New distribution record of *Myomenippe hardwickii* (Gray, 1831) (Crustacea: Decapoda: Brachyura) in Java, Indonesia

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Abstract. Indonesia is an archipelagic country with a very long coastline. The coastal area is home to many intertidal biota that have the ability to adapt to very rapid habitat changes. Cases of cryptic species are very common in crustaceans compared to other metazoan groups. This paper reports the congruence of recognizing members of the stone crab complex species of the genus Myomenippe (Decapoda; Brachyura) based on morphological characteristics and the CO1 gene of the mitochondrial genome. The stone crabs were collected from Ujung Kulon Bay and Indramayu Beach. Morphological descriptions were made based on photos which were then used as line drawings. The DNA material used was obtained from leg muscle biopsies. The results of morphological identification and the CO1 gene of the stone crab from Indramayu were confirmed as Myomenippe hardwickii. Meanwhile, two species were found from Ujung Kulon Bay, namely Myomenippe hardwickii and Menippe rumphii.

1. Introduction

True crabs (Brachyura) have diverse species. This infraorder has high diversity and lives in various habitats but is not followed by comprehensive species recording. The Brachyura infraorder in the world numbers 7200 species [1] and 1400 species have been reported in Indonesia [2].

Families in Brachyura are grouped according to the morphological characteristics such as the carapace surface, the claw shape, and the 4th ambulatory leg. However, grouping based on morphological characteristics often leads to misidentification. Mistakes in identification often occur in species that have similar characteristics and are difficult to distinguish. Many of these species are included in cryptic or complex groups that have high morphological similarities but are genetically different species [3].

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The Menippidae family, especially the *Menippe* and *Myomenippe* genus, represent similar morphologically; however, they turn out to be different species while being observed in more detail. *Menippe* and *Myomenippe* have been classified into different taxa [4]. Generally, the Menippidae family also has similar characteristics to some crabs in the Xanthidae family, such as *Leptodius exaratus* and *Etisus laevimanus*. This group has been classified based on morphology. Cases of cryptic species are very common in Crustacea compared to other metazoan groups [5].

The rock crab or thunder crab is the local name for *Myomenippe hardwickii*, which belongs to the Menippidae family and the Decapoda order. This rock crab is known by various names in various regions. The people on Dompak and Senggarang Islands in Riau Island call it the *okop* crab [6]. In the northern coastal areas of West Java, especially Indramayu, this crab is called the *krompol* or *prongkol* crab.

The habitat of *M. hardwickii* is in the mangrove area to the rocky intertidal zone full of shellfish [7]. Rock crabs have economic value as a source of additional income when mangrove crabs are hard to find. The demand for rock crabs is usually only for the claws because of their thick meat [6].

Molecular techniques such as DNA barcoding are useful for delineating species and explaining the biological relationships and function as species markers [8]. DNA barcoding is a taxonomic method to characterize and identify species using DNA sequences. The cytochrome oxidase subunit 1 (COI) gene is one of the common and standard species markers used. The short sequence of the COI gene, which is about 650 bp long, can be used as a barcoding tool to distinguish between species in taxonomy, ecology, and evolution. The use of the COI gene for DNA barcoding is very precise and accurate in identifying species in various marine organisms [9]. The COI gene is stable, deletions and insertions are rare, and has little variation, making it very suitable for use as a species marker [10]. This study aimed to identify the morphology of the *Myomenippe hardwickii* complex species from Indramayu and analyze the relationship based on the COI gene.

2. Materials and method

Sampling was conducted in February to March 2023. *Myomenippe hardwickii* (IM1) were obtained from the area around the fish pond in Pabean Ilir and the fish pond area in Karangsong, Indramayu (Figure 1). Sampling was carried out using the hand pick up and using traps. Then, all samples were documented, then fixed using 70% etanol. After 2 hours, the etanol was replaced with a concentration of 96%. The *Menippidae Myomenippe hardwickii* (UK3) and *Menippe rumphii* (UK2) samples were the collection of the Fungsi Hayati Laboratory, Department of Biology, FMIPA IPB, which came from Ujung Kulon.

