dian bhagawati <dian.bhagawati@unsoed.ac.id>

[biodiv] Submission Acknowledgement

Ahmad Dwi Setyawan <smujo.id@gmail.com> Mon, Apr 18, 2022 at 1:52 PM
To: Dian Bhagawati <dian.bhagawati@unsoed.ac.id>

Dian Bhagawati:

Thank you for submitting the manuscript, "Morphological and molecular characterization of mole crab (Genus: Emerita) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of Emerita sp. nov." to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/11019
Username: bhagawati

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

Biodiversitas Journal of Biological Diversity

dian bhagawati <dian.bhagawati@unsoed.ac.id>

Request revisions

Managing Editor <unsjournals@gmail.com> Wed, Apr 20, 2022 at 10:20 AM
To: dian.bhagawati@unsoed.ac.id

Dian Bhagawati:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Morphological and molecular characterization of mole crab (Genus: Emerita) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of Emerita sp. nov.".

Our decision is: Revisions Required

Reviewer A:
Recommendation: -

Thank you,
Regards,

Ahmad Dwi Setyawan
Managing Editor,

04-Morphological and molecular characterization of mole crab xo.doc
2466K

dian bhagawati <dian.bhagawati@unsoed.ac.id>

[biodiv] Editor Decision

dian bhagawati <dian.bhagawati@unsoed.ac.id> Wed, Apr 20, 2022 at 10:20 AM
To: Smujo Editors <smujo.id@gmail.com>

To: Biodiversity Journal Editor

Thank you for the response and the results of the review, we will revise it soon

Regards
Dian Bhagawati
[Quoted text hidden]

dian bhagawati <dian.bhagawati@unsoed.ac.id>

[biodiv] New notification from Biodiversitas Journal of Biological Diversity

DEWI NUR PRATIWI <smujo.id@gmail.com> Thu, Apr 21, 2022 at 12:14 PM
Reply-To: Ahmad Dwi Setyawan <editors@smujo.id>
To: Dian Bhagawati <dian.bhagawati@unsoed.ac.id>

You have a new notification from Biodiversitas Journal of Biological Diversity:

There is new activity in the discussion titled "BILLING" regarding the submission "Morphological and molecular characterization of mole crab (Genus: Emerita) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of Emerita sp. nov.".

[Quoted text hidden]

dian bhagawati <dian.bhagawati@unsoed.ac.id>

[biodiv] Editor Decision

Agustina Putri <smujo.id@gmail.com> Sat, Apr 23, 2022 at 1:06 AM
To: DIAN BHAGAWATI <dian.bhagawati@unsoed.ac.id>, AGUS NURYANTO <authors@smujo.id>

DIAN BHAGAWATI, AGUS NURYANTO, ELLY TUTY WINARNI, ANASTASIA ENDANG PULUNGSARI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Morphological and molecular characterization of mole crab (Genus: Emerita) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of Emerita sp. nov. ".

Our decision is to: Accept Submission

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COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

Title:

Morphological and molecular characterization of mole crab (Genus *Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of *Emerita* sp. nov.

Author(s) name:

Dian Bhagawati, Agus Nuryanto, Elly Tuti Winarni, Anastasia Endang Pulungsari

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(Fill in your institution's name and address, your cellular phone, and email)

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For possible publication in the journal:

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Biodiversitas

Novelty:

(state your claimed novelty of the findings versus current knowledge)

Previous studies reported *Emerita emeritus* as the only member of Genus *Emerita* in the Cilacap Coastlines, Central Java, Indonesia. A recent study reported the presence of possible cryptic species along Cilacap coastlines based on a small number of samples (four individuals). The result was no statistically reliable enough to make a comprehensive and robust conclusion. Furthermore, there was no population genetics of *Emerita emeritus* or *Emerita* sp., especially in the Cilacap Region.

This study evaluated the taxonomic status of *Emerita* samples based on a higher number of mole crab from Cilacap coastlines than samples utilized in the previous survey. It provides new and the first information that focuses on the genetic diversity of suspected new *Emerita* species (*Emerita* sp.) from Cilacap coastlines.

Statements:

This manuscript has not been published and is not under consideration for publication in any other journal or any type of publication (including web hosting) either by my co- authors or me.

The author (s) has been read and agree to the Ethical Guidelines.

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Dr. Hamid Toha, email: hamid.toha@gmail.com; SCOPUS ID: 56100224700
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Prof. Harry W Palm, email: haryy.palm@uni-rostock.de
Prof. Dr. Zainal Abidin Muchlisin; email: muchlisinza@unsyiah.ac.id

Place and date:

Purwokerto, 19 April 2022

Sincerely yours,

(fill in your name, no need for a scanned autograph)

Dian Bhagawati

Morphological and molecular characterization of mole crab (Genus: *Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of *Emerita* sp. nov.

Abstract. Previous studies reported *Emerita emeritus* is the only species of the Genus *Emerita* inhabiting the coastal ecosystem of the Cilacap Regency. A recent study reported the presence of suspected new *Emerita* species living on the Cilacap sandy beach but used a small number of specimens and no reports about genetic diversity. This study used more Emerita samples than previous study. This study aimed to identify *Emerita* specimens based on the morphology and the cytochrome c oxidase 1 gene and analyzed the genetic diversity of *Emerita* sp. nov. *Emerita* samples were collected from three different beaches in Cilacap Regency, Central Java, Indonesia. Morphological identification placed the samples into two different morphotypes. Morphotype A was identified as *Emerita emeritus*. Morphotype B was determined as *Emerita* sp. nov. Molecular data support the placement of *Emerita* samples into *Emerita emeritus*, and *Emerita* sp. nov. *Emerita* sp. nov. has haplotype diversity of 0.857 ± 0.057, indicating a high genetic diversity. Haplotype H2 was suggested as the most primitive one because other haplotypes radiated from it. This study concluded that two sympatric *Emerita* species inhabit Cilacap coastlines, and *Emerita* sp. nov. has high genetic diversity.

Keywords: *Albunea*, genetic variation, *Hyppa*, polymorphism, sand crabs

Abbreviations: COI= cytochrome c oxidase 1

Running title: Morphological and molecular characterization of mole crabs

INTRODUCTION

Classical taxonomy and systematic utilized morphological data during species characterization (Erlank et al., 2018; Shu et al., 2022). In some animal groups, morphology characteristics are entirely satisfactory (Chan et al. 2016; Mauroka et al. 2018; Korovchinsky 2019). However, in other groups, this character may lead to identification mistakes, especially in groups with limited morphological differences, such as in mole crab from the Genus *Albunea* (Boko and MacLaughlin 2010), cryptic species (Karanovic 2015; Bilgin et al. 2015; Bekker et al. 2016; Kusbiyanto 2020) or group with limited and undeveloped morphological characters, such as egg, larvae, and early juvenile (Ko et al. 2013; Palero et al. 2016; Palecanda 2020)

Mole crabs, locally known as 'yutuk,' belong to Decapoda from the superfamily Hippoidea. It consists of three different families of Albuneidae, Blepharipodidae, and Hippidae, which are divided into *Emerita* and *Hippa* genera. Moreover, ten species have been identified and described under Genus *Emerita* (Boyko and McLaughlin 2010). This crustacean group is widely distributed over the World (Boyko and McLaughlin 2010), and the distribution has been elaborated by Mahapatro et al. (2018). In Indonesia, these crabs inhibit sandy coastlines from the West Coast of Sumatera to Moluccas (Wardiatno et al., 2015; Boyko and Harvey, 1999).

Previous studies reported that the three genera of Hippoidea have been described from Indonesia waters (Bhagawati et al. 2016; Pramithasari et al. 2017; Nugroho et al. 2018; Butet et al. 2019; Hartoko et al. 2019; Bhagawati et al. 2020). Other studies described *Emerita emeritus* as the only species of genus *Emerita* found on the southern coastlines of Java (Nugroho et al. 2018; Dewi et al. 2019; Krisanti et al. 2020; Suryanti et al. 2020), including from Cilacap sandy beaches, such as Widarapayung beach, District of Binangun (Bhagawati et al. 2016; Haq et al. 2018). However, recent studies observed morphological and molecular deviations in some samples to the *Emerita emeritus* characteristics. The possible presence of the species complex's sympatric of the Cilacap coastlines was reported (Nuryanto et al., 2020). Even, Hanim et al. (2017) proposed a scientific name for the new suspected species of Emerita from Pangandaran beach, as *Emerita pangandarensis* sp. nov., but it has not been approved by the international commission of zoological nomenclature. However, the studies by Nuryanto et al. (2020) and Hanim et al. (2017) were conducted in few samples and only focused on species identification. These studies did not report genetic diversity in newly suspected *Emerita* species. Molecular characterization was performed in a higher number of specimens and data types. Additionally, it assessed the genetic diversity of new suspected *Emerita* species collected from the southern coast of Cilacap, Central Java, Indonesia.

Species identification and population genetic studies were conducted using various molecular markers (Nuryanto et al., 2017; Butet et al., 2019; Nuryanto et al., 2019; Elvyra et al., 2020; Setyaningrum et al., 2022; Riani et al. 2021). The cytochrome c oxidase 1 (COI) gene is a common marker used in species determination (Ko et al., 2013; Muchlisin et al.,

2013; Dahruddin et al., 2016; Irmawati et al., 2017; Syaifudin et al. 2020) and population genetic studies (Song et al. 2013; Zhang et al. 2014; Fahmi 2015; Nuryanto et al. 2019). Therefore, this research aimed to characterize samples of genus *Emerita* based on morphological and molecular characteristics, as well as assess the genetic diversity using the cytochrome c oxidase 1 gene.

MATERIALS AND METHODS

Research location and sampling sites

The samples of mole crabs were collected from the sandy coastal region of the Cilacap Regency, Central Java, Indonesia. Additionally, the sampling was carried out in Jetis beach in District of Nusawungu as well as Kenari Indah and Widarapayung beaches in District of Binangun, Cilacap Regency, Central Java, Indonesia (Figure 1).

Figure 1. Peta Sampling Location of *Emerita* at Jetis Beach, Kenari Indah and Widarapayung (source: google maps, modified by S.S. Asmarani, 2022)

Mole crabs sampling

Emerita specimens were collected manually using two traditional fishing gears called "sodo nets" and "sorok bamboo" (Figure 2). Furthermore, local fishermen performed samples collection and handling. Abdominal tissue samples were cut off for approximately 5 mm^2 and preserved using 96% alcohol in 2 ml screw lid tubes.

Figure 2. Fishing gears for collecting mole crab (*Emerita*) samples (A. sodo nets and B. sorok bamboo)

Procedures

Morphological characterization

Freshly collected crabs were brought to Animal Taxonomy Laboratory, Faculty of Biology Jenderal Sudirman University. The samples were washed thoroughly using freshwater, and morphological characterization was carried out based on the diagnostic character essential for identifying crustaceans. According to NG (1998), several diagnostic characteristics for identifying crustaceans are carapax, anterolateral side, dorsal surface, frontal side, buccal cavern/mouthpart, and locomotion (dactyl and pereopod), abdominal segments, and gonopods.

Observations on the genus *Emerita* were performed by referring to the diagnostic character used by Sankolli (1965), Bhagawati et al. (2016, 2020); Boyko & Harvey (1999), Haig, (1986); Osawa & Chan (2010), and Wardiatno et al. (2015).

These characteristics are the color and shape of the carapax; the position, number, and shape of the slits (Carapace Groove/CG) on the carapax surface; spines on the anterior carapax; a curved shape of the margin on the latero-anterior; and the shape and number of fine spines on the latero-anterior portion. Carapax height measurements were conducted on the front, middle, and back of the body, with the shape and size of the eyestalk. The merus distal and dactyl form on the maxilliped-3 and the first pereopod, while spines and hairs form on the margin of the first pereopod dactyl. Pleopods are formed in the abdominal segment as pleural.

DNA isolation and marker amplification

Genomic DNA was isolated from abdominal tissue samples using the Quick-DNA™ Miniprep Plus kit from Zymo's research. The processes were conducted based on the procedures provided by the company. The extracted DNA was migrated in 1% agarose electrophoresis and stained using ethidium bromide. The COI gene marker fragments were amplified using FishF2 and FishR2 primers (Ward et al. 2005) in Primus 25 Peqlab Thermocycler. Subsequently, the amplification reactions consisted of 1x buffer PCR, 2 mM MgCl2, 0.2 mM of each primer, 0.2 mM dNTP mix, 1 U Taq polymerase, and 2.0 ng / µl template DNA. The final volume to 50 µl of the mixtures was adjusted by adding DNA-RNA-free water. Thermal cycles were started by pre-denaturation step at 95°C for 4 minutes. The amplification reactions were performed for 35 cycles with denaturation steps lasted for 30 seconds at 95°C, followed by annealing at 53°C for 120 seconds, and terminated by extension steps 60 for minutes at 72°C. Additionally, a final elongation step terminated the cycles after 5 minutes, at 72°C. The amplified COI marker were stained using ethidium bromide in 1.5% agarose gel and documented using the GelDoc apparatus (BioRad).

Marker sequencing and editing

Nucleotide sequencing of the used marker was conducted in the Molecular Genetic Laboratory of PT Genetika Science Indonesia Jakarta, according to Sanger method. The study obtained consensus and multiple alignments by assembling the forward and reverse sequences using ClustalW ver.1.4 in Bioedit (Hall 2011). In addition, it obtained haplotype and Arlequin data files from its generating process in DnaSP 6 (Rozas et al. 2017).

Data analysis

The taxonomic status of *Emerita* samples was delineated based a sequence homology to the conspecific relative available in GenBank. This test was carried out using an essential local alignment search tool (BLAST). Additionally, this study was also used genetic distance genetic divergence or a gap of 5% (Candek and Kuntner, 2015; Setyaningrum et al., 2020). Variance analysis and fixation statistic (Fst) were conducted in Arlequin 3.5 (Excoffier & Lischer 2010) to estimate significant genetic divergence between the morphotypes. The diversity data was evaluated using Haplotypes (h) and nucleotide (π) diversities, calculated using Arlequin 3.5. Similarly, the neutrality of the used COI marker was tested using Fs and D values (Excoffier & Lischer 2010). Evolutionary relationships among haplotypes were estimated based on haplotype networks reconstructed using the median-joining method in NETWORK software (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Taxonomic status

Morphological identification

A total of 17 individual *Emerita* samples were analyzed during the study. Morphological identification separated the samples into two different morphotypes. The first (A) and second (B) morphotypes consisted of 3 and 14 individuals, respectively (Figures 3A and 3B). However, they have similar general morphological characteristics that lead to Genus *Emerita* placement. The body of the *Emerita* crab is light, dark to blackish gray, with a slightly cylindrical shape with a wider distal carapace area. The eyestalk is long and slender, extending beyond the second antenna segment. The antennae are very long with hairy setae, and the segment on the second antenna consists of 3 horn-like and median spines. There are two oblique elongated protrusions with distinct spines that can be moved at the edges. The carapace has 3 frontal lobes, with the median very pointed and triangular, separated from the lateral lobe. The surface of the carapace has visible line-shaped slits located post-frontal and post-gastric. The latero-frontal margin has fine spines with sparse hairs. It has a short abdomen with a long telson, almost half the carapace length. The first pereopods were simple with an oval, lamellate dactyl, less than twice the width. Dactylus of the first pereopod with 4-5 rigid spines is found in the distal half of the lower margin, while 1-3 with 2 spines is in the tip.

The morphotypes A and B were differentiated by the following characteristics. The frontal part of the carapace showed differences in the shape and length of the spines at the base of the second antenna segment, the shape of a hollow between the three spines at the tip of the carapace, and the post-frontal and post-gastric cleft forms (Fig. 3). Individuals with morphotype A have eye stalks longer than the spines at the second antenna base (Figure 3A). In contrast, morphotype B has eye stalks almost the same length as the spines at the second antenna base. Another performance is the concave shape between the three spines on the frontal carapace. Morphotype A does not form an angle, while B forms a curve. The shape of the gap found in the post-frontal area is a straight line, neat and flat in morphotype A, but it is elevated in morphotype B

with a curved line in the post-stomach. In morphotype A, the arch is not too deep, and its carapace's right and left ends have a thin curved slit. In contrast, morphotype B has a narrower curved line.

