Molecular Barcoding Reveals Possible Existence of Sympatric Species of Emerita emeritus in South Coast of Cilacap Central Java

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Abstract. Cilacap Regency resides in the southern part of Central Java. It faces the Indian Ocean and has a quite long coastline with sandy beaches as the favorable habitats for mole crabs. Careful examinations on previously identified as *Emerita emeritus* samples from Cilacap, the mole crabs showed slight morphological differences to *Emerita emeritus* Linnaeus. We assume that our samples are sympatric species of E. *emeritus* complex rather than E. *emeritus* Boyko. A length of 560 bp fragments of the cytochrome oxidase 1 was sequenced. Homology test resulted in 83 - 86% sequences similarity to E. *emeritus* sequence available in GenBank (KR047035). Our samples also had high genetic distances (0.152 - 0.155) to the sequence of KR047035. The phylogenetic tree showed a clear separation between our samples and reference sequence (*Emerita emeritus* KR047035) with a quite long branch. Those all three kinds of data prove that our Emerita samples are most likely not belong to previously identified *Emerita emeritus* Boyko although it shows only slight morphological differences. These results indicate that possible cryptic species of *Emerita emeritus* or E. *emeritus* complex inhabits sandy beaches in Cilacap coast. It has been described that cryptic species are common in aquatic organisms. However, we need more samples to examine and strengthen our finding.

1. Introduction

Sand crabs or mole crabs, also locally known as "undur-undur laut" or "yutuk" belong to Decapoda. They live in sandy habitats of the tidal areas. Geographic distributions of sand crabs span across the Indo-Pacific region. In Indonesia, these crabs inhabit the South Coast of Java, West Coast of Sumatera and Moluccas [1].

Previous observation confirmed that sand crabs were also live in the southern coastline of Cilacap Regency, especially in Widarapayung Wetan, Sidayu, and Widarapayung Kulon. It was reported that three different species of mole crabs, namely *Emerita emeritus* (yutuk jambe), *Hippa adactyla*, and *Albunea symmysta* inhabited those three areas [2].

The detail and careful examination on previously identified as E. *emeritus* samples from those mentioned areas demonstrated subtle morphological differences to the reference specimens of E. *emeritus* Linnaeus [1]. These species belong to Hippoidea [1]. It has been well-known that mole crabs from superfamily Hippoidea show high morphological variability [3-5]. A similar phenomenon is also observed on other mole crabs in the genus Albunea. The morphological variations in Albunea lead to an uncertain classification of the genus [6].

Morphological differences between our samples, which were previously identified as E. *emeritus*, and *Emerita emeritus* (Linnaeus 1767), indicate that our specimens are different species and possibly indicate sympatric species (sibling), or cryptic species, or complex species of E. *emeritus*. Cryptic species and complex species are common phenomena in aquatic biota [7], which might lead to misidentification when performed based on morphological characters solely. Cryptic species phenomenon occurs in stingray [8], whereas complex species phenomena have been reported in giant clams [9].

Misidentification in sympatric, cryptic, or complex species can be avoided through the application of DNA barcoding using a short fragment of DNA [10-12]. This technique has been successfully applied from species to subspecies levels, which is very hard to differentiate or identify based on morphological characters such as cryptic species [13,14]. The cytochrome c oxidase I (COI) gene is a common marker for species identification in animal [10]. Previous studies have determined that the COI has been successfully used in species identification of wide ranges of animal phyla [11,15–20]. Therefore, it is expected that the fragment of COI gene can be used as a barcode marker on species-level identification to strengthen the taxonomic status of "Emerita" samples from the south coast of Cilacap, especially from Widarapayung Wetan, Sidayu, and Widarapayung Kulon.

2. Methods

The study was run from April to November 2018 with samples taken from Widarapayung Wetan, Sidayu, and Widarapayung Kulon Coastlines Cilacap Regency, Central Java (Figure 1). Molecular analysis was performed in Animal Taxonomy Laboratory, Faculty of Biology, Universitas Jenderal Soedirman Purwokerto, Central Java.