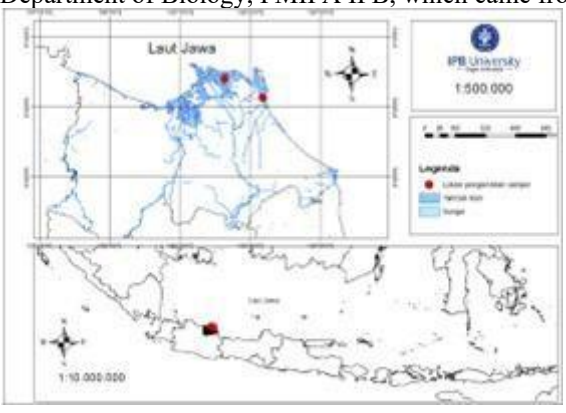

Fig.1. Sampling location map of *Myomenippe hardwickii* (IM1) in Indramayu Regency, West Java

2.1 Morphological identification

Captions Morphological identification and terminology followed Serène (1984) and Ng (1998) [7, 11] (Figures 2 and 3). The characteristics of *Myomenippe hardwickii* and *Menippe rumphii* were documented using a Xiaomi Redmi Note 10s smartphone. The photos were then corrected using Adobe Photoshop 2023 software. Morphological characters such as cheliped, carapace, anterolateral teeth, frontal node, and gastric area were sketched using Adobe Illustrator 2023 software. The sketches of the characteristics of *Myomenippe hardwickii* and *Menippe rumphii* were compared to identify the differences between the two species. The morphological characteristics of *Etisus laevimanus* and *Leptodius exaratus* were not sketched and followed the diagnosis of Abdelnaser-Amer *et al*. (2023) (Figure 4) [12].

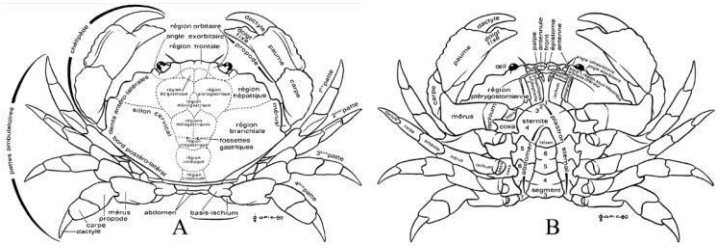

Fig.2. Morphological feature by Serène (1984). (A) Dorsal view, (B) Ventral view

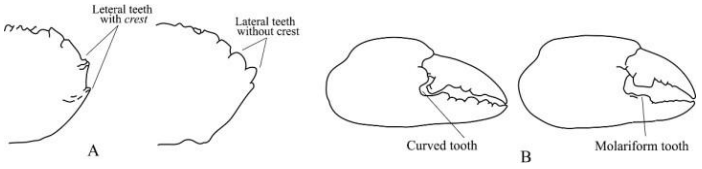

Fig. 3. Morphological feature by Ng (1998). (A) Anterolateral carapace. (B) Mayor surface of chela

Fig.4. (A) *Myomenippe hardwickii*, (B) *Menippe rumphii*, (C) *Leptodius exaratus* (Abdelnaser-Amer *et al*. 2023), (D) *Etisus laevimanus* (Abdelnaser-Amer *et al*. 2023)

2.2 Molecular identification

Specimens were preserved in 96% etanol. Muscle tissue was extracted and placed in tubes with sterile water. Samples were incubated at room temperature (25 °C) for 20 minutes, then centrifuged at 6000 rpm for 10 minutes. DNA extraction followed the protocol from the GENEAID genomic DNA Mini Kit (Tissue). DNA was amplified by PCR for 30 cycles using the forward primer AF 286 (5'-TCTACAAAYCATAAAGAYATYGG) and the reverse

primer AF 287 (5'- GTGGCRGANGTRAARTARGCTCG), which had been adapted for Crustacea with the addition of GoTaq® Green Mastermix. PCR was performed with predenaturation steps at 94°C for 3 minutes, followed by denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, elongation at 72°C for 1 minute, post-elongation at 72°C for 2 minutes, and finalization at 15°C for 10 minutes. PCR products were electrophoresed on 1.0% agarose gel with 1x TBE buffer and florosafe staining, then observed under UV light. Single and thick DNA bands were isolated for sequencing using sequencing company services.