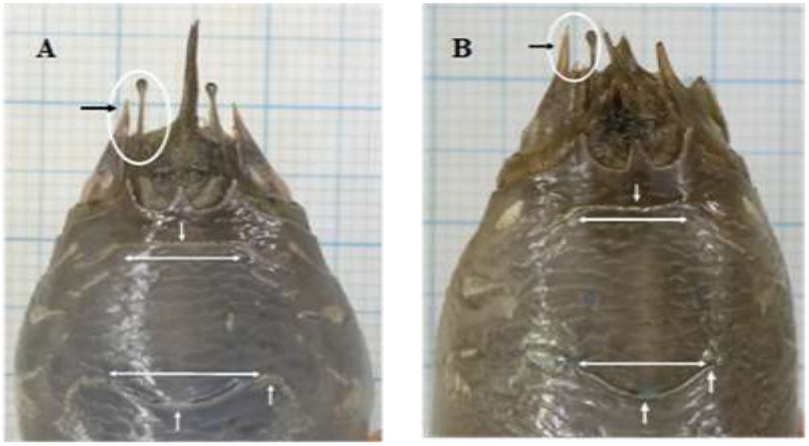

Figure 3. Frontal carapace on morphotype A and morphotype B

Based on the latero-frontal section on body height, morphotype A had a flatter body shape than B (Figure 4.). There are differences in the spines at the second antenna base from the lateral side between morphotypes A and B. Furthermore, Morphotype A has slightly curved outer spines, while B tends to be straight. The latero-frontal margin of the carapace, which contains fine spines with sparse hairs between the two morphotypes, has different spine shapes, arch, and the number of spines. The tip of the spine is not sharp; hence, when touched, it feels like a smooth protrusion. The anterior end of the margin does not have spines and has a shorter size than that of morphotype B. The shape of the posterior carapace margin curve in morphotype A is more prominent without spines. In contrast, it is more sloping in morphotype B, which has fine spines.

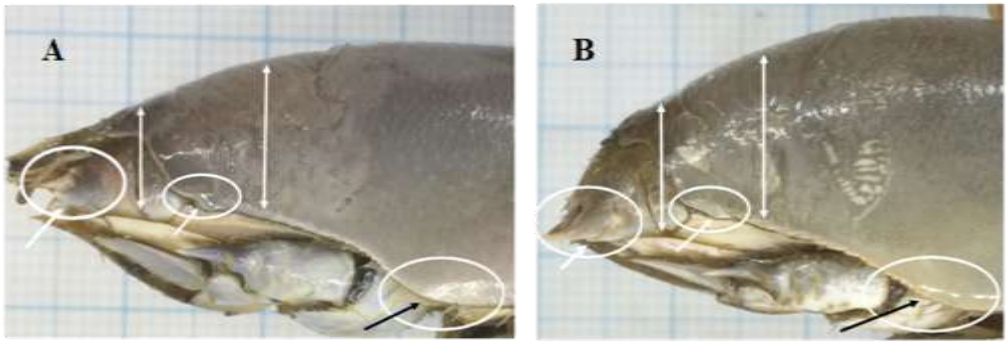

Figure 4. Latero-frontal carapace in morphotype A and morphotype B

Morphotypes A and B had different shapes on the distal part of the merus of the third maxilliped (Figure 5). Sankolli (1965), Kazmi and Siddiqui (2006), Boyko (2002), and Bhagawati et al. characterize morphotype A as having features similar to *Emerita emeritus* (Linnaeus, 1756) (2016; 2020). Morphotype A has the first pereopod dactyl oval, measuring less than twice the largest width. There are distinct spines on margins and occupy nearly the distal third of the lower part. Morphotype B has the character of the first pereopod dactyl, which is similar to morphotype A. However, the spines on the margins are smaller and possess the same size.

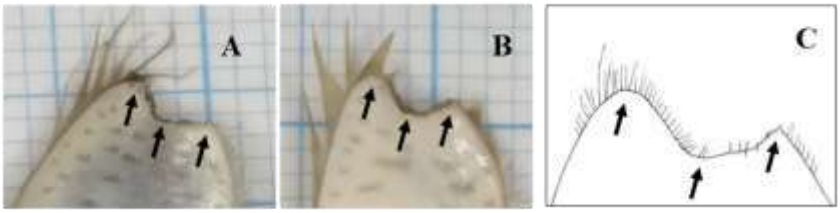

Figure 5. The distal part of the merus of the third maxilliped: Morphotype A; Morphotype B; and C. schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli 1965)

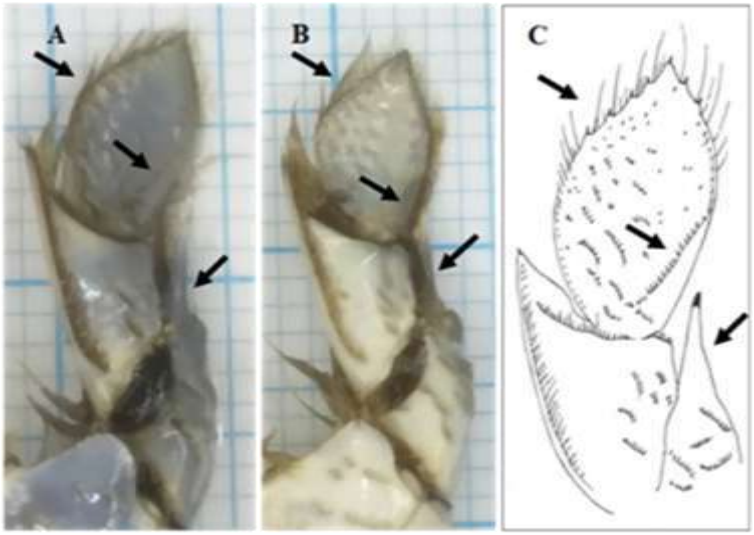

Figure 6. First pereopod on morphotype A, morphotype B, and schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli, 1965)

Based on their morphological characteristics, morphotypes A and B have many similarities (*Emerita*) and differences, suggesting the occurrence of new species. However, it has been well-known that mole crabs from the superfamily Hippoidea show high variability in morphology (Poore 2004; Ahyong et al. 2009; Schnabel and Ahyong 2009). This condition may lead to misidentification when performed based on morphological characters. Molecular data confirmed the possible occurrence of Sympatric species of *Emerita emeritus* in the Cilacap coastlines (Nuryanto et al. 2020) inferred from several specimens. Therefore, further study is still needed using more samples to strengthen the data on new *Emerita* species in the areas.

Molecular characterization

Sequence identity tests to the closest relative in GenBank revealed that three individuals (KI1, KI3, and WP9) of the morphotype A have high sequence identities to *Emerita emeritus* KR047035 ranging from 96.12 % to 96.25 %. In contrast, the sequence identities to *Emerita* sp. ranged from 85.60 % to 85.74 %. The remaining 14 individuals of the morphotype B showed low identities to *Emerita emeritus* KR 047035 in GenBank, ranging between 84.78% and 86.87%. The sequence identity of the remaining 14 specimens of the morphotype B to *Emerita* sp. MZ571198 was high ranging from 98.83 to 100% (Table 1).

Table 1. The BLAST parameters of *Emerita* samples from Cilacap coastlines to their conspecific relatives in GenBank

Samples	*Emerita emeritus* KR047035			*Emerita* sp. MZ571198		
	Coverage	Expect Value	Genetic identity (%)	Coverage	Expect Value	Genetic identity (%)
K1 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
K3 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
WP9 (A)	**99**	**0.00**	**96.25**	100	0.00	85.60
J3 (B)	96	2e179	86.87	**100**	**0.00**	**98.83**
J4 (B)	97	2e-125	84.78	**99**	**0.00**	**99.15**
J6 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
J7 (B)	99	0.00	86.44	**100**	**0.00**	**99.16**
J8 (B)	99	0.00	86.45	**99**	**0.00**	**100**
KI4 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
KI5 (B)	99	0.00	86.32	**100**	**0.00**	**99.67**
WP3 (B)	99	0.00	86.32	**100**	**0.00**	**99.51**
WP5 (B)	98	0.00	86.64	**99**	**0.00**	**99.83**
WP8 (B)	99	0.00	86.42	**100**	**0.00**	**99.85**
CLPE8* (B)	99	0.00	86.84	**100**	**0.00**	**99.84**
CLP4* (B)	98	2e-169	86.57	**100**	**0.00**	**99.82**
CLP11* (B)	98	5e-171	86.75	**100**	**0.00**	**100**
CLP15* (B)	98	5e-171	86.75	**100**	**0.00**	**100**

Genetic distance and genetic gap

Table 2 summarizes the genetic distance and gap between morphotype A and *E. emeritus* KR047035, as well as morphotype B and *Emerita* sp. MZ571198. The genetic gap was estimated based on the difference between the maximum and the minimum genetic distance of species.

Table 2. Genetic distance and gap within and among species (%)

Population	***Emerita emeritus***	***Emerita* sp.**
Emerita emeritus	0.00 – 3.20	16.80 – 19.00
Emerita sp.	16.80 – 0.190	0.00 – 1.70
The gap between *E. emeritus* and *Emerita* sp.	16.80-3.20 = **13.6**	

Genetic divergence

Variance analysis and Fst value indicated that the two morphotypes showed significant genetic differences with a p-value of 0.005 (Table 3). The significant genetic difference between the two indicated that both belong to two different species, proved by the BLAST result.

Table 3. Variance and Fst analysis indicate significant genetic divergence between two *Emerita* morphotypes

Source of variation	**d.f**	**Sum of squares**	**Variance components**	**Percentage of variation**
Between morphotypes	1	1.303	0.143^{Va}	26.53
Within morphotypes	17	6.750	0.397	73.47
Total	18	8.053	0.540	
Fixation index (Fst):	0.265			
p-value (Va and Fst)	0.0059			

Amino acid composition

The morphotypes were also subjected to amino acid composition to define molecular divergence, as summarized in Table 4.

Table 4. Amino acid composition of each morphotype (%)

Nucleotide	Morphotype	
	Morphotype A	Morphotype B
A	24.34	19.60
T	29.72	33.41
G	27.39	28.93
C	18.54	18.05

This research delineated the samples of morphotype A as *Emerita emeritus*. This is due to the strong genetic and conspecific identities of 96.12% to 96.25% and 85.60% to 85.74% for *Emerita* sp (MZ571198). Morphotype A was delineated into *E. emeritus* because genetic divergence within species may range from 0% to 4.6% (da Silva et al. 2014) or higher (Weis et al. 2014). Genetic divergence between morphotype A and *Emerita emeritus* KR047035 was below 4.6% (da Silva et al. 2014). The highest value was 3.88%, within the allowable range of 4% to 5%, as a moderate level of genetic identity for species delineation (Jeffery et al. 2011). This study has selected the value because the mutation rate of the COI gene is species-specific (Karanovic et al. 2015; Palecanda 2020). A genetic threshold between 4% and 5% is permissible for genetic species determination, although additional considerations should be accounted (Higashi et al. 2011; Jeffrey et al. 2011). Previous studies also utilized a genetic threshold of 5% during species determination (Candek and Kuntner 2015; Kusbiyanto et al. 2020; Riani et al. 2021).

The remaining 14 samples were identified as *Emerita* sp. nov. because of their high genetic identity (98.83% to 100%) to *Emerita* sp. MZ571198. In contrast, morphotype B had a low genetic identity (84.78% to 86.87%) to *Emerita emeritus* KR047035. This value is widely used as a genetic threshold in species delineation during animal barcoding (Hubert et al. 2010; Candek and Kuntner 2015).

The division of morphotypes A and B into two distinct species is due to a genetic distance ranging from 16.80% to 19.00%, with a genetic gap of % (Table 2). Moreover, the two morphotypes also showed significant genetic variances and fixation index ($p = 0.0059$, Table 3) with different compositions of nucleotide content, especially in Adenine (A) and Thymine (T) composition (Table 4). Amino acid AT was higher than GC in both morphotypes, but the content of A and T was different. The phenomena were also reported in fish (Elvyra et al. 2020). The molecular difference observed in this study is in line with morphotypes A and B morphology. Therefore, the delineation of morphotypes A and B as *Emerita emeritus* and *Emerita* sp. nov. was reliable.

This study also proved that the CO1 gene is a good marker for taxonomic identification into species level. The reliability the COI gene as a barcode because it is highly variable among animal species (Sachithanandam et al. 2012; Balkhis et al. 2011; Winarni et al., 2021). Similar phenomena were also reported from several locations across Indonesia (Muchlisin et al. 2013; Irmawati et al. 2017; Pramono et al. 2017) and other regions (Aquilino et al. al. 2011; Triantafyllidis et al. 2011).

Historical demography and genetic diversity of *Emerita* sp. nov.

Historical demography

Tajima's D value was -1.563 (p = 0.044). That statistically significant results assumed that the used COI marker was under selection pressure. Instead of accepting selection pressure on the used marker, the negative sign of the D value indicated a recent population bottleneck and neutrality of the marker (Tajima1989; Jong et al. 2011). The negative symptoms and non-significant Fs (-1.580, p= 0.147) could be used to assume that the marker was neutral marker and indicated population bottleneck (Table 5). The assumption was based on a fact that Fus' Fs values are believed to be more sensitive than Tajimas' D. According to Jong et al. (2011) and Mohammed et al. (2021), sensitivity of Fus' Fs values because it is calculated based on nucleotide diversity. A similar phenomenon was also reported in fish (Setyaningrum et al. 2022). Therefore, the used COI marker could be assumed as a neutral marker for assessing the genetic diversity of the Emerita sp population in the Cilacap coastlines.

Table 5. Species, number of individuals (N), number of haplotypes (nhp), haplotype diversity (h), nucleotide diversity (μ), Tajima'D, and Fu's Fs values

Species	N	nhp	h	μ	D	p-sig.	Fs	p-sig.
Emerita sp.	15	7	0.857 ± 0.057	0.005 ± 0.003	-1.563*	0.044	-1.580ns	0.147

Note: *= significant, ns= not significant

Genetic diversity

Multiple sequences alignment resulted s total of 418 bp COI gene fragment from 14 individuals *Emerita* sp. nov. collected from the coastlines of Widarapayung Wetan, Widarapayung Kulon, and Sedayu Villages, District of Binagun, Cilacap Regency, Central Java, Indonesia. Furthermore, 12 out of 418 bp were polymorphic, resulting in 7 haplotypes, and the haplotype diversity was 0.857 ± 0.057 (Table 5). This data indicates that the *Emerita* sp. nov. population in the Cilacap coastlines has high genetic diversity. The nucleotide diversity value (μ) was 0.005 ± 0.003, which revealed low nucleotide diversity and a relatively low rate of evolution in the *Emerita* sp. nov. population on the Cilacap coastlines. The haplotype network (Figure 3) shows that haplotypes were separated by 2 to 5 mutation steps. However, the mutation was widely distributed in the population, as indicated by high diversity (0.857 ± 0.057). High haplotype diversity assessed using the COI gene was widespread in animal phyla (Dorn et al. 2011; Dung et al. 2013; Song et al. 2013; Zhang et al. 2014; Nuryanto et al. 2019). At the same time, low haplotype diversity was also common in animal populations (Setyaningrum et al. 2022). The COI gene's study may show a complex pattern of diversity levels, even within species (Pavesi et al. 2011; Parmaksiz and Eksi 2017). The phenomena are also observed in population studies using other markers, such as microsatellites (Esa and Rahim 2013; Gouskov et al. 2016; Abbas et al. 2017; Achrem et al. 2017; Cheng et al. 2017) and d-loop (Zhong et al. 2013; Liu et al. 2016; Lau et al. 2018; Parmaksiz 2019; Ariyaraphong et al. 2021; Zhang et al. 2022).

