Biodiversity Journal, 2020,11 (2): 535–540 https://doi.org/10.31396/Biodiv.Jour.2020.11.2.535.540

Two morphotypes of tri-spine horseshoe crab, *Tachypleus tridentatus* (Leach, 1819) (Xiphosura Limulidae) in Indonesia and implications for species identification

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ABSTRACT Of the four extant species of horseshoe crabs in the world (Xiphosura Limulidae), Indonesia have three species, i.e., *Tachypleus tridentatus* (Leach, 1819), *T. gigas* (O.F. Müller, 1785) and *Carcinoscorpius rotundicauda* (Latreille, 1802). The species identification based on morphology of especially the females of the two *Tachypleus* species can be difficult. This study aimed at evaluating morphological characters used in species determination of *T. tridentatus* and *T. gigas*. Two morphological characters for separating the two species are the number and position of immovable spines and the spinnerets on the opisthosomatic carapace. The study was conducted in Balikpapan coastal area in Indonesia. Two morphotypes of *T. tridentatus* were found. Of the 120 collected specimens, 13.33% had one immovable spine, while 86.67% had three immovable spines on the opisthosomatic carapace. The two morphotypes were found in both males and females. DNA barcoding of both males and females with two morphotypes confirmed that the specimens were *T. tridentatus*. Thus, three immovable spines at opisthosoma can still be used as a morphological characters for *T. tridentatus* identification, but another morphotype with only one spine is present. This makes the distinction between the females of the two *Tachypleus* species more difficult. However, spinnerets on the dorsal opisthosoma can still be used to separate them.

KEY WORDS Horseshoe crab; morphogenetics; molecular identificatication; species complex.

Received 10.03.2020; accepted 13.05.2020; published online 30.06.2020

INTRODUCTION

The four species of extant horseshoe crabs are the American horseshoe crab, *Limulus polyphemus* (Linnaeus, 1758), restricted to Atlantic waters (Walls et al., 2002) and the three Asian horseshoe crabs (*Carcinoscorpius rotundicauda* (Latreille, 1802), *Tachypleus gigas* (O.F. Müller, 1785), and

Tachypleus tridentatus (Leach, 1819) (Lee & Morton, 2005; Sekiguchi & Shuster, 2009). All three species of Asian horseshoe crabs can be found in Indonesian coastal waters, distributed around Sumatra, Java, Kalimantan and Sulawesi (Rubiyanto, 2012; Mashar et al., 2017; John et al., 2018a, b; Vestbo et al., 2018).

The conservation status of *L. polyphemus* is vulnerable (VN) and the species has been intensively studied compared to *C. rotundicauda*, *T. gigas*, and *Tachypleus tridentatus*. Recently, *T. tridentatus* changed to endangered (EN) conservation status (IUCN, 2019) from data deficient (DD) (IUCN, 2015). The remaining two species of horseshoe crabs (*C. rotundicauda* and *T. gigas*) are categorised as data deficient conservation status (DD) (IUCN, 2015).

Since the number of studies of Asian horseshoe crabs are limited and their current geographic distribution is not fully known, it would be valuable to involve local communities living near the habitats of the horseshoe crabs in sampling and monitoring.

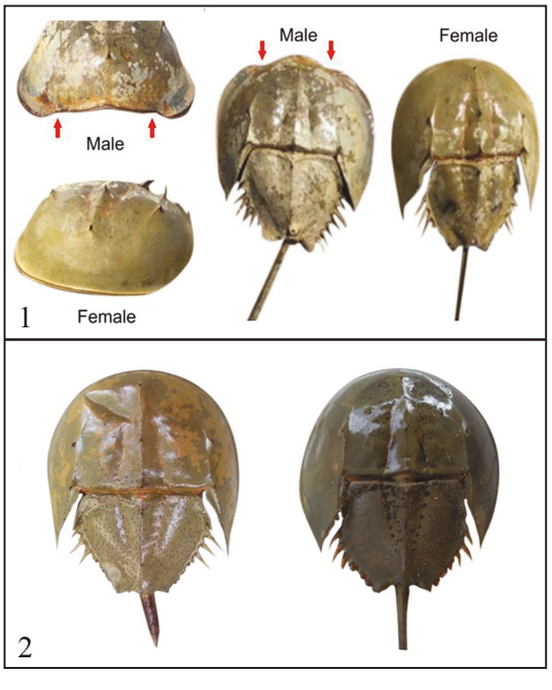


Figure 1. Male and female of *Tachypleus tridentatus* collected from Balikpapan coastal area, Indonesia. The prosoma in the male showed two clear symmetrical indentation as indicated by arrows. Figure 2. Dorsal view of female *T. tridentatus* (left) and *T. gigas* (right) showing smooth curves of prosoma which renders these two species as the same species.