2.3 COI gene analysis

The DNA sequencing results were opened using a text editor, then analyzed using MEGA 11 (Molecular Evolutionary Genetic Analysis) software [12]. For clarification, the DNA sequences were matched with the GenBank sequences as a reference using blastn. The obtained sequences were analyzed and aligned with the sequences listed in Table 1 from GenBank using ClustalW available in MEGA 11 with standard settings. The phylogeny tree was constructed using the Neighbor Joining method with the P-distance substitution model and verified using the Neighbor Joining method with the Kimura 2-Parameter substitution model, with 1000 bootstraps to support statistics.

Table 1. COI gene sequences used in analysis.

No.	Species	Accession Number	References
Ingroup			
1	*Myomenippe hardwickii* IM	-	This study
2	*Myomenippe hardwickii* UK3	-	This study
3	*Myomenippe hardwickii* voucher ZRC 2000.1136	HM638052	
4	*Myomenippe fornasinii*	NC024437	
5	*Menippe rumphii* UK2	-	This study
6	*Menippe rumphii* voucher 135cc06697	OL960453	
7	*Menippe rumphii* voucher ZRC 2003.0211	HM638051	
8	*Menippe mercenaria* voucher FTP_0247	KP255317	
9	*Menippe mercenaria* voucher FTP_0248	KP254868	

Table 2. COI gene sequences used in analysis (continue).

No.	Species	Accession Number	References
Ingroup			
10	*Leptodius exaratus* voucher RCAZUE-Crus-Br.91864.19	ON246345	Abdelnaser-Amer *et al.* (2023).
11	*Leptodius exaratus* voucher RCAZUE-Crus-Br.91864.17	ON246985	
12	*Etisus laevimanus* voucher RCAZUE-Crus-Br.914114.300	ON246340	
13	*Etisus laevimanus* voucher RCAZUE-Crus-Br.914114.20	ON246338	
Outgroup			
14	*Scylla serrata*	AF097019	

3. Results and discussion

Myomenippe hardwickii can be recognized by its round carapace and the 4th ambulatory leg that has a sharp tip, also a flat and long carpus (Figure 6A-B). The carapace has small granules with less clear area divisions, but the gastric area is still clearly visible on the carapace section (Figure 7B), including the mesogastric (S), metagastric (M), urogastric (U), and cardiac region (R). The mesogastric tip is long and the cardiac region slightly passes the urogastric area. The front section (Figure 8B) is separated by a deep notch, with three blunt teeth on each side. The anterolateral section (Figure 8F) of T2-T4 forms a fold (crest), with sharp teeth facing forward and an arrangement of small granules on the edge. The claws are large with variations in size. The carpus section (Figure 9B) has an arrangement of granules, while the major chela (Figure 9E-F) has a structure similar to a molariform. The fingers are black with blunt claw tips.

Fig. 5. Appearance of dorsal and ventral of *Myomenippe hardwickii*♂(A-B), Appearance of dorsal and ventral of *Menippe rumphii*♂ (C-D).

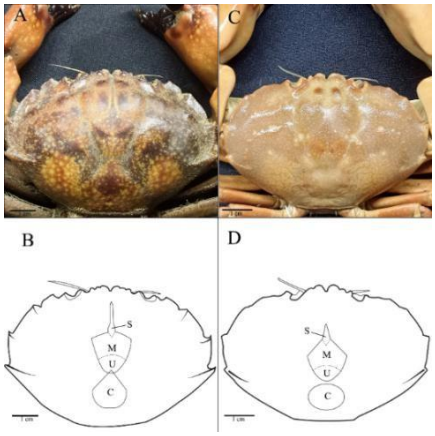

Fig. 6. Comparation of the carapace and the gastric region shape (A-B) *M. hardwickii* with (C-D) *M. rumphii*, (S) mesogastric, (M) metagastric, (U) urogastric, (C) cardiac region

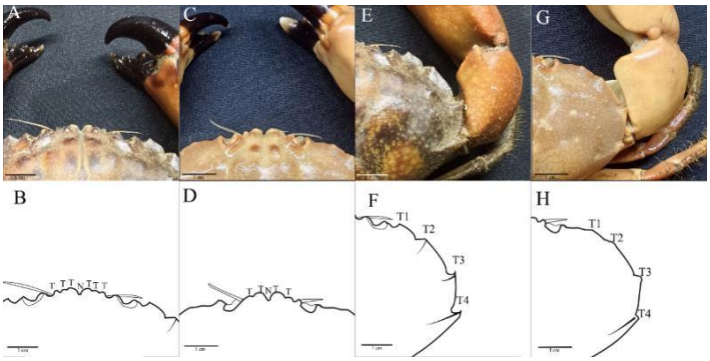

Fig. 7. Comparation of the front and anterolateral section (A-H). Front section (A-B) *M. hardwickii* with (C-D) *M. rumphii*, (T) teeth, (N) notch. Anterolateral section (E-F) *M. hardwickii* with (G-H) *M. rumphii*. (T1-T4) Teeth along the anterolateral margin.