This study cannot be compared with previous results because there is no population genetic study on mole crabs, especially on the presumable *Emerita* sp. nov. The only population study was conducted by Pramithasari et al. (2017), who compared mole crabs (*Albunea symmysta*) populations in Java and Sumatra. However, their study used morphological data, and the comparison to Pramithasari et al. (2017) was not congruent. This fact implies that more studies on the population genetics of mole crabs are needed.

Evolutionary relationships among *Emerita* sp. nov. individuals

The evolutionary process of the *Emerita* sp. nov. population on the southern coast of Cilacap is presented in the haplotype network (Figure 7). Star-like haplotype network in Figure 7 showed that haplotype 2 was the most primitive. Meanwhile, H2 was the center of the network, and other haplotypes evolved from (H2) as the most abundant (Balkhis et al., 2011; Song et al., 2012). The result contradicted the general acceptance that primitive haplotype has the highest abundance in the population (Adamson et al. 2012; Barasa et al. 2014; Basvar et al. 2018; 2019). The low frequency of H2 observed was assumed because of the small population (14 individuals). However, this assumption should be proven based on a further study using a high number of analyzed individuals.

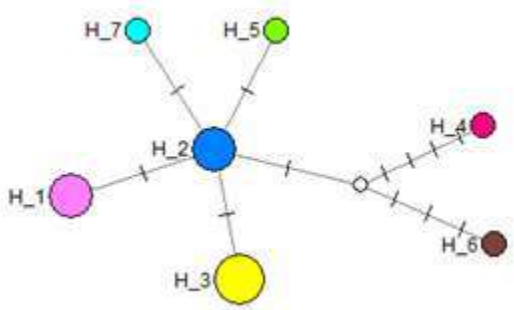

Figure 7. Haplotype networks indicating evolutionary relationships among *Emerita* sp. individuals

According to the analyzed data, this study concluded that mole crabs (Genus *Emerita*) in the Cilacap coastlines consisted of two distinct sympatric species (*Emerita emeritus* and *Emerita* sp. nov). *Emerita* sp. nov. had high haplotype diversity and was more abundant than *Emerita emeritus*. As a result, comprehensive research in terms of sampling site, number of samples, and other biological characteristics are needed to provide complete information for sympatric and taxa species of *Emerita* sp. nov.

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Morphological and molecular characterization of mole crab (Genus: *Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of *Emerita* sp. nov.

Abstract. Previous studies reported *Emerita emeritus* is the only species of the Genus *Emerita* inhabiting the coastal ecosystem of the Cilacap Regency. A recent study reported the presence of suspected new *Emerita* species living on the Cilacap sandy beach but used a small number of specimens and no reports about genetic diversity. This study used more Emerita samples than the previous study. This study aimed to identify *Emerita* specimens based on the morphology and the cytochrome c oxidase 1 gene and analyzed the genetic diversity of *Emerita* sp. nov. *Emerita* samples were collected from three different beaches in Cilacap Regency, Central Java, Indonesia. Morphological identification placed the samples into two different morphotypes. Morphotype A was identified as *Emerita emeritus*. Morphotype B was determined as *Emerita* sp. nov. Molecular data support the placement of *Emerita* samples into *Emerita emeritus*, and *Emerita* sp. nov. *Emerita* sp. nov. has haplotype diversity of 0.857 ± 0.057, indicating a high genetic diversity. Haplotype H2 was suggested as the most primitive one because other haplotypes radiated from it. This study concluded that two sympatric *Emerita* species inhabit Cilacap coastlines, and *Emerita* sp. nov. has high genetic diversity.

Keywords: *Albunea*, genetic variation, *Hyppa*, polymorphism, sand crabs

Abbreviations: COI= cytochrome c oxidase 1

Running title: Morphological and molecular characterization of mole crabs

INTRODUCTION

Classical taxonomy and systematic utilized morphological data during species characterization (Erlank et al., 2018; Shu et al., 2022). In some animal groups, morphology characteristics are entirely satisfactory (Chan et al. 2016; Mauroka et al. 2018; Korovchinsky 2019). However, in other groups, this character may lead to identification mistakes, especially in groups with limited morphological differences, such as in mole crab from the Genus *Albunea* (Boko and MacLaughlin 2010), cryptic species (Karanovic 2015; Bilgin et al. 2015; Bekker et al. 2016; Kusbiyanto 2020) or group with limited and undeveloped morphological characters, such as egg, larvae, and early juvenile (Ko et al. 2013; Palero et al. 2016; Palecanda 2020)

Mole crabs, locally known as 'yutuk,' belong to Decapoda from the superfamily Hippoidea. It consists of three different families of Albuneidae, Blepharipodidae, and Hippidae, which are divided into *Emerita* and *Hippa* genera. Moreover, ten species have been identified and described under Genus *Emerita* (Boyko and McLaughlin 2010). This crustacean group is widely distributed over the World (Boyko and McLaughlin 2010), and the distribution has been elaborated by Mahapatro et al. (2018). In Indonesia, these crabs inhibit sandy coastlines from the West Coast of Sumatera to Moluccas (Wardiatno et al., 2015; Boyko and Harvey, 1999).

Previous studies reported that the three genera of Hippoidea have been described from Indonesia waters (Bhagawati et al. 2016; Pramithasari et al. 2017; Nugroho et al. 2018; Butet et al. 2019; Hartoko et al. 2019; Bhagawati et al. 2020). Other studies described *Emerita emeritus* as the only species of genus *Emerita* found on the southern coastlines of Java (Nugroho et al. 2018; Dewi et al. 2019; Krisanti et al. 2020; Suryanti et al. 2020), including from Cilacap sandy beaches, such as Widarapayung beach, District of Binangun (Bhagawati et al. 2016; Haq et al. 2018). However, recent studies observed morphological and molecular deviations in some samples to the *Emerita emeritus* characteristics. The possible presence of the species complex's sympatric of the Cilacap coastlines was reported (Nuryanto et al., 2020). Even, Hanim et al. (2017) proposed a scientific name for the new suspected species of Emerita from Pangandaran beach, as *Emerita pangandarensis* sp. nov.~~, but~~. Still, the international commission has not approved its~~it has not been approved by the international commission of~~ zoological nomenclature. However, the studies by Nuryanto et al. (2020) and Hanim et al. (2017) were conducted in few samples and only focused on species identification. These studies did not report genetic diversity in newly suspected *Emerita* species. Molecular characterization was performed in a higher number of specimens and data types. Additionally, it assessed the genetic diversity of new suspected *Emerita* species collected from the southern coast of Cilacap, Central Java, Indonesia.

Species identification and population genetic studies were conducted using various molecular markers (Nuryanto et al., 2017; Butet et al., 2019; Nuryanto et al., 2019; Elvyra et al., 2020; Setyaningrum et al., 2022; Riani et al. 2021). The

cytochrome c oxidase 1 (COI) gene is a common marker used in species determination (Ko et al., 2013; Muchlisin et al., 2013; Dahruddin et al., 2016; Irmawati et al., 2017; Syaifudin et al. 2020) and population genetic studies (Song et al. 2013; Zhang et al. 2014; Fahmi 2015; Nuryanto et al. 2019). Therefore, this research aimed to characterize samples of genus *Emerita* based on morphological and molecular characteristics~~, as well as~~ and assess the genetic diversity using the cytochrome c oxidase 1 gene.

MATERIALS AND METHODS

Research location and sampling sites

The samples of mole crabs were collected from the sandy coastal region of the Cilacap Regency, Central Java, Indonesia. Additionally, the sampling was carried out in Jetis beach in the District of Nusawungu as well as Kenari Indah and Widarapayung beaches in the District of Binangun, Cilacap Regency, Central Java, Indonesia (Figure 1).

Figure 1. Peta Sampling Location of *Emerita* at Jetis Beach, Kenari Indah and Widarapayung (source: google maps, modified by S.S. Asmarani, 2022)

Mole crabs sampling

Emerita specimens were collected manually using two traditional fishing gears called "sodo nets" and "sorok bamboo" (Figure 2). Furthermore, local ~~fishermen~~ fishers performed samples collection and handling. Abdominal tissue samples were cut off for approximately 5 mm^2 and preserved using 96% alcohol in 2 ml screw lid tubes.

Figure 2. Fishing gears for collecting mole crab (*Emerita*) samples (A. sodo nets and B. sorok bamboo)

Procedures

Morphological characterization

Freshly collected crabs were brought to Animal Taxonomy Laboratory, Faculty of Biology Jenderal Sudirman University. The samples were washed thoroughly using freshwater, and morphological characterization was carried out based on the diagnostic character essential for identifying crustaceans. According to NG (1998), several diagnostic characteristics for identifying crustaceans are carapax, anterolateral side, dorsal surface, frontal side, buccal cavern/mouthpart, and locomotion (dactyl and pereopod), abdominal segments, and gonopods.

Observations on the genus *Emerita* were performed by referring to the diagnostic character used by Sankolli (1965), Bhagawati et al. (2016, 2020); Boyko & Harvey (1999), Haig, (1986); Osawa & Chan (2010), and Wardiatno et al. (2015). These characteristics are the color and shape of the carapax; the position, number, and shape of the slits (Carapace Groove/CG) on the carapax surface; spines on the anterior carapax; a curved shape of the margin on the latero-anterior; and the shape and number of fine spines on the latero-anterior portion. Carapax height measurements were conducted on the front, middle, and back of the body, with the shape and size of the eyestalk. The merus distal and dactyl form on the maxilliped-3 and the first pereopod, while spines and hairs form on the margin of the first pereopod dactyl. Pleopods are formed in the abdominal segment as pleural.

DNA isolation and marker amplification

Genomic DNA was isolated from abdominal tissue samples using the Quick-DNA™ Miniprep Plus kit from Zymo's research. The processes were conducted based on the procedures provided by the company. The extracted DNA was migrated in 1% agarose electrophoresis and stained using ethidium bromide. The COI gene marker fragments were amplified using FishF2 and FishR2 primers (Ward et al. 2005) in Primus 25 Peqlab Thermocycler. Subsequently, the amplification reactions consisted of 1x buffer PCR, 2 mM MgCl2, 0.2 mM of each primer, 0.2 mM dNTP mix, 1 U Taq polymerase, and 2.0 ng / µl template DNA. The final volume to 50 µl of the mixtures was adjusted by adding DNA-RNA-free water. The pre-denaturation step at 95°C started thermal cycles~~Thermal cycles were started by pre-denaturation step at 95°C~~ for 4 minutes. The amplification reactions were performed for 35 cycles with denaturation steps that lasted for 30 seconds at 95°C, followed by annealing at 53°C for 120 seconds, and terminated by extension steps 60 for minutes at 72°C. Additionally, a final elongation step terminated the cycles after 5 minutes, at 72°C. The amplified COI marker ~~were~~ was stained using ethidium bromide in 1.5% agarose gel and documented using the GelDoc apparatus (BioRad).

Marker sequencing and editing

Nucleotide sequencing of the used marker was conducted in the Molecular Genetic Laboratory of PT Genetika Science Indonesia Jakarta, according to the Sanger method. The study obtained consensus and multiple alignments by assembling the forward and reverse sequences using ClustalW ver.1.4 in Bioedit (Hall 2011). In addition, it obtained haplotype and Arlequin data files from its generating process in DnaSP 6 (Rozas et al., 2017).

Data analysis

The taxonomic status of *Emerita* samples was delineated based on ~~a~~ sequence homology to the conspecific relative available in GenBank. This test was carried out using an essential local alignment search tool (BLAST). ~~Additionally, this~~This study ~~was~~ also used genetic distance genetic divergence or a gap of 5% (Candek and Kuntner, 2015; Setyaningrum et al., 2020). Variance analysis and fixation statistic (Fst) were conducted in Arlequin 3.5 (Excoffier & Lischer 2010) to estimate significant genetic divergence between the morphotypes. The diversity data was evaluated using Haplotypes (h) and nucleotide (π) diversities, calculated using Arlequin 3.5. Similarly, the neutrality of the used COI marker was tested using Fs and D values (Excoffier & Lischer 2010). Evolutionary relationships among haplotypes were estimated based on haplotype networks reconstructed using the median-joining method in NETWORK software (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Taxonomic status

Morphological identification

A total of 17 individual *Emerita* samples were analyzed during the study. Morphological identification separated the samples into two different morphotypes. The first (A) and second (B) morphotypes consisted of 3 and 14 individuals, respectively (Figures 3A and 3B). However, they have similar general morphological characteristics that lead to Genus *Emerita* placement. The body of the *Emerita* crab is light, dark to blackish gray, with a slightly cylindrical shape with a wider distal carapace area. The eyestalk is long and slender, extending beyond the second antenna segment. The antennae are very long with hairy setae, and the segment on the second antenna consists of 3 horn-like and median spines. There are two oblique elongated protrusions with distinct spines that can be moved at the edges. The carapace has 3 frontal lobes, with the median very pointed and triangular, separated from the lateral lobe. The surface of the carapace has visible line-shaped slits located post-frontal and post-gastric. The latero-frontal margin has fine spines with sparse hairs. It has a short abdomen with a long telson, almost half the carapace length. The first pereopods were simple with an oval, lamellate dactyl, less than twice the width. Dactylus of the first pereopod with 4-5 rigid spines is found in the distal half of the lower margin, while 1-3 with 2 spines is in the tip.

The morphotypes A and B were differentiated by the following characteristics. The frontal part of the carapace showed differences in the shape and length of the spines at the base of the second antenna segment, the shape of a hollow between the three spines at the tip of the carapace, and the post-frontal and post-gastric cleft forms (Fig. 3). Individuals with morphotype A have eye stalks longer than the spines at the second antenna base (Figure 3A). In contrast, morphotype B

has eye stalks almost the same length as the spines at the second antenna base. Another performance is the concave shape between the three spines on the frontal carapace. Morphotype A does not form an angle, while B forms a curve. The shape of the gap found in the post-frontal area is a straight line, neat and flat in morphotype A, but it is elevated in morphotype B with a curved line in the post-stomach. In morphotype A, the arch is not too deep, and its carapace's right and left ends have a thin curved slit. In contrast, morphotype B has a narrower curved line.

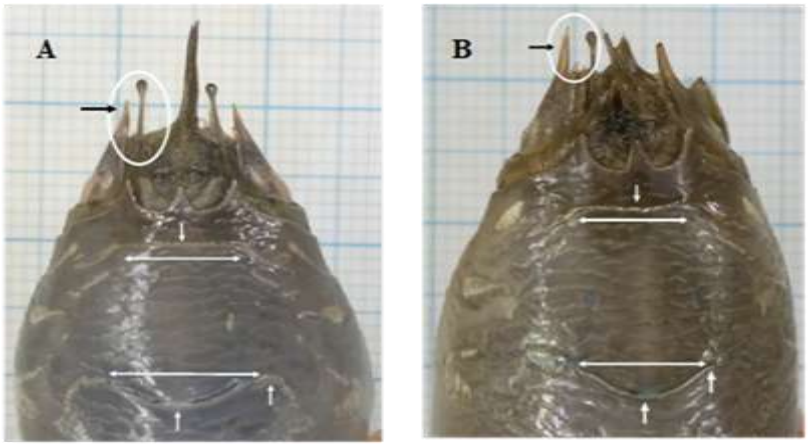

Figure 3. Frontal carapace on morphotype A and morphotype B

Based on the latero-frontal section on body height, morphotype A had a flatter body shape than B (Figure 4.). There are differences in the spines at the second antenna base from the lateral side between morphotypes A and B. Furthermore, Morphotype A has slightly curved outer spines, while B tends to be straight. The latero-frontal margin of the carapace, which contains fine spines with sparse hairs between the two morphotypes, has different spine shapes, arch, and the number of spines. The tip of the spine is not sharp; hence, when touched, it feels like a smooth protrusion. The anterior end of the margin does not have spines and has a shorter size than that of morphotype B. The shape of the posterior carapace margin curve in morphotype A is more prominent without spines. In contrast, it is more sloping in morphotype B, which has fine spines.