However, in order to do this, knowledge on how to differentiate between the three Asian species using morphological characters is required. Determining sex for *T. tridentatus* can be done by abscertaining the presence of two clear symmetrical indentations in prosoma for males and their absences of it for females (Fig. 1). The indentations can be used to distinguish between male *Tachypleus tridentatus* and *T. gigas*. However, the frontal margin (smooth curve of prosoma) in the female of the two species look the same (Fig. 2).

Monophyly in the genus *Tachypleus* is strongly supported by phylogenetic analysis using molecular data (Obst el al., 2012), and the two species of *Tachypleus* are charactarized by a triangular telson in cross-section with a dorsal keel and a ventral groove and can easily be distinguished from the mangrove horseshoe crab *Carcinoscorpius rotundicauda* that has instead a subtriangular telson with round ridges in cross-section. Also, *C. rotundicauda* is small-sized compared to the two *Tachypleus* species (Yamasaki, 1988). For *T. tridentatus*, morphological characters used in previous studies are three immovable spines located on the dorsal posterior margin of ophisthosoma and spinnerets scattered on the dorsal opisthosomatic carapace (Pocock, 1902; Waterman, 1958; Yamasaki, 1988; Liao et al., 2002; Chiu & Morton, 2003). The common name tri-spine horsehoe crab used for *T. tridentatus* actually refers to the presence of the three immovable spines. The morphological characters used for identifying *T. gigas* is the presence of only one median immoveable spine pointed posteriorly, and a smooth opisthosomatic carapace except for the cardiac region (Yamasaki, 1988). This study aimed at evaluating the morphological characters used to differentiate between *T. tridentatus* and *T. gigas* and to provide a practical guide for species determination in voluntary monitoring by local communities living near the horseshoe crab habitats.

MATERIAL AND METHODS

Study area and specimen collection

The study was conducted in at fish landing sites of Balikpapan coastal area, Indonesia (Fig. 3). Horseshoe crabs were collected with help from

local fishermen using gillnet and trawl-like fishing gear. A total of 105 individuals of *T. tridentatus* were collected from 1 January to 31 March 2018 and 15 additional individuals were obtained from 23 October to18 November 2019. Two specimens of *Tachypleus gigas* were collected for morphological comparison with *T. tridentatus.*

Morphological identification and DNA barcoding

All collected specimens were identified morphologically following Pocock (1902) and Yamasaki (1988). Hemolymph from four specimens of *T. tridentatus* were sampled to validate the species identification using molecular approach. The four specimens included males and females with one and three spines on the posteriormost dorsal opisthosoma. Genomic DNA was extracted using the GeneAID Kit as instructed from the supplier. A cytochrome c oxidase I (CO1) mitochondrial gene fragment were amplified using the primer pairs designed by Nurlisa Alias Butet (2013; unpublished data). PCR amplification was performed in a SEDI Thermo Cycler RM G with 5 µl genomic DNA, 4.5 µl H2O, 12.5 taq polymerase 12.5 µl, CO1 forward primers 1.5 µl, reverse CO1 primers 1.5 µl. Stages of PCR include pre denaturation (95ºC for 5 min), denaturation (94ºC for 45 sec), annealing (54ºC for 1 min), elongation (72ºC for 1 min) and post elongation (72ºC for 1 min). The stages of denaturation, annealing and elongation were carried out in 35 cycles. The quality of PCR products were tested on 1.2% agarose gels, and those with amplified products were sent to sequencing facility by Genetika Science Indonesia. Forward and reverse nucleotide sequences were aligned with ClustalW in MEGA version 7 software (Kumar et al., 2016). The aligned sequences were blasted in GenBank using the National Center for Biotechnology Information (NCBI) (Zhang et al., 2000, Morgulis et al., 2008) for species identification and comparison.

RESULTS AND DISCUSSIONS

Of the 120 collected specimens of *T. tridentatus* from Balikpapan coastal waters, 13.33% of them had one mid-dorsal immovable spine on the posterior margin of the opisthosomatic carapace, while 86.67% of them had three immovable spines in the posterior of opisthosoma. The two morphotypes were found in both males and females (Fig. 4).

Figure 3. Sampling location in Balikpapan coastal area, Indonesia as indicated by the red arrow.

Successful PCR and sequence data was obtained from four *T. tridentatus* representing two males and two females of the two morphotypes. Blasting of 551–593 bp long CO1 gene fragments of all four samples showed 99% similarity with sequences of *Tachypleus tridentatus* in the NCBI database (access code JQ739210.1, FJ860267.1, and U09387.1). Consequently, the presence of the three immovable spines at the posteriormost opisthosoma of *T. tridentatus* could be potentially be used as a morphological character for species identification with precaution, although it was used succesfully in many previous studies (Pocock, 1902; Waterman, 1958; Yamasaki, 1988; Liao et al., 2002; Chiu & Morton 2003; Muslihah, 2004; Yang & Ko, 2015; Dolejš & Vaňousová, 2015; Hu et al., 2015). However, the presence of spinnerets on the dorsal opisthosomatic carapace of *T. tridentatus* compared to *T. gigas* with a smooth surface except for the cardiac region was consistently species-specific (Figs. 5, 6).