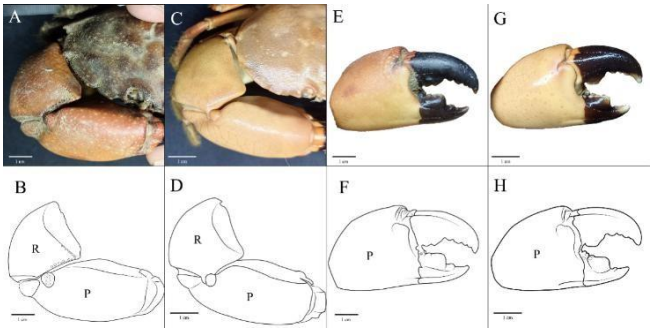

Fig. 8. Difference of cheliped and major cheliped (A-B) *M. hardwickii* with (C-D) *M. rumphii*, (R) carpus, (P) palm. Major chela (E-F) *M. hardwickii* with (G-H) *M. rumphii* (P) palm.

Menippe rumphii could be characterized by a slightly oval carapace laterally with a smooth surface and the 4th ambulatory leg as a walking leg with a pointed tip and the carpus

part is quite flat and long (Figure 6C-D). The carapace area division is not clearly visible but it could be recognized from the gastric part. In (Figure 7D), it shows that the mesogastric (S), the metagastric (M), the urogastric (U), and the cardiac region (R) in *Menippe rumphii* has a short mesogastric tip and the cardiac region does not intersect with the urogastric. The front section (Figure 8D) is separated by a deep notch, each side has 2 blunt teeth. The anterolateral part (Figure 8H) only T4 forms a fold (crest), has a blunt teeth arrangement slightly pointing forward and on the edge section there is no granule arrangement found. Having large claws with different sizes on the major chela (Figure 9G-H) there is a structure that resembles a graham (molariform). The black finger section has a blunt claw tip.

If the morphology of *M. hardwickii* and *M. rumphii* is compared to the two genes of the Xanthidae Family, that is represented by the *Leptodius exaratus* and the *Etisus laevimanus* based on the description of (Abdelnaser-amer *et al*. 2023) [3], both crabs also have several characteristics that are similar to those found in *M. hardwickii* and *M. rumphii*. The comparation of these characteristics could be seen in Table 2.

Table 2. Morphological comparison between *M. hardwickii* and *M. rumphii* with *L. exaratus* and *E. laevimanus*

No	Differentiation character	*Myomenippe hardwickii*	*Menippe rumphii*	*Leptodius exaratus* (Abdelnaser-Amer *et al.* 2023)	*Etisus laevimanus* (Abdelnaser-Amer *et al.* 2023)
1	Carapace	•Shape: Convex, rounded • Surface: granular, division of areas on the carapace is not clearly visible	• Shape: Flat, tapering to the side • Surface: smooth with the division of the carapace area not clearly visible	• Shape: flat, elongated to the side • Surface: granular or smooth with visible and raised areas	• Shape: Convex, tapering to the side • Surface: smooth with areas on the carapace that are slightly visible and tend to be flat
2	Frontal margin	• Front separated by a deep notch • Front section from notch to orbital has 3 teeth on each side	• Front separated by a deep notch • Front section from notch to orbital has 2 teeth on each side	• Front separated by a deep notch • Flat frontal section passes through supraorbital part	• Front separated by a slightly deep notch • Front section forms an angle passing through the supraorbital part
3	Anterolateral margin	Shape: Convex, granular at the edges and contains four pointed teeth	Shape: Convex without granules with four pointed teeth	Shape: Convex, slightly granular with four blunt teeth.	Shape: Convex. without granules with four pointed teeth

Table 2. Morphological comparison between *M. hardwickii* and *M. rumphii* with *L. exaratus* and *E. laevimanus* (continue)