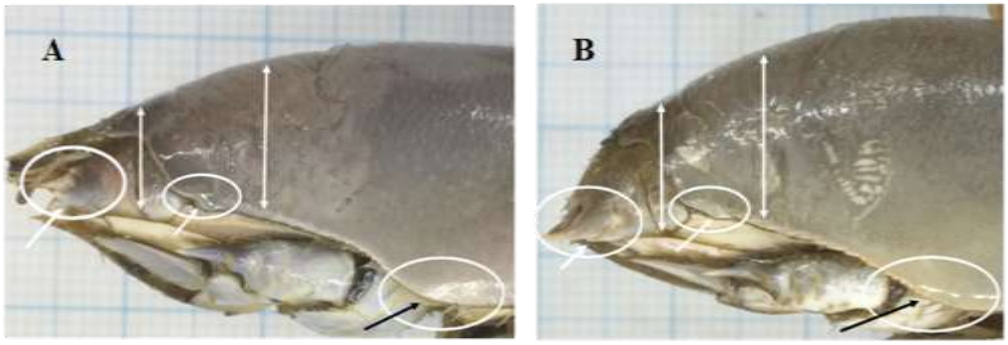

Figure 4. Latero-frontal carapace in morphotype A and morphotype B

Morphotypes A and B had different shapes on the distal part of the merus of the third maxilliped (Figure 5). Sankolli (1965), Kazmi and Siddiqui (2006), Boyko (2002), and Bhagawati et al. characterize morphotype A as having features similar to *Emerita emeritus* (Linnaeus, 1756) (2016; 2020). Morphotype A has the first pereopod dactyl oval, measuring less than twice the largest width. There are distinct spines on margins and occupy nearly the distal third of the lower part. Morphotype B has the character of the first pereopod dactyl, which is similar to morphotype A. However, the spines on the margins are smaller and possess the same size.

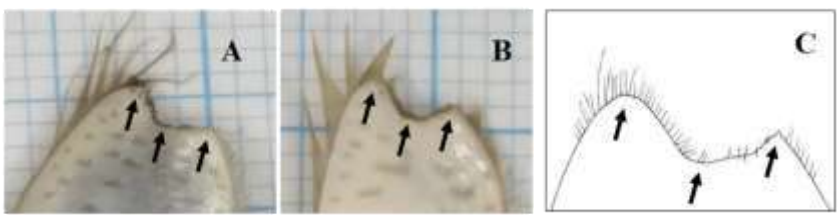

Figure 5. The distal part of the merus of the third maxilliped: Morphotype A; Morphotype B; and C. schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli 1965)

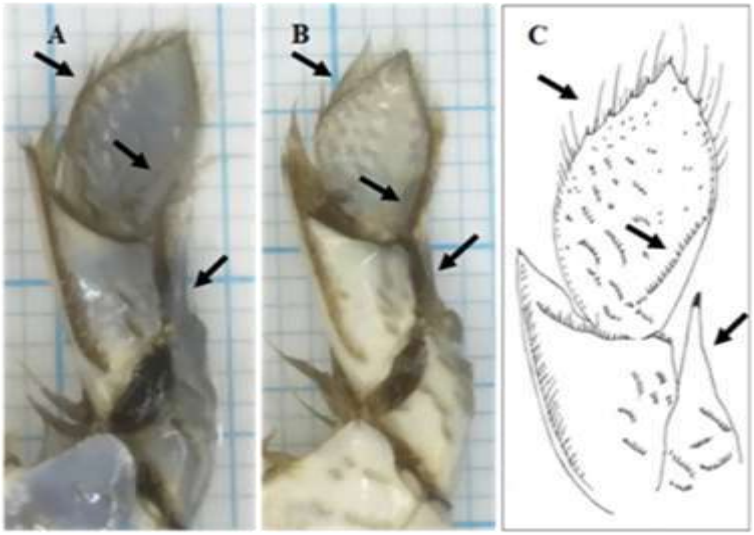

Figure 6. First pereopod on morphotype A, morphotype B, and schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli, 1965)

Based on their morphological characteristics, morphotypes A and B have many similarities (*Emerita*) and differences, suggesting the occurrence of new species. However, it has been well-known that mole crabs from the superfamily Hippoidea show high variability in morphology (Poore 2004; Ahyong et al. 2009; Schnabel and Ahyong 2009). This condition may lead to misidentification when performed based on morphological characters. Molecular data confirmed the possible occurrence of Sympatric species of *Emerita emeritus* in the Cilacap coastlines (Nuryanto et al. 2020) inferred from several specimens. Therefore, further study is still needed using more samples to strengthen the data on new *Emerita* species in the areas.

Molecular characterization

Sequence identity tests to the closest relative in GenBank revealed that three individuals (KI1, KI3, and WP9) of the morphotype A have high sequence identities to *Emerita emeritus* KR047035 ranging from 96.12 % to 96.25 %. In contrast, the sequence identities to *Emerita* sp. ranged from 85.60 % to 85.74 %. The remaining 14 individuals of the morphotype B showed low identities to *Emerita emeritus* KR 047035 in GenBank, ranging between 84.78% and 86.87%. The sequence identity of the remaining 14 specimens of the morphotype B to *Emerita* sp. MZ571198 was high ranging from 98.83 to 100% (Table 1).

Table 1. The BLAST parameters of *Emerita* samples from Cilacap coastlines to their conspecific relatives in GenBank

Samples	*Emerita emeritus* KR047035			*Emerita* sp. MZ571198		
	Coverage	Expect Value	Genetic identity (%)	Coverage	Expect Value	Genetic identity (%)
K1 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
K3 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
WP9 (A)	**99**	**0.00**	**96.25**	100	0.00	85.60
J3 (B)	96	2e179	86.87	**100**	**0.00**	**98.83**
J4 (B)	97	2e-125	84.78	**99**	**0.00**	**99.15**
J6 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
J7 (B)	99	0.00	86.44	**100**	**0.00**	**99.16**
J8 (B)	99	0.00	86.45	**99**	**0.00**	**100**
KI4 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
KI5 (B)	99	0.00	86.32	**100**	**0.00**	**99.67**
WP3 (B)	99	0.00	86.32	**100**	**0.00**	**99.51**
WP5 (B)	98	0.00	86.64	**99**	**0.00**	**99.83**
WP8 (B)	99	0.00	86.42	**100**	**0.00**	**99.85**
CLPE8* (B)	99	0.00	86.84	**100**	**0.00**	**99.84**
CLP4* (B)	98	2e-169	86.57	**100**	**0.00**	**99.82**
CLP11* (B)	98	5e-171	86.75	**100**	**0.00**	**100**
CLP15* (B)	98	5e-171	86.75	**100**	**0.00**	**100**

Genetic distance and genetic gap

Table 2 summarizes the genetic distance and gap between morphotype A and *E. emeritus* KR047035~~, as well as~~ and morphotype B and *Emerita* sp. MZ571198. The genetic gap was estimated based on the difference between the maximum and the minimum genetic distance of species.

Table 2. Genetic distance and gap within and among species (%)

Population	***Emerita emeritus***	***Emerita* sp.**
Emerita emeritus	0.00 – 3.20	16.80 – 19.00
Emerita sp.	16.80 – 0.190	0.00 – 1.70
The gap between *E. emeritus* and *Emerita* sp.	16.80-3.20 = **13.6**	

Genetic divergence

Variance analysis and Fst value indicated that the two morphotypes showed significant genetic differences with a p-value of 0.005 (Table 3). The significant genetic difference between the two indicated that both belong to two different species, proved by the BLAST result.

Table 3. Variance and Fst analysis indicate significant genetic divergence between two *Emerita* morphotypes

Source of variation	**d.f**	**Sum of squares**	**Variance components**	**Percentage of variation**
Between morphotypes	1	1.303	0.143^{Va}	26.53
Within morphotypes	17	6.750	0.397	73.47
Total	18	8.053	0.540	
Fixation index (Fst):	0.265			
p-value (Va and Fst)	0.0059			

Amino acid composition

The morphotypes were also subjected to amino acid composition to define molecular divergence, as summarized in Table 4.

Table 4. Amino acid composition of each morphotype (%)

Nucleotide	Morphotype	
	Morphotype A	Morphotype B
A	24.34	19.60
T	29.72	33.41
G	27.39	28.93
C	18.54	18.05

This research delineated the samples of morphotype A as *Emerita emeritus*. This is due to the strong genetic and conspecific identities of 96.12% to 96.25% and 85.60% to 85.74% for *Emerita* sp (MZ571198). Morphotype A was delineated into *E. emeritus* because genetic divergence within species may range from 0% to 4.6% (da Silva et al. 2014) or higher (Weis et al. 2014). Genetic divergence between morphotype A and *Emerita emeritus* KR047035 was below 4.6% (da Silva et al. 2014). The highest value was 3.88%, within the allowable range of 4% to 5%, as a moderate level of genetic identity for species delineation (Jeffery et al. 2011). This study has selected the value because the mutation rate of the COI gene is species-specific (Karanovic et al., 2015; Palecanda, 2020). A genetic threshold between 4% and 5% is permissible for genetic species determination, although additional considerations should be accounted for (Higashi et al., 2011; Jeffrey et al., 2011). Previous studies also utilized a genetic threshold of 5% during species determination (Candek and Kuntner 2015; Kusbiyanto et al. 2020; Riani et al. 2021).

The remaining 14 samples were identified as *Emerita* sp. nov. because of their high genetic identity (98.83% to 100%) to *Emerita* sp. MZ571198. In contrast, morphotype B had a low genetic identity (84.78% to 86.87%) to *Emerita emeritus* KR047035. This value is widely used as a genetic threshold in species delineation during animal barcoding (Hubert et al., 2010; Candek and Kuntner, 2015).

The division of morphotypes A and B into two distinct species is due to a genetic distance ranging from 16.80% to 19.00%, with a genetic gap of % (Table 2). Moreover, the two morphotypes also showed significant genetic variances and fixation index (p = 0.0059, Table 3) with different compositions of nucleotide content, especially in Adenine (A) and Thymine (T) composition (Table 4). Amino acid AT was higher than GC in both morphotypes, but the content of A and T was different. The phenomena were also reported in fish (Elvyra et al., 2020). The molecular difference observed in this study is in line with morphotypes A and B morphology. Therefore, ~~the delineation of morphotypes A and B~~ morphotypes A and B delineated as *Emerita emeritus* and *Emerita* sp. nov. was reliable.

This study also proved that the CO1 gene is a good marker for taxonomic identification ~~into~~ at the species level. The ~~reliability the COI gene as a barcode because it~~COI gene's reliability as a barcode is highly variable among animal species (Sachithanandam et al., 2012; Balkhis et al., 2011; Winarni et al., 2021). Similar phenomena were also reported from

several locations across Indonesia (Muchlisin et al. 2013; Irmawati et al. 2017; Pramono et al. 2017) and other regions (Aquilino et ~~al.~~ al. 2011; Triantafyllidis et al. 2011).

Historical demography and genetic diversity of *Emerita* sp. nov.

Historical demography

Tajima's D value was -1.563 (p = 0.044). That statistically significant results assumed that the used COI marker was under selection pressure. Instead of accepting selection pressure on the used marker, the negative sign of the D value indicated a recent population bottleneck and neutrality of the marker (Tajima1989; Jong et al. 2011). The negative symptoms and non-significant Fs (-1.580, p= 0.147) could ~~be used to~~ assume that the marker was neutral~~neutral marker~~ and indicated a population bottleneck (Table 5). The assumption was based on ~~a~~ the fact that Fus' Fs values are believed to be more sensitive than Tajimas' D. According to Jong et al. (2011) and Mohammed et al. (2021), the sensitivity of Fus' Fs values because it is calculated based on nucleotide diversity. A similar phenomenon was also reported in fish (Setyaningrum et al., 2022). Therefore, the used COI marker could be assumed as a neutral marker for assessing the genetic diversity of the Emerita sp population in the Cilacap coastlines.

Table 5. Species, number of individuals (N), number of haplotypes (nhp), haplotype diversity (h), nucleotide diversity (μ), Tajima'D, and Fu's Fs values

Species	N	nhp	h	μ	D	p-sig.	Fs	p-sig.
Emerita sp.	15	7	0.857 ± 0.057	0.005 ± 0.003	-1.563*	0.044	-1.580^{ns}	0.147

Note: *= significant, ns= not significant

Genetic diversity

Multiple sequences alignment resulted in s total of 418 bp COI gene ~~fragment~~ fragments from 14 individuals *Emerita* sp. nov. collected from the coastlines of Widarapayung Wetan, Widarapayung Kulon, and Sedayu Villages, District of Binagun, Cilacap Regency, Central Java, Indonesia. Furthermore, 12 out of 418 bp were polymorphic, resulting in 7 haplotypes, and the haplotype diversity was 0.857 ± 0.057 (Table 5). This data indicates that the *Emerita* sp. nov. population in the Cilacap coastlines has high genetic diversity. The nucleotide diversity value (μ) was 0.005 ± 0.003, which revealed low nucleotide diversity and a relatively low rate of evolution in the *Emerita* sp. nov. population on the Cilacap coastlines. The haplotype network (Figure 3) shows that haplotypes were separated by 2 to 5 mutation steps. However, the mutation was widely distributed in the population, as indicated by high diversity (0.857 ± 0.057). High haplotype diversity assessed using the COI gene was widespread in animal phyla (Dorn et al., 2011; Dung et al., 2013; Song et al., 2013; Zhang et al., 2014; Nuryanto et al., 2019). At the same time, low haplotype diversity was also common in animal populations (Setyaningrum et al., 2022). The COI gene's study may show a complex pattern of diversity levels, even within species (Pavesi et al., 2011; Parmaksiz and Eksi, 2017). The phenomena are also observed in population studies using other markers, such as microsatellites (Esa and Rahim 2013; Gouskov et al. 2016; Abbas et al. 2017; Achrem et al. 2017; Cheng et al. 2017) and d-loop (Zhong et al. 2013; Liu et al. 2016; Lau et al. 2018; Parmaksiz 2019; Ariyaraphong et al. 2021; Zhang et al. 2022).

This study cannot be compared with previous results because there is no population genetic study on mole crabs, especially on the presumable *Emerita* sp. nov. The only population study was conducted by Pramithasari et al. (2017), who compared mole crabs (*Albunea symmysta*) populations in Java and Sumatra. However, their study used morphological data, and the comparison to Pramithasari et al. (2017) was not congruent. This fact implies that more studies on the population genetics of mole crabs are needed.

Evolutionary relationships among *Emerita* sp. nov. individuals

The evolutionary process of the *Emerita* sp. nov. population on the southern coast of Cilacap is presented in the haplotype network (Figure 7). Star-like haplotype network in Figure 7 showed that haplotype 2 was the most primitive. Meanwhile, H2 was the center of the network, and other haplotypes evolved from (H2) as the most abundant (Balkhis et al., 2011; Song et al., 2012). The result contradicted the general acceptance that primitive haplotype has the highest abundance in the population (Adamson et al., 2012; Barasa et al., 2014; Basvar et al., 2018; 2019). The low frequency of H2 observed was assumed because of the small population (14 individuals). However, this assumption should be proven based on a further study using a high number of analyzed individuals.