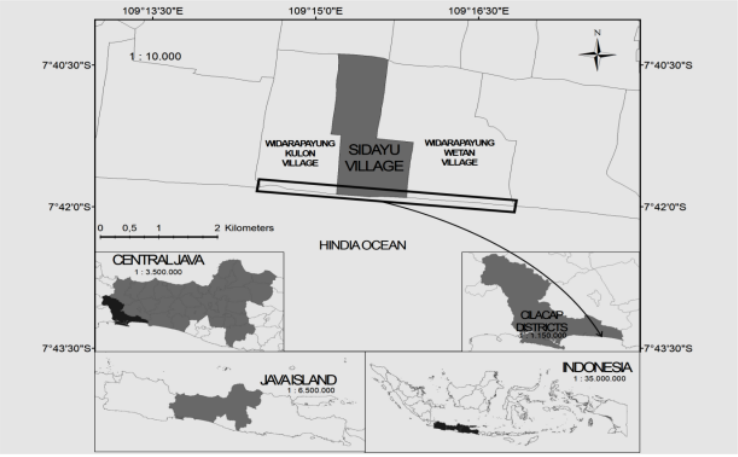

Figure 1. Sampling sites (source: Indonesia 2018©DigitalGlobe, Google Earth © 2018 Google, modified S.S. Asmarani, 2018).

This study used a survey method with incidental sampling. Mole crab samples were collected using two different fishing gears, "sodo net" and "sorok bamboo." The local fishermen assisted the sample collections. Small pieces of pereopod tissues were collected and preserved in 96% ethanol for DNA analysis.

Genomic DNA was extracted from the tissue samples using Chelex100® technique [21] with subtle modification in incubation time. The fragment of the COI gene was amplified using a pair of universal primer of LCO 1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO 2198:

5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (22). The PCR reaction was set up to 50 µl of total volume. The PCR mixtures contain 29.8 µl of ddH_2O; 1X PCR buffer, 5 mM of $MgCl_2$, 0.01 mM of each primer 0.05 mM of dNTPs, 1 U Taq polymerase, and 4 µl of template DNA. The arranged thermal condition was as follow: 95 °C pre-denaturation for 5 minutes, continued 35 cycles with denaturation phase for 1 minute on 94 °C, annealing on 54 °C for one minute, extension for 1.5 minutes on 72 °C. The final extension was performed for 10 minutes on 72 °C [23]. The PCR products were then migrated in 1% agarose gel and visualized over UV-transilluminator. Strong and clear PCR products were shipped to 1st BASE for sequencing (www.base-asia.com).

Nucleotide sequences of the COI gene were aligned and edit manually in Bioedit (Ver. 7.0.5.; Hall, 1999). Multiple alignments of all sequences were done in bioedit. The nucleotide sequences of the samples were submitted to the database to obtain the accession number. Species-level identification was based on the homology values of the samples with references conspecific sequences *Emerita emeritus* KR047035 and *Emerita emeritus* AF246159. The result of homology test was strengthening by phylogenetic analysis through reconstructing the taxonomic tree. The taxonomic tree was reconstructed in MEGA 6.0 [24] based on the neighbor-joining algorithm and substitution model of Kimura-2 Parameter (K2P). Branching pattern of the taxonomic tree was supported by 1000 non-parametric pseudo-replicates. The polarity of the branching pattern was resulted by using Blepharipoda_occidentalis_AF437625 as an out-group comparison.

3. Results

A total of four sequences have been resulted from morphologically divergences samples of previously identified as *Emerita emeritus*. Multiple sequences aligned together with references species and the out-group species resulted in a length of 507 bp fragment of the COI gene. Homology test based on 3% cutoff genetic divergences either to references sequences in bold system or GenBank database resulted in sequences similarity values of 83% - 86% (KR047035). This means that genetic divergences between our samples and references species (KR047035) ranged from 14% up to 17%. Average genetic divergences among our samples were 0.000. Genetic divergences among our samples and between our samples and reference species (KR047035) and also out-group species (AF437625) are presented in Table 1.

Table 1. The Kimura 2 parameter genetic divergences among samples and between samples and references species and also out-group species

Specimens	1	2	3	4	5	6	7
Sample_Clp_4		0.000	0.000	0.000	0.018	0.083	0.083
Sample_Clp_11	0.000		0.000	0.000	0.018	0.083	0.083
Sample_Clp_15	0.000	0.000		0.000	0.018	0.083	0.083
Sample_Clp_E8	0.000	0.000	0.000		0.018	0.083	0.083
Emerita emeritus KR047035	0.142	0.142	0.142	0.142		0.107	0.089
Emerita emeritus AF246159	0.934	0.934	0.934	0.934	1.075		0.025
*Blepharipoda_occidentalis*_AF437625	0.922	0.922	0.922	0.922	0.977	0.229	

The Kimura 2 parameter neighbor-joining tree showed that our samples formed the monophyletic group. The tree also indicates that our samples are becoming sister taxa to *Emerita emeritus* (Figure 2).

