison, the average male horseshoe crab has a length of about 28–30.5 cm (11.0–12.0 inches), including a tail measuring about 15–17.5 cm (5.9–6.9 inches),

and a carapace width of about 14.5–15 cm (5.7–5.9 inches). [23] stated that the total length of *C. rotundicauda* is 31.7 cm while the *Tachypleus gigas* species is 36.01 cm [24]. [25] said that the increase in the length of the prosoma is influenced by the availability of food and population density in the habitat environment.

The results of sample morphological observations can be seen in Table 1.

Table 1. Morphological Characteristics of Mimi from Madura Waters.

Number	Characteristics	Result
X1	total length	28 cm
X2	phone length	15.5 cm
X3	body length	13.5 cm
X4	prosoma length	7.5 cm
X5	median ridge length	4 cm
X6	occeli front length	3.5 cm
X7	opistoma length	6 cm
X8	ventral messel thickness	3.5 cm
X9	the maximum width of the prosoma	13.5 cm
X10	the distance between the compound eyes	6 cm
X11	the distance between the auricular spines	8.8 cm
X12	distance between marginal processes	93.3 mm
X13	the distance between the posterior corners	115.0 mm
X14	distance between anal angles	32.6 mm
X15	mid telson high	3.9 mm
X16	marginal spine length 1	3.1 mm
X17	marginal spine length 2	4.8 mm
X18	marginal spine length 3	6.6 mm
X19	marginal spine length 4	9.00 mm
X20	marginal spine length 5	6.40 mm
X21	marginal spine length 6	2.1 mm
X22	pedipalp pincer diameter	1.3 mm
X23	chelicerae pincer diameter	0.5 mm
X24	pedipalp pincer diameter	1.4 mm
X25	diameter of claw foot 1	2.2 mm
X26	diameter of claw foot 2	1.9 mm
X27	diameter of claw foot 3	1.9 mm
X28	diameter of claw foot 4	3.1 mm
	weight	152 gram

Fig. 2. Photo of Horseshoe Crab sample from Bangkalan Waters.

3.2 DNA of hosrshoe crab

The results of alignment of the sequence with the forward and reserve primers obtained a nucleotide base length of 640 bp. This is almost the same as [16] who stated that the size range of *C. rotundicauda* fragments in Madura is in the size range of 200 to 2400 pb. The primary selection in this study is CO1 which is different from [26] which uses influential RAPD analysis because each primer has its own attachment site.

The results of BLAST-n obtained 99% closeness with several species of *Carcinoscorpius rotundicauda* in the NCBI code database. The phylogeny tree construction shows three clades. *Carcinoscorpius rotundicauda* from Madura Island is in the same group as *Carcinoscorpius rotundicauda* and different group from *Tachypleus tridentatus*. In the construction of this phylogeny tree, *Limulus polyphemus* is used as an outgroup.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *1 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Carcinoscorpius rotundicauda voucher CR_2_Madura cytochrome c oxidase subunit I (COX1) gene, partial cds;...	Carcinoscorpius ...	1171	1171	99%	0.0	99.84%	659	MW454812.1
Carcinoscorpius rotundicauda voucher CR_4_Madura cytochrome c oxidase subunit I (COX1) gene, partial cds;...	Carcinoscorpius ...	1171	1171	99%	0.0	99.84%	659	MW454811.1
Carcinoscorpius rotundicauda voucher CR_3_Madura cytochrome c oxidase subunit I (COX1) gene, partial cds;...	Carcinoscorpius ...	1171	1171	99%	0.0	99.84%	659	MW454803.1
Carcinoscorpius rotundicauda voucher CR_5_Madura cytochrome c oxidase subunit I (COX1) gene, partial cds;...	Carcinoscorpius ...	1171	1171	99%	0.0	99.84%	659	MW454801.1
Carcinoscorpius rotundicauda voucher CR_1_Madura cytochrome c oxidase subunit I (COX1) gene, partial cds;...	Carcinoscorpius ...	1171	1171	99%	0.0	99.84%	659	MW454802.1
Carcinoscorpius rotundicauda isolate Johor_b1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitoc...	Carcinoscorpius ...	1142	1142	99%	0.0	99.21%	658	MF469062.1
Carcinoscorpius rotundicauda mitochondrion, complete genome	Carcinoscorpius ...	1140	1140	99%	0.0	98.90%	15033	NC_019623.1
Carcinoscorpius rotundicauda voucher AKB124 cytochrome oxidase subunit I (COX1) gene, partial cds; mitoch...	Carcinoscorpius ...	1134	1134	99%	0.0	98.90%	647	JF896106.1
Carcinoscorpius rotundicauda cytochrome oxidase subunit I gene, partial cds; mitochondrial gene for mitochond...	Carcinoscorpius ...	1134	1134	99%	0.0	98.90%	658	AF370828.1
Carcinoscorpius rotundicauda mitochondrion, complete genome	Carcinoscorpius ...	1127	1127	99%	0.0	98.59%	15037	JX437074.1
Carcinoscorpius rotundicauda isolate Pahang_b51 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; m...	Carcinoscorpius ...	1125	1125	99%	0.0	98.74%	658	MF469060.1
Carcinoscorpius rotundicauda voucher SDK10 mitochondrion, complete genome	Carcinoscorpius ...	1105	1105	99%	0.0	97.96%	15029	OP973936.1

Fig. 3. Results of BLAST-n with several species of *Carcinoscorpius rotundicauda* on the NCBI code database.

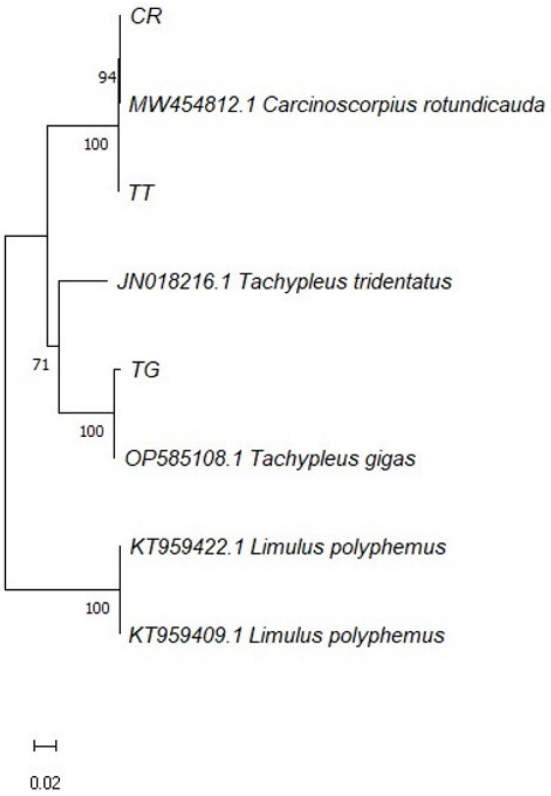

Fig. 4. Construction of the phylogeny tree *Carcinoscorpius rotundicauda*.

4 Conclusion

DNA barcoding analysis was employed to identify horseshoe crab species on Madura Island. Using the genetic marker Cytochrome Oxidase I from mitochondrial DNA (mtDNA), we explored the genetic diversity of these ancient animals. Our research revealed a close relationship between the samples and *Carcinoscorpius rotundicauda*, with a remarkable 99% similarity. This pioneering effort sheds light on Madura Island's potential as a source of horseshoe crab genetic diversity and provides valuable insights for sustainable management of horseshoe crab resources.

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