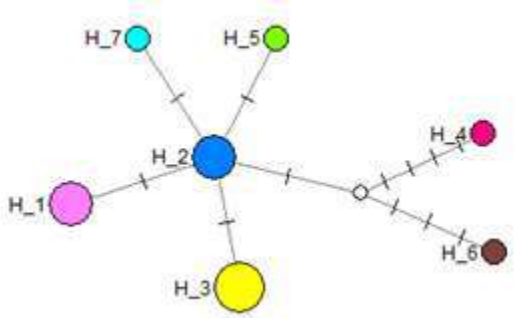

Figure 7. Haplotype networks indicating evolutionary relationships among *Emerita* sp. individuals

According to the analyzed data, this study concluded that mole crabs (Genus *Emerita*) in the Cilacap coastlines consisted of two distinct sympatric species (*Emerita emeritus* and *Emerita* sp. nov). *Emerita* sp. nov. had high haplotype diversity and was more abundant than *Emerita emeritus*. As a result, comprehensive research in terms of sampling site, number of samples, and other biological characteristics are needed to provide complete information for sympatric and taxa species of *Emerita* sp. nov.

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Morphological and molecular characterization of mole crab (Genus: *Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of *Emerita* sp. nov.

Abstract. Previous studies reported *Emerita emeritus* is the only species of the Genus *Emerita* inhabiting the coastal ecosystem of the Cilacap Regency. A recent study reported the presence of suspected new *Emerita* species living on the Cilacap sandy beach but used a small number of specimens and no reports about genetic diversity. This study used more Emerita samples than the previous study. This study aimed to identify *Emerita* specimens based on the morphology and the cytochrome c oxidase 1 gene and analyzed the genetic diversity of *Emerita* sp. nov. *Emerita* samples were collected from three different beaches in Cilacap Regency, Central Java, Indonesia. Morphological identification placed the samples into two different morphotypes. Morphotype A was identified as *Emerita emeritus*. Morphotype B was determined as *Emerita* sp. nov. Molecular data support the placement of *Emerita* samples into *Emerita emeritus*, and *Emerita* sp. nov. *Emerita* sp. nov. has haplotype diversity of 0.857 ± 0.057, indicating a high genetic diversity. Haplotype H2 was suggested as the most primitive one because other haplotypes radiated from it. This study concluded that two sympatric *Emerita* species inhabit Cilacap coastlines, and *Emerita* sp. nov. has high genetic diversity.

Keywords: *Albunea*, genetic variation, *Hyppa*, polymorphism, sand crabs

Abbreviations: COI= cytochrome c oxidase 1

Running title: Morphological and molecular characterization of mole crabs

INTRODUCTION

Classical taxonomy and systematic utilized morphological data during species characterization (Erlank et al., 2018; Shu et al., 2022). In some animal groups, morphology characteristics are entirely satisfactory (Chan et al. 2016; Mauroka et al. 2018; Korovchinsky 2019). However, in other groups, this character may lead to identification mistakes, especially in groups with limited morphological differences, such as in mole crab from the Genus *Albunea* (Boko and MacLaughlin 2010), cryptic species (Karanovic 2015; Bilgin et al. 2015; Bekker et al. 2016; Kusbiyanto 2020) or group with limited and undeveloped morphological characters, such as egg, larvae, and early juvenile (Ko et al. 2013; Palero et al. 2016; Palecanda 2020)

Mole crabs, locally known as 'yutuk,' belong to Decapoda from the superfamily Hippoidea. It consists of three different families of Albuneidae, Blepharipodidae, and Hippidae, which are divided into *Emerita* and *Hippa* genera. Moreover, ten species have been identified and described under Genus *Emerita* (Boyko and McLaughlin 2010). This crustacean group is widely distributed over the World (Boyko and McLaughlin 2010), and the distribution has been elaborated by Mahapatro et al. (2018). In Indonesia, these crabs inhibit sandy coastlines from the West Coast of Sumatera to Moluccas (Wardiatno et al., 2015; Boyko and Harvey, 1999).

Previous studies reported that the three genera of Hippoidea have been described from Indonesia waters (Bhagawati et al. 2016; Pramithasari et al. 2017; Nugroho et al. 2018; Butet et al. 2019; Hartoko et al. 2019; Bhagawati et al. 2020). Other studies described *Emerita emeritus* as the only species of genus *Emerita* found on the southern coastlines of Java (Nugroho et al. 2018; Dewi et al. 2019; Krisanti et al. 2020; Suryanti et al. 2020), including from Cilacap sandy beaches, such as Widarapayung beach, District of Binangun (Bhagawati et al. 2016; Haq et al. 2018). However, recent studies observed morphological and molecular deviations in some samples to the *Emerita emeritus* characteristics. The possible presence of the species complex's sympatric of the Cilacap coastlines was reported (Nuryanto et al., 2020). Even, Hanim et al. (2017) proposed a scientific name for the new suspected species of Emerita from Pangandaran beach, as *Emerita pangandarensis* sp. nov.. Still, the international commission has not approved its zoological nomenclature. However, the studies by Nuryanto et al. (2020) and Hanim et al. (2017) were conducted in few samples and only focused on species identification. These studies did not report genetic diversity in newly suspected *Emerita* species. Molecular characterization was performed in a higher number of specimens and data types. Additionally, it assessed the genetic diversity of new suspected *Emerita* species collected from the southern coast of Cilacap, Central Java, Indonesia.

Species identification and population genetic studies were conducted using various molecular markers (Nuryanto et al., 2017; Butet et al., 2019; Nuryanto et al., 2019; Elvyra et al., 2020; Setyaningrum et al., 2022; Riani et al. 2021). The cytochrome c oxidase 1 (COI) gene is a common marker used in species determination (Ko et al., 2013; Muchlisin et al.,

2013; Dahruddin et al., 2016; Irmawati et al., 2017; Syaifudin et al. 2020) and population genetic studies (Song et al. 2013; Zhang et al. 2014; Fahmi 2015; Nuryanto et al. 2019). Therefore, this research aimed to characterize samples of genus *Emerita* based on morphological and molecular characteristics and assess the genetic diversity using the cytochrome c oxidase 1 gene.

MATERIALS AND METHODS

Research location and sampling sites

The samples of mole crabs were collected from the sandy coastal region of the Cilacap Regency, Central Java, Indonesia. Additionally, the sampling was carried out in Jetis beach in the District of Nusawungu as well as Kenari Indah and Widarapayung beaches in the District of Binangun, Cilacap Regency, Central Java, Indonesia (Figure 1).

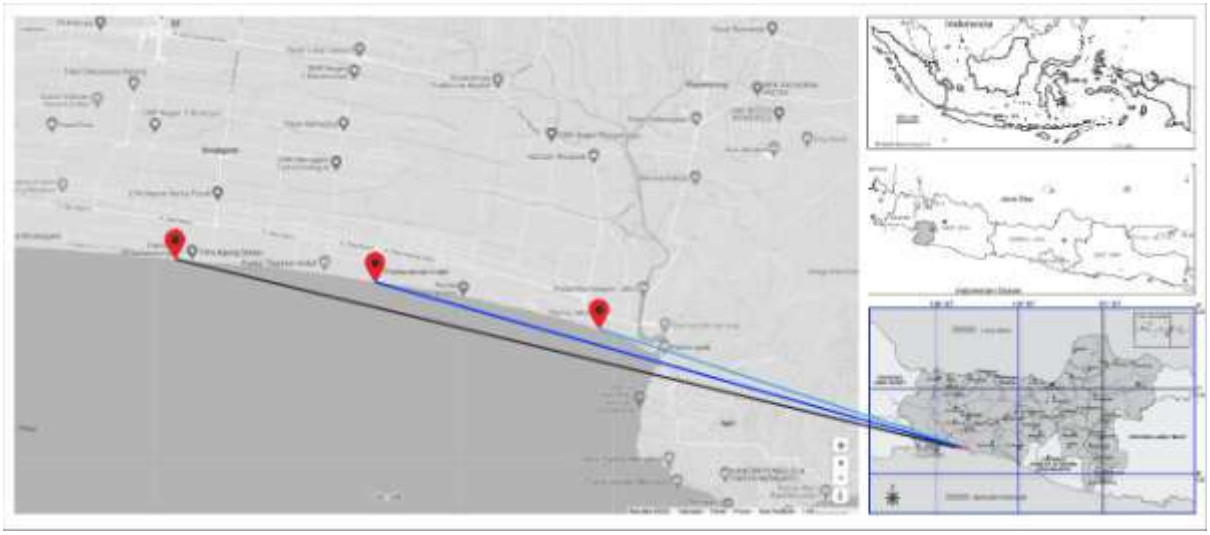

Figure 1. Peta Sampling Location of *Emerita* at Jetis Beach, Kenari Indah and Widarapayung (source: google maps, modified by S.S. Asmarani, 2022)

Mole crabs sampling

Emerita specimens were collected manually using two traditional fishing gears called "sodo nets" and "sorok bamboo" (Figure 2). Furthermore, local fishers performed samples collection and handling. Abdominal tissue samples were cut off for approximately 5 mm^2 and preserved using 96% alcohol in 2 ml screw lid tubes.

Figure 2. Fishing gears for collecting mole crab (*Emerita*) samples (A. sodo nets and B. sorok bamboo)

Procedures

Morphological characterization

Freshly collected crabs were brought to Animal Taxonomy Laboratory, Faculty of Biology Jenderal Sudirman University. The samples were washed thoroughly using freshwater, and morphological characterization was carried out based on the diagnostic character essential for identifying crustaceans. According to NG (1998), several diagnostic characteristics for identifying crustaceans are carapax, anterolateral side, dorsal surface, frontal side, buccal cavern/mouthpart, and locomotion (dactyl and pereopod), abdominal segments, and gonopods.

Observations on the genus *Emerita* were performed by referring to the diagnostic character used by Sankolli (1965), Bhagawati et al. (2016, 2020); Boyko & Harvey (1999), Haig, (1986); Osawa & Chan (2010), and Wardiatno et al. (2015).

These characteristics are the color and shape of the carapax; the position, number, and shape of the slits (Carapace Groove/CG) on the carapax surface; spines on the anterior carapax; a curved shape of the margin on the latero-anterior; and the shape and number of fine spines on the latero-anterior portion. Carapax height measurements were conducted on the front, middle, and back of the body, with the shape and size of the eyestalk. The merus distal and dactyl form on the maxilliped-3 and the first pereopod, while spines and hairs form on the margin of the first pereopod dactyl. Pleopods are formed in the abdominal segment as pleural.

DNA isolation and marker amplification

Genomic DNA was isolated from abdominal tissue samples using the Quick-DNA™ Miniprep Plus kit from Zymo's research. The processes were conducted based on the procedures provided by the company. The extracted DNA was migrated in 1% agarose electrophoresis and stained using ethidium bromide. The COI gene marker fragments were amplified using FishF2 and FishR2 primers (Ward et al. 2005) in Primus 25 Peqlab Thermocycler. Subsequently, the amplification reactions consisted of 1x buffer PCR, 2 mM MgCl2, 0.2 mM of each primer, 0.2 mM dNTP mix, 1 U Taq polymerase, and 2.0 ng / µl template DNA. The final volume to 50 µl of the mixtures was adjusted by adding DNA-RNA-free water. The pre-denaturation step at 95°C started thermal cycles for 4 minutes. The amplification reactions were performed for 35 cycles with denaturation steps that lasted for 30 seconds at 95°C, followed by annealing at 53°C for 120 seconds, and terminated by extension steps 60 for minutes at 72°C. Additionally, a final elongation step terminated the cycles after 5 minutes, at 72°C. The amplified COI marker was stained using ethidium bromide in 1.5% agarose gel and documented using the GelDoc apparatus (BioRad).

Marker sequencing and editing

Nucleotide sequencing of the used marker was conducted in the Molecular Genetic Laboratory of PT Genetika Science Indonesia Jakarta, according to the Sanger method. The study obtained consensus and multiple alignments by assembling the forward and reverse sequences using ClustalW ver.1.4 in Bioedit (Hall 2011). In addition, it obtained haplotype and Arlequin data files from its generating process in DnaSP 6 (Rozas et al., 2017).

Data analysis

The taxonomic status of *Emerita* samples was delineated based on sequence homology to the conspecific relative available in GenBank. This test was carried out using an essential local alignment search tool (BLAST). This study also used genetic distance, genetic divergence or a gap of 5% (Candek and Kuntner, 2015; Setyaningrum et al., 2020). Variance analysis and fixation statistic (Fst) were conducted in Arlequin 3.5 (Excoffier & Lischer 2010) to estimate significant genetic divergence between the morphotypes. The diversity data was evaluated using Haplotypes (h) and nucleotide (π) diversities, calculated using Arlequin 3.5. Similarly, the neutrality of the used COI marker was tested using Fs and D values (Excoffier & Lischer 2010). Evolutionary relationships among haplotypes were estimated based on haplotype networks reconstructed using the median-joining method in NETWORK software (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Taxonomic status

Morphological identification

A total of 17 individual *Emerita* samples were analyzed during the study. Morphological identification separated the samples into two different morphotypes. The first (A) and second (B) morphotypes consisted of 3 and 14 individuals, respectively (Figures 3A and 3B). However, they have similar general morphological characteristics that lead to Genus *Emerita* placement. The body of the *Emerita* crab is light, dark to blackish gray, with a slightly cylindrical shape with a wider distal carapace area. The eyestalk is long and slender, extending beyond the second antenna segment. The antennae are very long with hairy setae, and the segment on the second antenna consists of 3 horn-like and median spines. There are two oblique elongated protrusions with distinct spines that can be moved at the edges. The carapace has 3 frontal lobes, with the median very pointed and triangular, separated from the lateral lobe. The surface of the carapace has visible line-shaped slits located post-frontal and post-gastric. The latero-frontal margin has fine spines with sparse hairs. It has a short abdomen with a long telson, almost half the carapace length. The first pereopods were simple with an oval, lamellate dactyl, less than twice the width. Dactylus of the first pereopod with 4-5 rigid spines is found in the distal half of the lower margin, while 1-3 with 2 spines is in the tip.

The morphotypes A and B were differentiated by the following characteristics. The frontal part of the carapace showed differences in the shape and length of the spines at the base of the second antenna segment, the shape of a hollow between the three spines at the tip of the carapace, and the post-frontal and post-gastric cleft forms (Fig. 3). Individuals with morphotype A have eye stalks longer than the spines at the second antenna base (Figure 3A). In contrast, morphotype B has eye stalks almost the same length as the spines at the second antenna base. Another performance is the concave shape between the three spines on the frontal carapace. Morphotype A does not form an angle, while B forms a curve. The shape of the gap found in the post-frontal area is a straight line, neat and flat in morphotype A, but it is elevated in morphotype B

with a curved line in the post-stomach. In morphotype A, the arch is not too deep, and its carapace's right and left ends have a thin curved slit. In contrast, morphotype B has a narrower curved line.

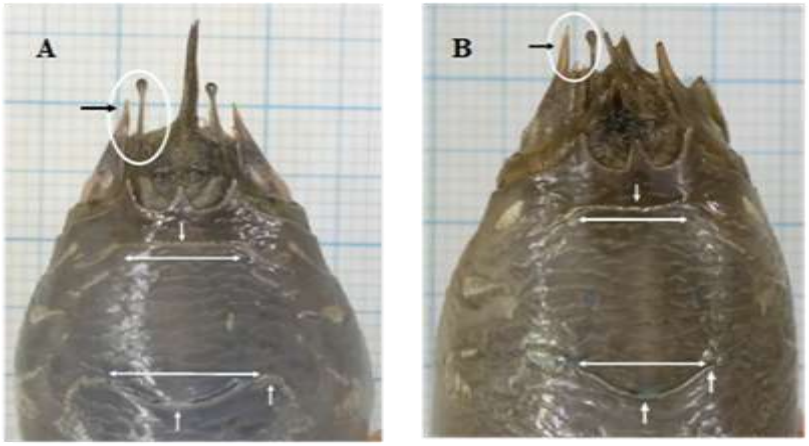

Figure 3. Frontal carapace on morphotype A and morphotype B

Based on the latero-frontal section on body height, morphotype A had a flatter body shape than B (Figure 4.). There are differences in the spines at the second antenna base from the lateral side between morphotypes A and B. Furthermore, Morphotype A has slightly curved outer spines, while B tends to be straight. The latero-frontal margin of the carapace, which contains fine spines with sparse hairs between the two morphotypes, has different spine shapes, arch, and the number of spines. The tip of the spine is not sharp; hence, when touched, it feels like a smooth protrusion. The anterior end of the margin does not have spines and has a shorter size than that of morphotype B. The shape of the posterior carapace margin curve in morphotype A is more prominent without spines. In contrast, it is more sloping in morphotype B, which has fine spines.