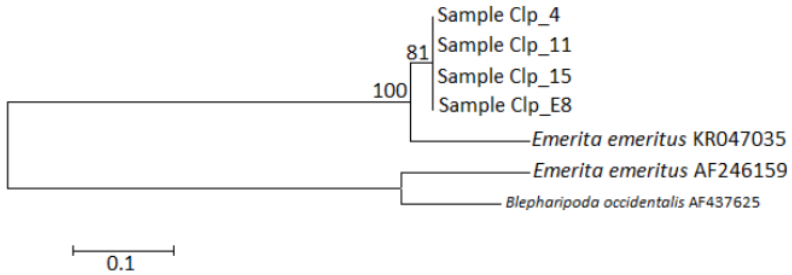

Figure 2. The K2P neighbor-joining tree showing monophyly of our samples and polyphyly between our samples and *Emerita emeritus* KR047035.

4. Discussion

Genetic divergence between our samples and reference species of *Emerita emeritus* (KR0470335) was much higher than 3% cutoff genetic divergence as a species border implemented in the bold system. According to this cutoff value, our specimen cannot be referred to the same species as reference species (*E. emeritus*). As high as 5.41% genetic divergences are observed between the west and east Americas populations of *E. talpoida* and of 3.47% among *E. analoga* populations [25]. Both genetic divergence values strengthen our decision that those genetic divergences of 14 – 17% between our samples and previously described *E. emeritus* are different species. Even, if we use recent standard, i.e., species level can be defined based on maximum genetic divergence of 8.5% [26], our samples cannot be identified as a single species as referred to bold system database of *Emerita emeritus* (KR047035). High genetic divergence between our samples and the reference species indicates that our samples differ from *Emerita emeritus*. However, since our samples have high similarity in their morphological characteristics to the reference, and collected in the same habitat as *E. emeritus*, they can be referred as sympatric species or sibling species from *E. emeritus*.

Although our samples genetically identified as a different species, according to 14 – 17% of genetic divergences between our mole crab samples and reference species of *Emerita emeritus*, we are convinced that our samples belong to the same genus as *Emerita emeritus*. We refer to several previous studies which provide evidences that genetic divergences between species within a genus can be more than 6% [18,26–28].

In Figure 2, we observe that our mole crab samples are separated from the reference species *E. emerita* with branch length more than 0.1 genetic divergences when we compared to the scale as indicated below the NJ tree. The tree also demonstrates that all our mole crabs form a monophyletic group and place as sister taxa of *E. emeritus*. Strong bootstraps values support the grouping of all our samples (81) and separation between our samples and the reference species (100). This clear separation between our mole crabs and *E. emeritus* confirms that our mole crabs are different species from *E. emeritus*. However, in a higher rank, our samples form a monophyletic group together to the reference species with branch length only slightly higher than the scale below the figure. This finding indicates that our samples can be identified as the same genus as *E.emeritus*.

Another interesting finding was that genetic divergence among our samples was 0.000. This value provides strong evidence that our samples belong to a single species. Individuals can be referred to as a single species if they have a genetic divergence between 1% and 3% [28]. Similar results report that genetic divergence among individuals within species Acanthuridae ranges from 0.000 up to 0.010, and between 0.000 and 0.009 in Holocentridae depending on the genera [29]. Moreover, genetic divergences between species range from 0.081±0.014 to 0.110±0.018 with maximum values between 0.09 and 0.140 in Acanthuridae, while in Holocentridae the mean value ranges from 0.063±0.012 up to 0.102±0.038 with maximum divergences between 0.080 and 0.138. Those results strengthen our

decision that all of our four samples belong to a single species, and they are different species from *Emerita emeritus* KR047035.

It has been explained in numerous publications that the superfamily Hippoidea shows high morphological variability, and also in Albunea [3–5]. These morphological variations lead to an uncertain classification of those mole crabs [6]. Our finding on highly genetic divergences specimen from the south coast of Cilacap that is compared to previously identify *Emerita emeritus* proves and increases the complexity of mole crab classification.

5. Conclusion

Based on their genetic divergences, we conclude that morphologically similar samples of mole crabs from Cilacap are genetically different species from *Emerita emeritus*. High morphological similarities between our samples and *Emerita emeritus* indicate that our samples can be referred to as sympatric or sibling species of *Emerita emeritus*. Our samples belong to a singles species and form sister taxa to *E. emeritus*. The mole crabs from the south coast of Cilacap can be placed into single genus as *Emerita emeritus*.

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