No	Differentiation character	*Myomenippe hardwickii*	*Menippe rumphii*	*Leptodius exaratus* (Abdelnaser-Amer *et al.* 2023)	*Etisus laevimanus* (Abdelnaser-Amer *et al.* 2023)
4	Posterolateral margin	• Convex and smooth	• Concave and smooth	• Concave and smooth	• Concave and smooth
5	Cheliped	• The left and right sides of the upper surface of the palm are slightly rough and the tips of both fingers are blunt.	• The left and right side is that the upper surface of the palm is smooth, the tips of both fingers are blunt.	• Different left and right, the upper surface of the palm is rough, both ends of the fingers are blunt, resembling footprints.	• Slightly different left-right palm surface smooth dactylus concave forming a spoon
6	4thAmbulatory leg (kaki ambulatori ke-4)	• The shape of the carpus is slightly flat and long.	• The shape of the carpus is slightly flat and long.	• Short flat carpus shape	• Short flat carpus shape

Myomenippe hardwickii (IM1) and *Myomenippe hardwickii* (UK3) have 0 genetic distance. This indicates that both are identical species with *M. hardwickii* (HM638052) which has a distance of 0.014 (P-dist) and 0.015 (K2P). This confirmed that *M. hardwickii* (IM1) and (UK3) can be grouped as one species with *M. hardwickii* in the GenBank. *Myomenippe fornasinii* has a genetic distance of 0.081 P-dist and 0.087 K2P (Table 3) with *M. hardwickii.* This indicates that both are different species. *Menippe rumphii* (UK2) has a genetic distance of 0.002 K2P and 0.002 P-dist with *M. rumphii* in GenBank, confirming that *M. rumphii* (UK2) is an identical species with *M. rumphii* in GenBank. The genetic distance between *M. hardwickii* and *M. rumphii* was around 0.134 (P-dist) and 0.150 (K2P).

The species used in this study were compared with *Etisus laevimanus* and *Leptodius exaratus*, which have similar morphological characteristics, based on molecular analysis showed a genetic distance of around 0.190 - 0.192 P-dist and 0.220 - 0.224 K2P (Table 3). When compared with the outgroup species *Scylla serrata* (AF097019), it showed a large genetic distance, namely 0.530 - 0.566 (Substitution P-distance) and 0.924 - 1.097 (Kimura 2-Parameter model) (Table 3).

Table 3. The genetic distance of COI gene using 2 substitution method

No	Spesies	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***Myomenippe hardwickii* UK3**		0.000	0.015	0.087	0.150	0.147	0.147	0.152	0.149	0.221	0.221	0.221	0.221	1.021
2	***Myomenippe hardwickii* IM1**	0.000		0.015	0.087	0.150	0.147	0.147	0.152	0.149	0.221	0.221	0.221	0.221	1.021
3	*Myomenippe hardwickii* (HM638052)	0.014	0.014		0.097	0.150	0.147	0.147	0.147	0.144	0.236	0.236	0.221	0.219	1.020
4	*Myomenippe fornasinii* (NC 024437)	0.081	0.081	0.089		0.152	0.150	0.150	0.139	0.137	0.187	0.187	0.193	0.195	0.989
5	***Menippe rumphii UK2***	0.134	0.134	0.135	0.136		0.002	0.002	0.122	0.120	0.224	0.224	0.221	0.224	1.089
6	*Menippe rumphii* (OL960453)	0.132	0.132	0.133	0.134	0.002		0.000	0.120	0.117	0.221	0.221	0.218	0.221	1.077
7	*Menippe rumphii* (HM638051)	0.132	0.132	0.133	0.134	0.002	0.000		0.120	0.117	0.221	0.221	0.218	0.221	1.077
8	*Menippe mercenaria*(KP255317)	0.136	0.136	0.133	0.126	0.112	0.110	0.110		0.002	0.184	0.184	0.201	0.199	0.932
9	*Menippe mercenaria* (KP254868)	0.134	0.134	0.130	0.124	0.110	0.107	0.107	0.002		0.182	0.182	0.199	0.196	0.924
10	*Leptodius exaratus* (ON246345)	0.190	0.190	0.201	0.165	0.192	0.190	0.190	0.163	0.161		0.000	0.193	0.188	0.958
11	*Leptodius exaratus* (ON246985)	0.190	0.190	0.201	0.165	0.192	0.190	0.190	0.163	0.161	0.000		0.193	0.188	0.958
12	*Etisus laevimanus* (ON246338)	0.190	0.190	0.190	0.169	0.190	0.188	0.188	0.176	0.174	0.169	0.169		0.008	1.094
13	*Etisus laevimanus* (ON246340)	0.190	0.190	0.188	0.171	0.192	0.190	0.190	0.174	0.171	0.165	0.165	0.008		1.097
14	*Scylla serrata* (AF097019)	0.549	0.549	0.548	0.543	0.564	0.562	0.562	0.530	0.528	0.536	0.536	0.566	0.566	