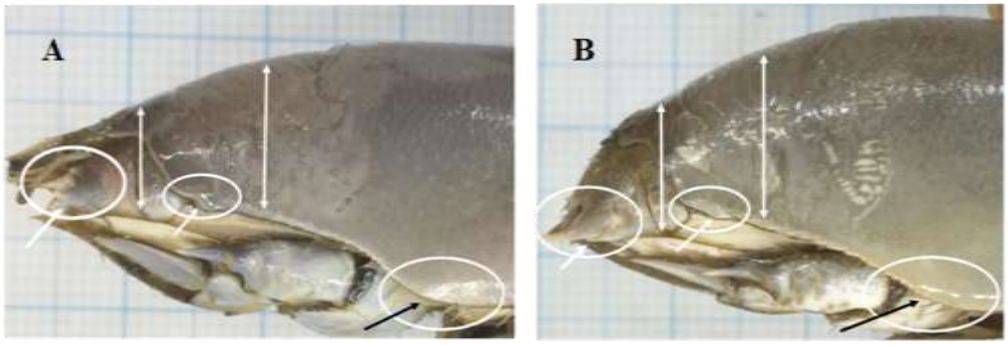

Figure 4. Latero-frontal carapace in morphotype A and morphotype B

Morphotypes A and B had different shapes on the distal part of the merus of the third maxilliped (Figure 5). Sankolli (1965), Kazmi and Siddiqui (2006), Boyko (2002), and Bhagawati et al. characterize morphotype A as having features similar to *Emerita emeritus* (Linnaeus, 1756) (2016; 2020). Morphotype A has the first pereopod dactyl oval, measuring less than twice the largest width. There are distinct spines on margins and occupy nearly the distal third of the lower part. Morphotype B has the character of the first pereopod dactyl, which is similar to morphotype A. However, the spines on the margins are smaller and possess the same size.

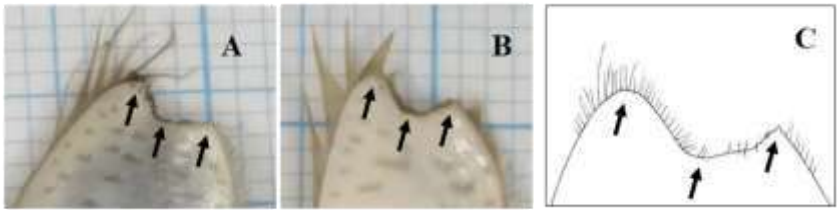

Figure 5. The distal part of the merus of the third maxilliped: Morphotype A; Morphotype B; and C. schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli 1965)

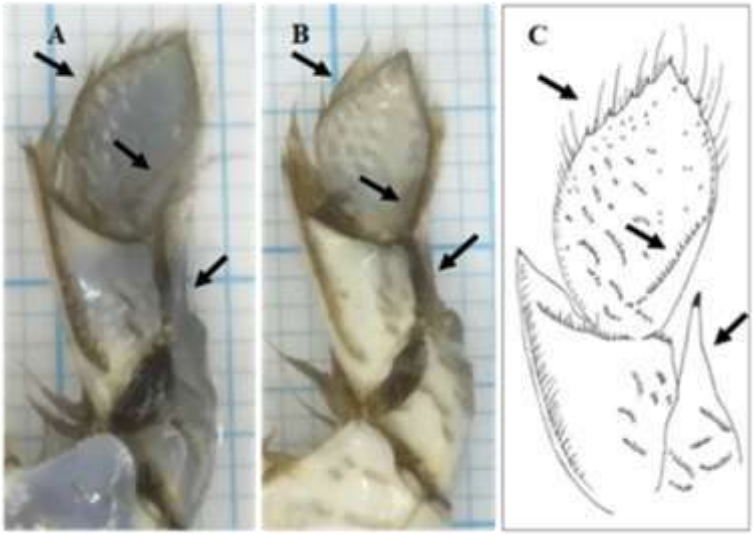

Figure 6. First pereopod on morphotype A, morphotype B, and schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli, 1965)

Based on their morphological characteristics, morphotypes A and B have many similarities (*Emerita*) and differences, suggesting the occurrence of new species. However, it has been well-known that mole crabs from the superfamily Hippoidea show high variability in morphology (Poore 2004; Ahyong et al. 2009; Schnabel and Ahyong 2009). This condition may lead to misidentification when performed based on morphological characters. Molecular data confirmed the possible occurrence of Sympatric species of *Emerita emeritus* in the Cilacap coastlines (Nuryanto et al. 2020) inferred from several specimens. Therefore, further study is still needed using more samples to strengthen the data on new *Emerita* species in the areas.

Molecular characterization

Sequence identity tests to the closest relative in GenBank revealed that three individuals (KI1, KI3, and WP9) of the morphotype A have high sequence identities to *Emerita emeritus* KR047035 ranging from 96.12 % to 96.25 %. In contrast, the sequence identities to *Emerita* sp. ranged from 85.60 % to 85.74 %. The remaining 14 individuals of the morphotype B showed low identities to *Emerita emeritus* KR 047035 in GenBank, ranging between 84.78% and 86.87%. The sequence identity of the remaining 14 specimens of the morphotype B to *Emerita* sp. MZ571198 was high ranging from 98.83 to 100% (Table 1).

Table 1. The BLAST parameters of *Emerita* samples from Cilacap coastlines to their conspecific relatives in GenBank

Samples	*Emerita emeritus* KR047035			*Emerita* sp. MZ571198		
	Coverage	Expect Value	Genetic identity (%)	Coverage	Expect Value	Genetic identity (%)
K1 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
K3 (A)	**100**	**0.00**	**96.12**	99	0.00	85.74
WP9 (A)	**99**	**0.00**	**96.25**	100	0.00	85.60
J3 (B)	96	2e179	86.87	**100**	**0.00**	**98.83**
J4 (B)	97	2e-125	84.78	**99**	**0.00**	**99.15**
J6 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
J7 (B)	99	0.00	86.44	**100**	**0.00**	**99.16**
J8 (B)	99	0.00	86.45	**99**	**0.00**	**100**
KI4 (B)	99	0.00	86.48	**100**	**0.00**	**99.84**
KI5 (B)	99	0.00	86.32	**100**	**0.00**	**99.67**
WP3 (B)	99	0.00	86.32	**100**	**0.00**	**99.51**
WP5 (B)	98	0.00	86.64	**99**	**0.00**	**99.83**
WP8 (B)	99	0.00	86.42	**100**	**0.00**	**99.85**
CLPE8* (B)	99	0.00	86.84	**100**	**0.00**	**99.84**
CLP4* (B)	98	2e-169	86.57	**100**	**0.00**	**99.82**
CLP11* (B)	98	5e-171	86.75	**100**	**0.00**	**100**
CLP15* (B)	98	5e-171	86.75	**100**	**0.00**	**100**

Genetic distance and genetic gap

Table 2 summarizes the genetic distance and gap between morphotype A and *E. emeritus* KR047035 and morphotype B and *Emerita* sp. MZ571198. The genetic gap was estimated based on the difference between the maximum and the minimum genetic distance of species.

Table 2. Genetic distance and gap within and among species (%)

Population	***Emerita emeritus***	***Emerita* sp.**
Emerita emeritus	0.00 – 3.20	16.80 – 19.00
Emerita sp.	16.80 – 0.190	0.00 – 1.70
The gap between *E. emeritus* and *Emerita* sp.	16.80-3.20 = **13.6**	

Genetic divergence

Variance analysis and Fst value indicated that the two morphotypes showed significant genetic differences with a p-value of 0.005 (Table 3). The significant genetic difference between the two indicated that both belong to two different species, proved by the BLAST result.

Table 3. Variance and Fst analysis indicate significant genetic divergence between two *Emerita* morphotypes

Source of variation	**d.f**	**Sum of squares**	**Variance components**	**Percentage of variation**
Between morphotypes	1	1.303	0.143^{Va}	26.53
Within morphotypes	17	6.750	0.397	73.47
Total	18	8.053	0.540	
Fixation index (Fst):	0.265			
p-value (Va and Fst)	0.0059			

Amino acid composition

The morphotypes were also subjected to amino acid composition to define molecular divergence, as summarized in Table 4.

Table 4. Amino acid composition of each morphotype (%)

Nucleotide	Morphotype	
	Morphotype A	Morphotype B
A	24.34	19.60
T	29.72	33.41
G	27.39	28.93
C	18.54	18.05

This research delineated the samples of morphotype A as *Emerita emeritus*. This is due to the strong genetic and conspecific identities of 96.12% to 96.25% and 85.60% to 85.74% for *Emerita* sp (MZ571198). Morphotype A was delineated into *E. emeritus* because genetic divergence within species may range from 0% to 4.6% (da Silva et al. 2014) or higher (Weis et al. 2014). Genetic divergence between morphotype A and *Emerita emeritus* KR047035 was below 4.6% (da Silva et al. 2014). The highest value was 3.88%, within the allowable range of 4% to 5%, as a moderate level of genetic identity for species delineation (Jeffery et al. 2011). This study has selected the value because the mutation rate of the COI gene is species-specific (Karanovic et al. 2015; Palecanda 2020). A genetic threshold between 4% and 5% is permissible for genetic species determination, although additional considerations should be accounted for (Higashi et al. 2011; Jeffrey et al. 2011). Previous studies also utilized a genetic threshold of 5% during species determination (Candek and Kuntner 2015; Kusbiyanto et al. 2020; Riani et al. 2021).

The remaining 14 samples were identified as *Emerita* sp. nov. because of their high genetic identity (98.83% to 100%) to *Emerita* sp. MZ571198. In contrast, morphotype B had a low genetic identity (84.78% to 86.87%) to *Emerita emeritus* KR047035. This value is widely used as a genetic threshold in species delineation during animal barcoding (Hubert et al., 2010; Candek and Kuntner, 2015).

The division of morphotypes A and B into two distinct species is due to a genetic distance ranging from 16.80% to 19.00%, with a genetic gap of % (Table 2). Moreover, the two morphotypes also showed significant genetic variances and fixation index ($p = 0.0059$, Table 3) with different compositions of nucleotide content, especially in Adenine (A) and Thymine (T) composition (Table 4). Amino acid AT was higher than GC in both morphotypes, but the content of A and T was different. The phenomena were also reported in fish (Elvyra et al., 2020). The molecular difference observed in this study is in line with morphotypes A and B morphology. Therefore, morphotypes A and B delineated as *Emerita emeritus* and *Emerita* sp. nov. was reliable.

This study also proved that the CO1 gene is a good marker for taxonomic identification at the species level. The COI gene's reliability as a barcode is highly variable among animal species (Sachithanandam et al. 2012; Balkhis et al. 2011; Winarni et al. 2021). Similar phenomena were also reported from several locations across Indonesia (Muchlisin et al. 2013; Irmawati et al. 2017; Pramono et al. 2017) and other regions (Aquilino et al. 2011; Triantafyllidis et al. 2011).

Historical demography and genetic diversity of *Emerita* sp. nov.

Historical demography

Tajima's D value was -1.563 (p = 0.044). That statistically significant results assumed that the used COI marker was under selection pressure. Instead of accepting selection pressure on the used marker, the negative sign of the D value indicated a recent population bottleneck and neutrality of the marker (Tajima1989; Jong et al. 2011). The negative symptoms and non-significant Fs (-1.580, p= 0.147) could assume that the marker was neutral and indicated a population bottleneck (Table 5). The assumption was based on the fact that Fus' Fs values are believed to be more sensitive than Tajimas' D. According to Jong et al. (2011) and Mohammed et al. (2021), the sensitivity of Fus' Fs values because it is calculated based on nucleotide diversity. A similar phenomenon was also reported in fish (Setyaningrum et al. 2022). Therefore, the used COI marker could be assumed as a neutral marker for assessing the genetic diversity of the Emerita sp population in the Cilacap coastlines.

Table 5. Species, number of individuals (N), number of haplotypes (nhp), haplotype diversity (h), nucleotide diversity (μ), Tajima'D, and Fu's Fs values

Species	N	nhp	h	μ	D	p-sig.	Fs	p-sig.
Emerita sp.	15	7	0.857 ± 0.057	0.005 ± 0.003	-1.563*	0.044	-1.580ns	0.147

Note: *= significant, ns= not significant

Genetic diversity

Multiple sequences alignment resulted in a total of 418 bp COI gene fragments from 14 individuals *Emerita* sp. nov. collected from the coastlines of Widarapayung Wetan, Widarapayung Kulon, and Sedayu Villages, District of Binagun, Cilacap Regency, Central Java, Indonesia. Furthermore, 12 out of 418 bp were polymorphic, resulting in 7 haplotypes, and the haplotype diversity was 0.857 ± 0.057 (Table 5). This data indicates that the *Emerita* sp. nov. population in the Cilacap coastlines has high genetic diversity. The nucleotide diversity value (μ) was 0.005 ± 0.003, which revealed low nucleotide diversity and a relatively low rate of evolution in the *Emerita* sp. nov. population on the Cilacap coastlines. The haplotype network (Figure 3) shows that haplotypes were separated by 2 to 5 mutation steps. However, the mutation was widely distributed in the population, as indicated by high diversity (0.857 ± 0.057). High haplotype diversity assessed using the COI gene was widespread in animal phyla (Dorn et al. 2011; Dung et al. 2013; Song et al. 2013; Zhang et al. 2014; Nuryanto et al. 2019). At the same time, low haplotype diversity was also common in animal populations (Setyaningrum et al. 2022). The COI gene's study may show a complex pattern of diversity levels, even within species (Pavesi et al. 2011; Parmaksiz and Eksi 2017). The phenomena are also observed in population studies using other markers, such a microsatellite (Esa and Rahim 2013; Gouskov et al. 2016; Abbas et al. 2017; Achrem et al. 2017; Cheng et al. 2017) and d-loop (Zhong et al. 2013; Liu et al. 2016; Lau et al. 2018; Parmaksiz 2019; Ariyaraphong et al. 2021; Zhang et al. 2022).

This study cannot be compared with previous results because there is no population genetic study on mole crabs, especially on the presumable *Emerita* sp. nov. The only population study was conducted by Pramithasari et al. (2017), who compared mole crabs (*Albunea symmysta*) populations in Java and Sumatra. However, their study used morphological data, and the comparison to Pramithasari et al. (2017) was not congruent. This fact implies that more studies on the population genetics of mole crabs are needed.

Evolutionary relationships among *Emerita* sp. nov. individuals

The evolutionary process of the *Emerita* sp. nov. population on the southern coast of Cilacap is presented in the haplotype network (Figure 7). Star-like haplotype network in Figure 7 showed that haplotype 2 was the most primitive. Meanwhile, H2 was the center of the network, and other haplotypes evolved from (H2) as the most abundant (Balkhis et al. 2011; Song et al. 2012). The result contradicted the general acceptance that primitive haplotype has the highest abundance in the population (Adamson et al. 2012; Barasa et al. 2014; Basvar et al. 2018; 2019). The low frequency of H2 observed was assumed because of the small population (14 individuals). However, this assumption should be proven based on a further study using a high number of analyzed individuals.