This study was based on specimens collected in the Balikpapan coastal area, Indonesia. It would be interesting to study possibly variability in the number and position of posterior immovable spines in *T. tridentatus* from other geographic areas to investigate their variation, either general or a local variation. A previous study that used morphometric analysis of body shape in three species of horseshoe crabs failed to show regional intraspecific variation in females of *C. rotundicauda* and *T. gigas*, however males of *C. rotundicauda* did show variation (Faurby et al., 2011).

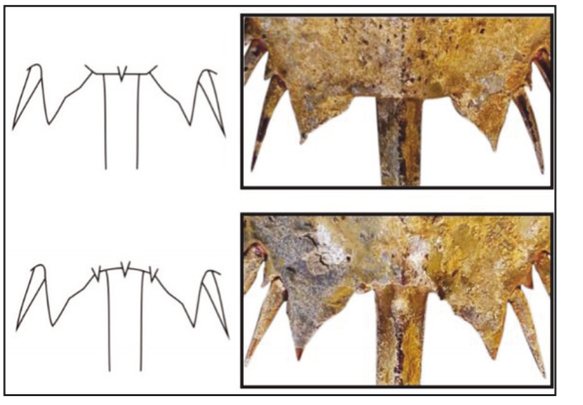
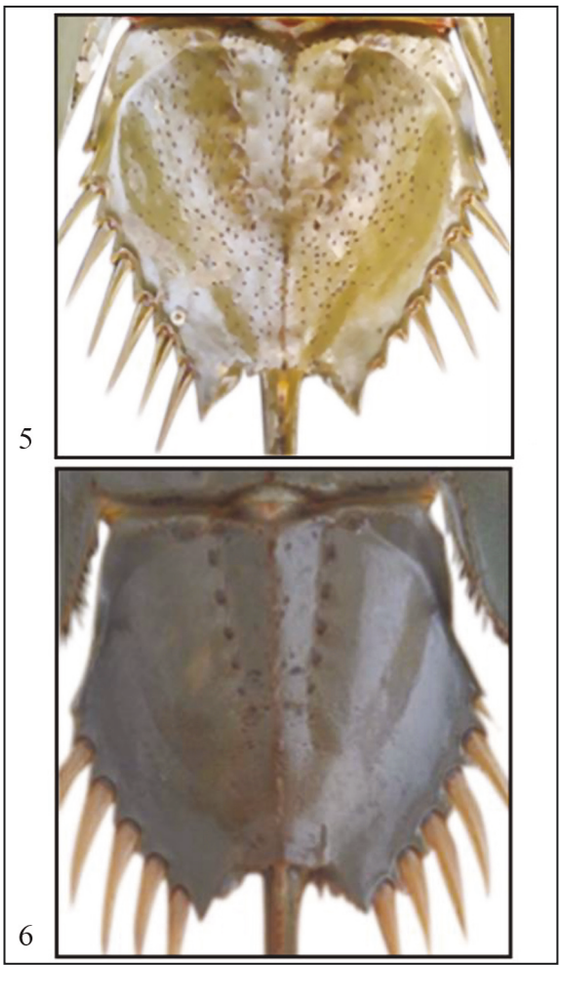


Figure 4. Two morphotypes of *Tachypleus tridentatus* from Balikpapan coastal area, Indonesia. The dorsal posteriormost opisthosoma carries either one or three spines.



Figures 5, 6. Comparison of the dorsal opisthosoma of *Tachypleus tridentatus* (Fig. 5) and *T. gigas* (Fig. 6) from Balikpapan coastal area, Indonesia.

CONCLUSIONS

It can be concluded that the distribution pattern of spinnerets on the dorsal opisthosomatic carapace could be potential to be used as a morphological character to distinguish between *T. tridentatus* from *T. gigas*, while the number and position of the immoveable spines as a brace character.

ACKNOWLEDGEMENTS

The research is funded by Research Center for Oceanography, Indonesian Institute of Sciences (PPO-LIPI) through Demand-Driven Research

Fund Contract No. B-5063/IPK.2/KS.02/III/2019 to YW and IPB University through PMDSU scheme Contract No. 6623/IT3.L1/PN/2019 to YW (following contract between IPB University and Ministry of Research, Technology and Higher Education No. 261/SP2H/LT/DRPM/20190). The authors want to thank to those who helped in sample collection and laboratory works: Heri Seputro, Rani Nuraisah, and Ananingtyas Septia Darmarini. Fahresa Nugraheni Supadminingsih is thanked for providing the photo of a female *T. gigas.*

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