Information: Kimura 2-Parameter/K2P (bottom diagonal) P-distance/P-dist (upper diagonal)
(…) is the genbank access code

3.1 Phylogenetic Analysis

The phylogenetic trees generated from the two substitution methods show differences. The phylogenetic tree constructed using the Neighbor Joining statistical method with the P-distance substitution model and 1000 bootstraps is more reliable than the tree constructed using the Neighbor Joining method with the Kimura 2-Parameter substitution model and 1000 bootstraps. Based on Putri's research (2010) [14], the P-distance model is quite reliable for constructing phylogenetic trees. From a total of 493 base pairs of sequence data, there are 313 variable sites, 171 conserved sites, and 141 parsimony informative sites.

Based on the formed phylogenetic tree (Figure 10), *M. hardwickii* (IM1) and *M. hardwickii* (UK3), form one cluster with *M. hardwickii* from GenBank in both models with bootstrap values of 100% and 99%, respectively. *Menippe rumphii* (UK2) also formed a cluster with *M. rumphii* and *M. rumphii* from GenBank. In the P-distance substitution model, the Menippidae family (genus *Menippe* and *Myomenippe*) formed a monophyletic group with a bootstrap value of 96%. In the Kimura 2-Parameter substitution model, *M. mercenaria* was not monophyletic with *M. rumphii* and the genus *Myomenippe*. Bootstrap values above 70% were considered valid for analysis using Neighbor Joining [15].

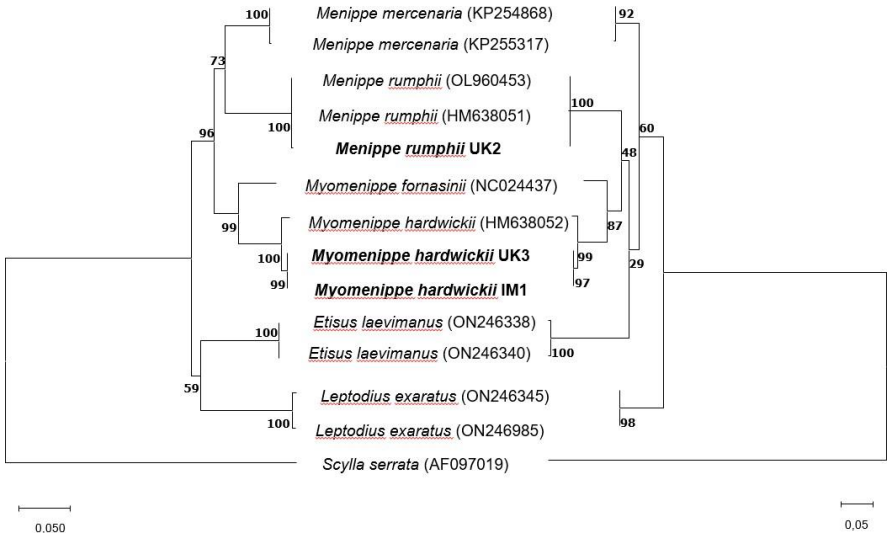

Fig. 9. Phylogenetic tree using the P-distance substitution method (left) with Kimura 2-Parameter (right) *Myomenippe hardwickii* with the Menippidae family and two genes from the Xanthidae family.

4. Conclusion

Based on the morphological and molecular identification, the Indramayu stone crab can be confirmed as the *Myomenippe hardwickii* species. *Myomenippe hardwickii* and *Menippe rumphii* from the Menippidae family were more closely related to *Etisus laevimanus* and *Leptodius exaratus* from the Xanthidae family, compared to *Scylla serrata* from the *Portunidae* family. The record of *Myomenippe hardwickii* in this study was the first record from Java.

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