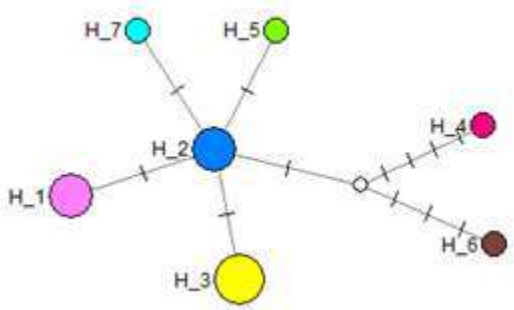

Figure 7. Haplotype networks indicating evolutionary relationships among *Emerita* sp. individuals

According to the analyzed data, this study concluded that mole crabs (Genus *Emerita*) in the Cilacap coastlines consisted of two distinct sympatric species (*Emerita emeritus* and *Emerita* sp. nov). *Emerita* sp. nov. had high haplotype diversity and was more abundant than *Emerita emeritus*. As a result, comprehensive research in terms of sampling site, number of samples, and other biological characteristics are needed to provide complete information for sympatric and taxa species of *Emerita* sp. nov.

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Morphological and molecular characterization of mole crab (Genus: *Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of *Emerita* sp. nov.

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Abstract. *Bhagawati D, Nuryanto A, Winarni ET, Pulungsari AE. 2022. Morphological and molecular characterization of mole crab (Genus:* Emerita*) in the Cilacap coastlines of Indonesia, with particular focus on genetic diversity of* Emerita *sp. nov. Biodiversitas 23: 2395-2404.* Previous studies reported *Emerita emeritus* is the only species of the Genus *Emerita* inhabiting the coastal ecosystem of the Cilacap District. A recent study reported the presence of suspected new *Emerita* species living on the Cilacap sandy beach but used a small number of specimens and no reports about genetic diversity. This study used more Emerita samples than the previous study. This study aimed to identify *Emerita* specimens based on the morphology and the cytochrome c oxidase 1 gene and analyzed the genetic diversity of *Emerita* sp. nov. *Emerita* samples were collected from three different beaches in Cilacap District, Central Java, Indonesia. Morphological identification placed the samples into two different morphotypes. Morphotype A was identified as *Emerita emeritus*. Morphotype B was determined as *Emerita* sp. nov. Molecular data support the placement of *Emerita* samples into *Emerita emeritus*, and *Emerita* sp. nov. *Emerita* sp. nov. has haplotype diversity of 0.857±0.057, indicating a high genetic diversity. Haplotype H2 was suggested as the most primitive one because other haplotypes radiated from it. This study concluded that two sympatric *Emerita* species inhabit Cilacap coastlines, and *Emerita* sp. nov. has high genetic diversity.

Keywords: *Albunea*, genetic variation, *Hyppa*, polymorphism, sand crabs

Abbreviations: COI: cytochrome c oxidase 1

INTRODUCTION

Classical taxonomy and systematic utilized morphological data during species characterization (Erlank et al. 2018; Shu et al. 2022). In some animal groups, morphology characteristics are entirely satisfactory (Chan et al. 2016; Mauroka et al. 2018; Korovchinsky 2019). However, in other groups, this character may lead to identification mistakes, especially in groups with limited morphological differences, such as in mole crab from the Genus *Albunea* (Boko and MacLaughlin 2010), cryptic species (Karanovic 2015; Bilgin et al. 2015; Bekker et al. 2016; Kusbiyanto et al. 2020) or group with limited and undeveloped morphological characters, such as egg, larvae, and early juvenile (Ko et al. 2013; Palero et al. 2016; Palecanda et al. 2020)

Mole crabs, locally known as 'yutuk,' belong to Decapoda from the superfamily Hippoidea. It consists of three different families of Albuneidae, Blepharipodidae, and Hippidae, which are divided into *Emerita* and *Hippa* genera. Moreover, ten species have been identified and described under Genus *Emerita* (Boyko and McLaughlin 2010). This crustacean group is widely distributed over the World (Boyko and McLaughlin 2010), and the distribution has been elaborated by Mahapatro et al. (2018). In Indonesia, these crabs inhibit sandy coastlines from the West Coast of Sumatera to Moluccas (Wardiatno et al. 2015; Boyko and Harvey 1999).

Previous studies reported that the three genera of Hippoidea have been described from Indonesia waters (Bhagawati et al. 2016; Pramithasari et al. 2017; Nugroho et al. 2018; Butet et al. 2019; Hartoko et al. 2019; Bhagawati et al. 2020). Other studies described *Emerita emeritus* as the only species of genus *Emerita* found on the southern coastlines of Java (Nugroho et al. 2018; Dewi et al. 2019; Krisanti et al. 2020; Desi et al. 2020), including from Cilacap sandy beaches, such as Widarapayung beach, Sub-district of Binangun (Bhagawati et al. 2016; Haq et al. 2018). However, recent studies observed morphological and molecular deviations in some samples to the *Emerita emeritus* characteristics. The possible presence of the sympatric species complex of the Cilacap coastlines was reported (Nuryanto et al. 2020). Even, Hanim et al. (2017) proposed a scientific name for the new suspected species of *Emerita* from Pangandaran beach, as *Emerita pangandarensis* sp. nov.. Still, the international commission has not approved its zoological nomenclature. However, the studies by Hanim et al. (2017) and Nuryanto et al. (2020) were conducted in few samples and only focused on species identification. These studies did not report genetic diversity in newly suspected *Emerita* species. Molecular characterization was performed in a higher number of specimens and data types. Additionally, it assessed the

genetic diversity of new suspected *Emerita* species collected from the southern coast of Cilacap, Central Java, Indonesia.

Species identification and population genetic studies were conducted using various molecular markers (Nuryanto et al. 2017; Butet et al. 2019; Nuryanto et al. 2019; Elvyra et al. 2020; Riani et al. 2021; Setyaningrum et al. 2022). The cytochrome c oxidase 1 (COI) gene is a common marker used in species determination (Ko et al. 2013; Muchlisin et al. 2013; Dahruddin et al. 2016; Irmawati et al. 2017; Syaifudin et al. 2020) and population genetic studies (Song et al. 2013; Zhang et al. 2014; Fahmi 2015; Nuryanto et al. 2019). Therefore, this research aimed to characterize samples of genus *Emerita* based on morphological and molecular characteristics and assess the genetic diversity using the cytochrome c oxidase 1 gene.

MATERIALS AND METHODS

Research location and sampling sites

The samples of mole crabs were collected from the sandy coastal region of the Cilacap District, Central Java, Indonesia. Additionally, the sampling was carried out in Jetis beach in the Sub-district of Nusawungu as well as Kenari Indah and Widarapayung beaches in the Sub-district of Binangun, Cilacap District, Central Java, Indonesia (Figure 1).

Mole crabs sampling

Emerita specimens were collected manually using two traditional fishing gears called "sodo nets" and "sorok bamboo" (Figure 2). Furthermore, local fishers performed samples collection and handling. Abdominal tissue samples were cut off for approximately 5 mm^2 and preserved using 96% alcohol in 2 ml screw lid tubes.

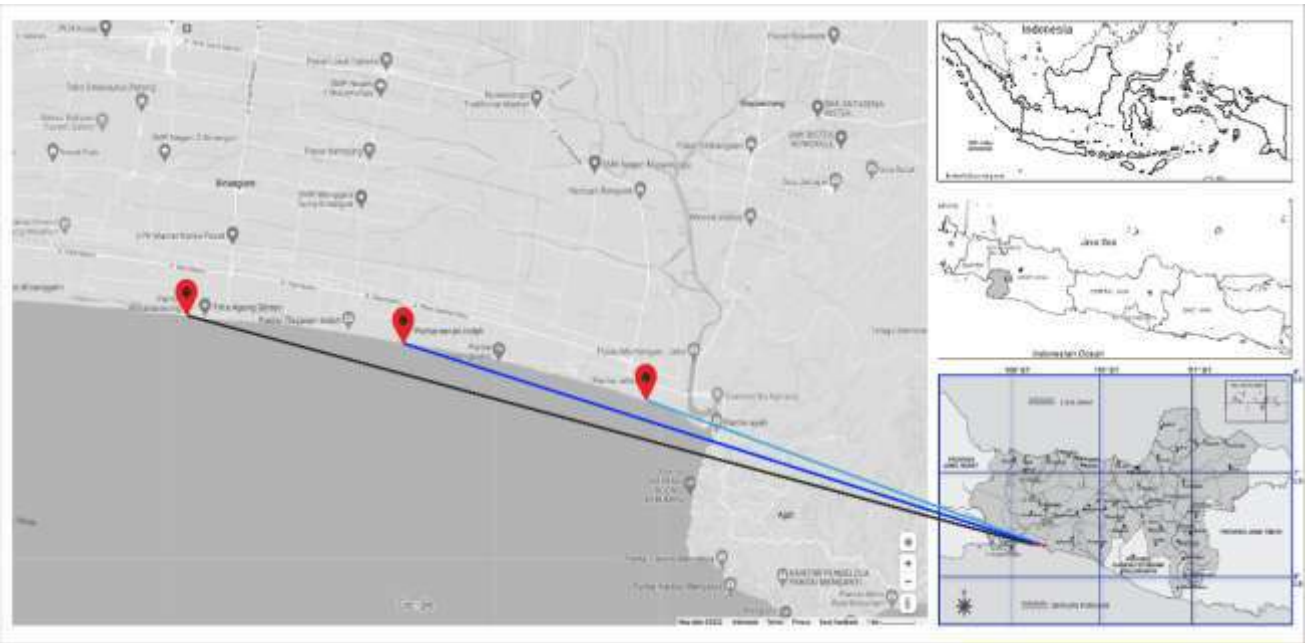

Figure 1. Sampling location map of *Emerita* at Jetis, Kenari Indah and Widarapayung Beaches (source: google maps, modified by S.S. Asmarani 2022)

Figure 2. Fishing gears for collecting mole crab (*Emerita*) samples. A. sodo nets. B. sorok bamboo

Procedures

Morphological characterization

Freshly collected crabs were brought to Animal Taxonomy Laboratory, Faculty of Biology Jenderal Soedirman University. The samples were washed thoroughly using freshwater, and morphological characterization was carried out based on the diagnostic character essential for identifying crustaceans. According to Ng (1998), several diagnostic characteristics for identifying crustaceans are carapax, anterolateral side, dorsal surface, frontal side, buccal cavern/mouthpart, and locomotion (dactyl and pereopod), abdominal segments, and gonopods.

Observations on the genus *Emerita* were performed by referring to the diagnostic character used by Sankolli (1965), Haig (1986); Boyko and Harvey (1999), Osawa and Chan (2010), Wardiatno et al. (2015) and Bhagawati et al. (2016, 2020). These characteristics are the color and shape of the carapax; the position, number, and shape of the slits (Carapace Groove/CG) on the carapax surface; spines on the anterior carapax; a curved shape of the margin on the latero-anterior; and the shape and number of fine spines on the latero-anterior portion. Carapax height measurements were conducted on the front, middle, and back of the body, with the shape and size of the eyestalk. The merus distal and dactyl form on the maxilliped-3 and the first pereopod, while spines and hairs form on the margin of the first pereopod dactyl. Pleopods are formed in the abdominal segment as pleural.

DNA isolation and marker amplification

Genomic DNA was isolated from abdominal tissue samples using the Quick-DNA™ Miniprep Plus kit from Zymo's research. The processes were conducted based on the procedures provided by the company. The extracted DNA was migrated in 1% agarose electrophoresis and stained using ethidium bromide. The COI gene marker fragments were amplified using FishF2 and FishR2 primers (Ward et al. 2005) in Primus 25 Peqlab Thermocycler. Subsequently, the amplification reactions consisted of 1x buffer PCR, 2 mM MgCl2, 0.2 mM of each primer, 0.2 mM dNTP mix, 1 U Taq polymerase, and 2.0 ng/µL template DNA. The final volume to 50 µl of the mixtures was adjusted by adding DNA-RNA-free water. The pre-denaturation step at 95°C started thermal cycles for 4 minutes. The amplification reactions were performed for 35 cycles with denaturation steps that lasted for 30 seconds at 95°C, followed by annealing at 53°C for 120 seconds, and terminated by extension steps 60 for minutes at 72°C. Additionally, a final elongation step terminated the cycles after 5 minutes, at 72°C. The amplified COI marker was stained using ethidium bromide in 1.5% agarose gel and documented using the GelDoc apparatus (BioRad).

Marker sequencing and editing

Nucleotide sequencing of the used marker was conducted in the Molecular Genetic Laboratory of PT Genetika Science Indonesia Jakarta, according to the Sanger method. The study obtained consensus and multiple alignments by assembling the forward and reverse sequences using ClustalW ver.1.4 in Bioedit (Hall 2011). In addition, it obtained haplotype and Arlequin data files from its generating process in DnaSP 6 (Rozas et al. 2017).

Data analysis

The taxonomic status of *Emerita* samples was delineated based on sequence homology to the conspecific relative available in GenBank. This test was carried out using an essential local alignment search tool (BLAST). This study also used genetic distance, genetic divergence or a gap of 5% (Candek and Kuntner 2015; Setyaningrum et al. 2022). Variance analysis and fixation statistic (Fst) were conducted in Arlequin 3.5 (Excoffier and Lischer 2010) to estimate significant genetic divergence between the morphotypes. The diversity data was evaluated using Haplotypes (h) and nucleotide (π) diversities, calculated using Arlequin 3.5. Similarly, the neutrality of the used COI marker was tested using Fs and D values (Excoffier and Lischer 2010). Evolutionary relationships among haplotypes were estimated based on haplotype networks reconstructed using the median-joining method in NETWORK software (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Taxonomic status

Morphological identification

A total of 17 individual *Emerita* samples were analyzed during the study. Morphological identification separated the samples into two different morphotypes. The first (A) and second (B) morphotypes consisted of 3 and 14 individuals, respectively (Figures 3A and 3B). However, they have similar general morphological characteristics that lead to Genus *Emerita* placement. The body of the *Emerita* crab is light, dark to blackish gray, with a slightly cylindrical shape with a wider distal carapace area. The eyestalk is long and slender, extending beyond the second antenna segment. The antennae are very long with hairy setae, and the segment on the second antenna consists of 3 horn-like and median spines. There are two oblique elongated protrusions with distinct spines that can be moved at the edges. The carapace has 3 frontal lobes, with the median very pointed and triangular, separated from the lateral lobe. The surface of the carapace has visible line-shaped slits located post-frontal and post-gastric. The latero-frontal margin has fine spines with sparse hairs. It has a short abdomen with a long telson, almost half the carapace length. The first pereopods were simple with an oval, lamellate dactyl, less than twice the width. Dactylus of the first pereopod with 4-5 rigid spines is found in the distal half of the lower margin, while 1-3 with 2 spines is in the tip.

The morphotypes A and B were differentiated by the following characteristics. The frontal part of the carapace showed differences in the shape and length of the spines at the base of the second antenna segment, the shape of a hollow between the three spines at the tip of the carapace, and the post-frontal and post-gastric cleft forms (Figure 3).

Individuals with morphotype A have eye stalks longer than the spines at the second antenna base (Figure 3A). In contrast, morphotype B has eye stalks almost the same length as the spines at the second antenna base. Another performance is the concave shape between the three spines on the frontal carapace. Morphotype A does not form an angle, while B forms a curve. The shape of the gap found in the post-frontal area is a straight line, neat and flat in morphotype A, but it is elevated in morphotype B with a curved line in the post-stomach. In morphotype A, the arch is not too deep, and its carapace's right and left ends have a thin curved slit. In contrast, morphotype B has a narrower curved line.

Based on the latero-frontal section on body height, morphotype A had a flatter body shape than B (Figure 4.). There are differences in the spines at the second antenna base from the lateral side between morphotypes A and B. Furthermore, morphotype A has slightly curved outer spines, while B tends to be straight. The latero-frontal margin of the carapace, which contains fine spines with sparse hairs between the two morphotypes, has different spine shapes, arch, and the number of spines. The tip of the spine is not sharp; hence, when touched, it feels like a smooth protrusion. The anterior end of the margin does not have spines and has a shorter size than that of morphotype B. The shape of the posterior carapace margin curve in morphotype A is more prominent without spines. In contrast, it is more sloping in morphotype B, which has fine spines.

Morphotypes A and B had different shapes on the distal part of the merus of the third maxilliped (Figure 5). Sankolli (1965), Kazmi and Siddiqui (2006), Boyko (2002), and Bhagawati et al. characterize morphotype A as having features similar to *Emerita emeritus* (Linnaeus, 1756) (2016; 2020). Morphotype A has the first pereopod dactyl oval, measuring less than twice the largest width. There are distinct spines on margins and occupy nearly the distal third of the lower part. Morphotype B has the character of the first pereopod dactyl, which is similar to morphotype A. However, the spines on the margins are smaller and possess the same size (Figure 6).

Based on their morphological characteristics, morphotypes A and B have many similarities (*Emerita*) and differences, suggesting the occurrence of new species. However, it has been well-known that mole crabs from the superfamily Hippoidea show high variability in morphology (Poore 2004; Ahyong et al. 2009; Schnabel and Ahyong 2010). This condition may lead to misidentification when performed based on morphological characters. Molecular data confirmed the possible occurrence of Sympatric species of *Emerita emeritus* in the Cilacap coastlines (Nuryanto et al. 2020) inferred from several specimens. Therefore, further study is still needed using more samples to strengthen the data on new *Emerita* species in the areas.

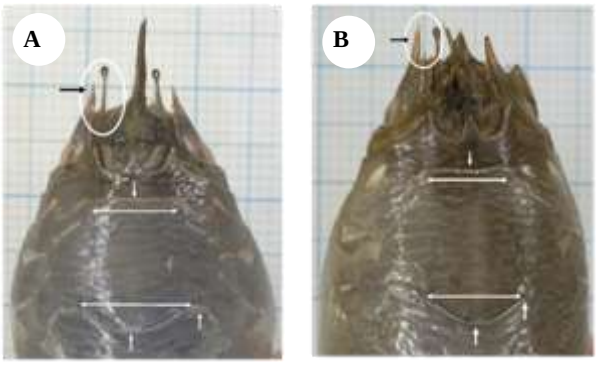

Figure 3. Frontal carapace on morphotype A and morphotype B

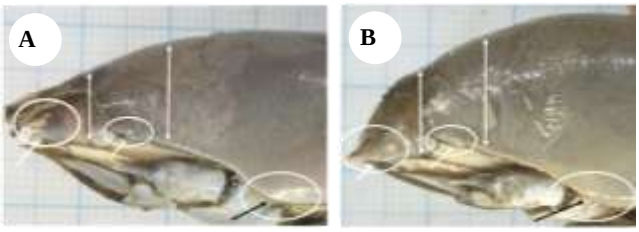

Figure 4. Latero-frontal carapace in morphotype A and morphotype B

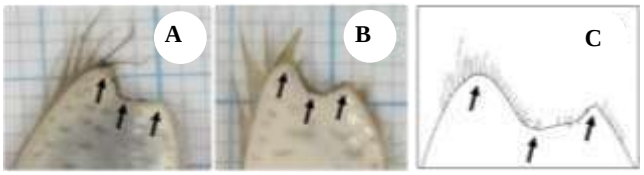

Figure 5. The distal part of the merus of the third maxilliped: Morphotype A; Morphotype B; and C. schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli 1965)

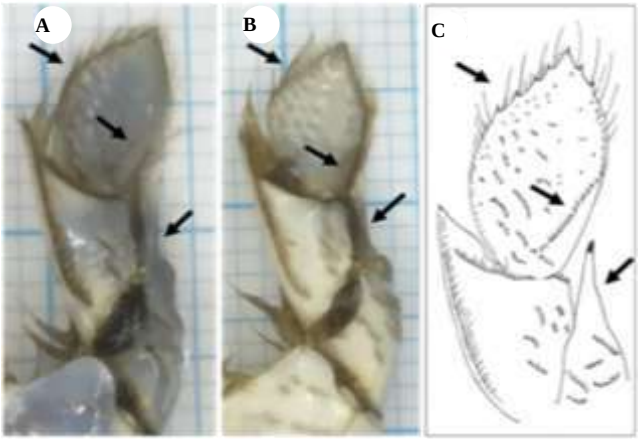

Figure 6. First pereopod on morphotype A, morphotype B, and schematic of *Emerita emeritus* (Linnaeus 1756.), ovigerous females from Madras (Sonkolli 1965)

Molecular characterization

Sequence identity tests to the closest relative in GenBank revealed that three individuals (KI1, KI3, and WP9) of the morphotype A have high sequence identities to *Emerita emeritus* KR047035 ranging from 96.12% to 96.25%. In contrast, the sequence identities to *Emerita* sp. ranged from 85.60% to 85.74%. The remaining 14 individuals of the morphotype B showed low identities to *Emerita emeritus* KR 047035 in GenBank, ranging between 84.78% and 86.87%. The sequence identity of the remaining 14 specimens of the morphotype B to *Emerita* sp. MZ571198 was high ranging from 98.83 to 100% (Table 1).

Genetic distance and genetic gap

Table 2 summarizes the genetic distance and gap between morphotype A and *E. emeritus* KR047035 and morphotype B and *Emerita* sp. MZ571198. The genetic gap was estimated based on the difference between the maximum and the minimum genetic distance of species.

Genetic divergence

Variance analysis and Fst value indicated that the two morphotypes showed significant genetic differences with a p-value of 0.005 (Table 3). The significant genetic difference between the two indicated that both belong to two different species, proved by the BLAST result.

Amino acid composition

The morphotypes were also subjected to amino acid composition comparison to define molecular divergence, as summarized in Table 4.

This research delineated the samples of morphotype A as *Emerita emeritus*. This is due to the strong genetic and conspecific identities of 96.12% to 96.25% and 85.60% to 85.74% for *Emerita* sp (MZ571198). Morphotype A was delineated into *E. emeritus* because genetic divergence within species may range from 0% to 4.6% (da Silva et al. 2011) or higher (Weis et al. 2014). Genetic divergence between morphotype A and *Emerita emeritus* KR047035 was below 4.6% (da Silva et al. 2011). The highest value was 3.88%, within the allowable range of 4% to 5%, as a moderate level of genetic identity for species delineation (Jeffery et al. 2011). This study has selected the value because the mutation rate of the COI gene is species-specific (Karanovic et al. 2015; Palecanda et al. 2020). A genetic threshold between 4% and 5% is permissible for genetic species determination, although additional considerations should be accounted for (Higashi et al. 2011; Jeffrey et al. 2011). Previous studies also utilized a genetic threshold of 5% during species determination (Candek and Kuntner 2015; Kusbiyanto et al. 2020; Riani et al. 2021).

The remaining 14 samples were identified as *Emerita* sp. nov. because of their high genetic identity (98.83% to 100%) to *Emerita* sp. MZ571198. In contrast, morphotype B had a low genetic identity (84.78% to 86.87%) to *Emerita emeritus* KR047035. This value is widely used as a genetic threshold in species delineation during animal barcoding (Hubert et al. 2010; Candek and Kuntner 2015).

The division of morphotypes A and B into two distinct species is due to a genetic distance ranging from 16.80% to 19.00%, with a genetic gap of 13.6% (Table 2). Moreover, the two morphotypes also showed significant genetic variances and fixation index (p=0.0059, Table 3) with different compositions of nucleotide content, especially in Adenine (A) and Thymine (T) composition (Table 4). Amino acid AT was higher than GC in both morphotypes, but the content of A and T was different. The phenomena were also reported in fish (Elvyra et al. 2020). The molecular difference observed in this study is in line with morphotypes A and B morphology. Therefore, morphotypes A and B delineated as *Emerita emeritus* and *Emerita* sp. nov. was reliable.

This study also proved that the CO1 gene is a good marker for taxonomic identification at the species level. The COI gene's reliability as a barcode is highly variable among animal species (Balkhis et al. 2011; Sachithanandam et al. 2012; Winarni et al. 2021). Similar phenomena were also reported from several locations across Indonesia (Muchlisin et al. 2013; Irmawati et al. 2017; Pramono et al. 2017) and other regions (Aquilino et al. 2011; Triantafyllidis et al. 2011).

Historical demography and genetic diversity of *Emerita* sp. nov.

Historical demography

Tajima's D value was -1.563 (p=0.044). That statistically significant results assumed that the used COI marker was under selection pressure. Instead of accepting selection pressure on the used marker, the negative sign of the D value indicated a recent population bottleneck and neutrality of the marker (Tajima 1989; Jong et al. 2011). The negative symptoms and non-significant Fs (-1.580, p=0.147) could assume that the marker was neutral and indicated a population bottleneck (Table 5). The assumption was based on the fact that Fus' Fs values are believed to be more sensitive than Tajimas' D. According to Jong et al. (2011) and Mohammed et al. (2021), the sensitivity of Fus' Fs values because it is calculated based on nucleotide diversity. A similar phenomenon was also reported in fish (Setyaningrum et al. 2022). Therefore, the used COI marker could be assumed as a neutral marker for assessing the genetic diversity of the *Emerita* sp. population in the Cilacap coastlines.

Table 1. The BLAST parameters of *Emerita* samples from Cilacap coastlines to their conspecific relatives in GenBank

Samples	*Emerita emeritus* KR047035			*Emerita* sp. MZ571198		
	Coverage	Expect Value	Genetic identity (%)	Coverage	Expect Value	Genetic identity (%)
K1 (A)	100	0.00	96.12	99	0.00	85.74
K3 (A)	100	0.00	96.12	99	0.00	85.74
WP9 (A)	99	0.00	96.25	100	0.00	85.60
J3 (B)	96	2e179	86.87	100	0.00	98.83
J4 (B)	97	2e-125	84.78	99	0.00	99.15
J6 (B)	99	0.00	86.48	100	0.00	99.84
J7 (B)	99	0.00	86.44	100	0.00	99.16
J8 (B)	99	0.00	86.45	99	0.00	100
KI4 (B)	99	0.00	86.48	100	0.00	99.84
KI5 (B)	99	0.00	86.32	100	0.00	99.67
WP3 (B)	99	0.00	86.32	100	0.00	99.51
WP5 (B)	98	0.00	86.64	99	0.00	99.83
WP8 (B)	99	0.00	86.42	100	0.00	99.85
CLPE8* (B)	99	0.00	86.84	100	0.00	99.84
CLP4* (B)	98	2e-169	86.57	100	0.00	99.82
CLP11* (B)	98	5e-171	86.75	100	0.00	100
CLP15* (B)	98	5e-171	86.75	100	0.00	100

Table 2. Genetic distance and gap within and among species (%)

Population	*Emerita emeritus*	*Emerita* sp.
Emerita emeritus	0.00-3.20	16.80-19.00
Emerita sp.	16.80-0.190	0.00-1.70
The gap between *E. emeritus* and *Emerita* sp.	16.80-3.20 = **13.6**	

Table 4. Amino acid composition of each morphotype (%)

Nucleotide	Morphotype	
	Morphotype A	Morphotype B
A	24.34	19.60
T	29.72	33.41
G	27.39	28.93
C	18.54	18.05

Table 3. Variance and Fst analysis indicate significant genetic divergence between two *Emerita* morphotypes

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Between morphotypes	1	1.303	0.143^{Va}	26.53
Within morphotypes	17	6.750	0.397	73.47
Total	18	8.053	0.540	
Fixation index (Fst):	0.265			
p-value (Va and Fst)	0.0059			

Table 5. Species, number of individuals (N), number of haplotypes (nhp), haplotype diversity (h), nucleotide diversity (μ), Tajima'D, and Fu's Fs values

Species	N	nhp	h	μ	D	p-sig.	Fs	p-sig.
Emerita sp. nov	15	7	0.857 ± 0.057	0.005 ± 0.003	-1.563^{*}	0.044	-1.580^{ns}	0.147

Note: *significant, ns: not significant

Genetic diversity

Multiple sequences alignment resulted in a total of 418 bp COI gene fragments from 14 individuals *Emerita* sp. nov. collected from the coastlines of Jetis, Sub-district of Nusawungu, Kenari Indah and Widarapayung, Sub-district of Binagun, Cilacap District, Central Java, Indonesia. Furthermore, 12 out of 418 bp were polymorphic, resulting in 7 haplotypes, and the haplotype diversity was 0.857±0.057 (Table 5). This data indicates that the *Emerita* sp. nov. population in the Cilacap coastlines has high genetic diversity. The nucleotide diversity value (μ) was 0.005±0.003, which revealed low nucleotide diversity and a relatively low rate of evolution in the *Emerita* sp. nov. population on the Cilacap coastlines. The haplotype network (Figure 3) shows that haplotypes were separated by 2 to 7 mutation steps. However, the mutation was widely distributed in the population, as indicated by high haplotype diversity (0.857±0.057). High haplotype

diversity assessed using the COI gene was widespread in animal phyla (Dorn et al. 2011; Dung et al. 2013; Song et al. 2013; Zhang et al. 2014; Nuryanto et al. 2019). At the same time, low haplotype diversity was also common in animal populations (Setyaningrum et al. 2022). The COI gene's study may show a complex pattern of diversity levels, even within species (Pavesi et al. 2011; Parmaksiz and Eksi 2017). The phenomena are also observed in population studies using other markers, such as microsatellite (Esa and Rahim 2013; Gouskov et al. 2016; Abbas et al. 2017; Achrem et al. 2017; Cheng et al. 2017) and d-loop (Zhong et al. 2013; Liu et al. 2016; Lau et al. 2018; Parmaksiz 2019; Ariyaraphong et al. 2021; Zhang et al. 2022).

This study cannot be compared with previous results because there is no population genetic study on mole crabs, especially on the presumable *Emerita* sp. nov. The only population study was conducted by Pramithasari et al. (2017), who compared mole crabs (*Albunea symmysta*) populations in Java and Sumatra. However, their study used morphological data, and the comparison to Pramithasari et al. (2017) was not congruent. This fact implies that more studies on the population genetics of mole crabs are needed.

Evolutionary relationships among *Emerita* sp. nov. individuals

The evolutionary process of the *Emerita* sp. nov. population on the southern coast of Cilacap is presented in the haplotype network (Figure 7). Star-like haplotype network in Figure 7 showed that haplotype 2 was the most primitive. Meanwhile, H2 was the center of the network, and other haplotypes evolved from (H2) as the most abundant (Balkhis et al. 2011; Song et al. 2012). The result contradicted the general acceptance that primitive haplotype has the highest abundance in the population (Adamson et al. 2012; Barasa et al. 2014; Baisvar et al. 2018, 2019). The low frequency of H2 observed was assumed because of the small population (14 individuals). However, this assumption should be proven based on a further study using a high number of analyzed individuals.

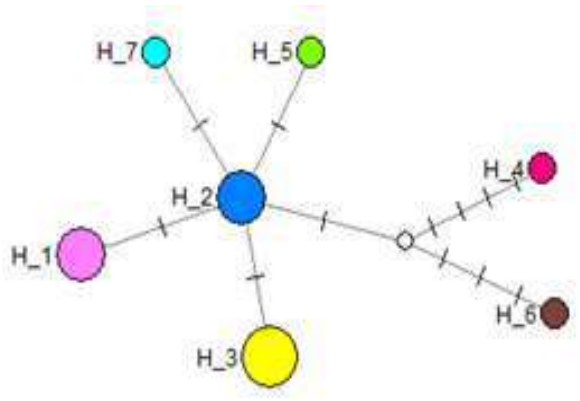

Figure 7. Haplotype networks indicating evolutionary relationships among *Emerita* sp. nov. individuals

According to the analyzed data, this study concluded that mole crabs (Genus *Emerita*) in the Cilacap coastlines consisted of two distinct sympatric species (*Emerita emeritus* and *Emerita* sp. nov). *Emerita* sp. nov. had high haplotype diversity and was more abundant than *Emerita emeritus*. As a result, comprehensive research in terms of sampling site, number of samples, and other biological characteristics are needed to provide complete information for sympatric and taxa species of *Emerita* sp. nov.

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