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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF BLUE SWIMMING CRAB (*Portunus* sp.) IN THE NORTHERN WATERS OF JEPARA USED DNA BARCODING

IDENTIFIKASI DAN ANALISIS FILOGENETIK RAJUNGAN (*Portunus* sp.) DI PERAIRAN UTARA JEPARA MENGGUNAKAN BARCODING DNA

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Abstract

The Awur Bay and Kartini Beach in Jepara, Central Java, are fishing areas with blue swimming crab as a main catch. This study aims to examine the species of blue swimming crabs captured in the Northern Waters of Jepara from different locations, analyze the molecular characteristics based on gender differences, obtain GenBank accession numbers as genetic conservation, and construct their phylogenetic tree. The research used a quantitative descriptive method. Samples were collected using purposive sampling from fishermen's catches and selected based on the difference in crab claws and carapace color, with a total of 4 samples. The samples were extracted using a DNA extraction kit and tested using Polymerase Chain Reaction (PCR) to visualize mitochondrial DNA (COI gene) and processed using MEGA 11. BLAST results showed that the similarity of the samples to *Portunus pelagicus* ranged from 97.02% to 99.42%. The different colors of claws and carapace did not affect the DNA sequence composition, hence indicating sexual dimorphisms. The DNA sequences from this study were deposited to GenBank with accession number LC836055.1-LC836058.1. Based on the phylogenetic tree construction, the clade of all samples shows a close relationship with the species *Portunus pelagicus*.

Keywords: Awur bay; Dimorphism; Jepara; Kartini beach; Polymerase Chain Reaction

Abstrak

Teluk Awur dan Pantai Kartini merupakan daerah penangkapan ikan di Kabupaten Jepara, Jawa Tengah dengan salah satu hasil tangkapannya adalah rajungan. Penelitian ini bertujuan untuk mengkaji jenis spesies rajungan yang ditangkap di Perairan Utara Jepara dari tempat yang berbeda, menganalisis perbedaan karakteristik molekuler dari rajungan dengan jenis kelamin berbeda, mendapatkan kode akses GenBank dalam upaya konservasi genetik dari rajungan asal perairan Jepara, dan menyusun pohon filogenetiknya. Metode penelitian yang digunakan adalah metode deskriptif kuantitatif. Rajungan diambil menggunakan metode purposive sampling melalui hasil tangkapan nelayan dan dipilih berdasarkan perbedaan warna capit dan karapas rajungan sebanyak 4 sampel. Sampel diekstrak menggunakan DNA Extraction kit dan diuji menggunakan Polymerase Chain Reaction (PCR) untuk memudahkan visualisasi DNA mitokondria (gen Cytochrome C Oxidase subunit I) dan diolah menggunakan aplikasi MEGA 11. Hasil BLAST menunjukkan bahwa tingkat kemiripan sampel dengan Portunus pelagicus berkisar antara 97,02% hingga 99,42%. Warna capit yang berbeda antara jantan dan betina tidak memengaruhi komposisi sekuens DNA, kondisi tersebut dinamakan dimorfisme seksual. Urutan DNA telah disimpan pada database global GenBank dengan kode nomor akses LC836055.1-LC836058.1. Berdasarkan konstruksi pohon filogenetik, jarak clade semua sampel menunjukkan kekerabatan yang erat dengan spesies Portunus pelagicus.

***Kata Kunci**: Dimorfisme; Jepara; Pantai Kartini; Polymerase Chain Reaction; Teluk Awur*

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INTRODUCTION

Teluk Awur and Kartini Beach are places where fishermen carry out their capture activities in the Northern Waters of Jepara. One of the aquatic resources captured by fishermen due to its high market value in the Northern Waters of Jepara is the catch of crab species (Setiyowati & Sulistyawati, 2019). The export value of crabs and mud crabs, according to the Ministry of Marine Affairs and Fisheries (KKP), increased by 66.31% from 2020 to 2021. The export value of crabs and mud crabs in 2020 was US$ 367.52 million, while in 2021 it reached US$ 611.225 million (Pambudi, 2023). The high economic value of crabs has led to increased capture, which can trigger overfishing. The knowledge of the crab species captured in the area is needed to present genetic information, otherwise, this species will continue being exploited and potentially endangering the crab stock as increasing in fishing gear that catch this commodity (Mustofa et al., 2021).

The report of crabs in Jepara Regency is relatively minimal. This could pose difficulties for the Jepara Regency government if, at some point, one of the crab species becomes endangered due to a lack of information about the crab species in Teluk Awur and Kartini Beach. The scarcity of crab documentation is attributed to the absence of official crab landing sites (Setiyowati, 2016). The identification of crab species in the Northern Waters of Jepara can be determined through species identification. Identification is divided into two types: morphological identification and molecular identification. Identifying a species is commonly done by observing its morphology comprehensively and matching it with identification books, known as conventional identification (Andriyanto & Yulianti, 2020). Conventional identification that relies on morphological observation is generally very limited and not accurate enough to identify until species level that have very similar morphology. Another weakness of conventional identification is that the time required tends to be longer if the number of samples observed is large. It also needs the expertise to identify morphological characters and potentially cause high misidentified if there is no experience (Indriati & Hidayat, 2023).

The morphology of a species can have similarities with others because the species falls into the category of cryptic species. Errors in the identification process are fatal as they can lead to incorrect treatment of the species, potentially causing it to become extinct faster in the face of high resource exploitation. Molecular identification provides a solution for obtaining more accurate identification results. This is because molecular identification uses DNA Barcoding, which traces nucleotide base variations in each species using the Cytochrome c oxidase I (COI) gene marker located in the mitochondria. According to Aznardi and Madduppa (2020), the advantage of DNA Barcoding lies in its higher accuracy in identifying various organisms. Therefore, the objectives of this research include determining the crab species caught in the Northern Waters of Jepara, Central Java, Indonesia from different locations, analyzing the molecular characteristics difference between males and females, obtaining GenBank accession number as genetic conservation to swimming blue crab in Jepara waters, as well as construct their phylogenetic tree.

MATERIALS AND METHODS

This research was conducted in Teluk Awur and Pantai Kartini. The sampling method used for collecting crab samples was purposive sampling. Samples were collected from local fishermen, with 2 individuals selected from each location with different color variations on its claws and carapace (Figure 1). The samples encoded TAB (male crab from Teluk Awur), TAM (female crab from Teluk Awur), KB (male crab from Pantai Kartini), and KM (female crab from Pantai Kartini).

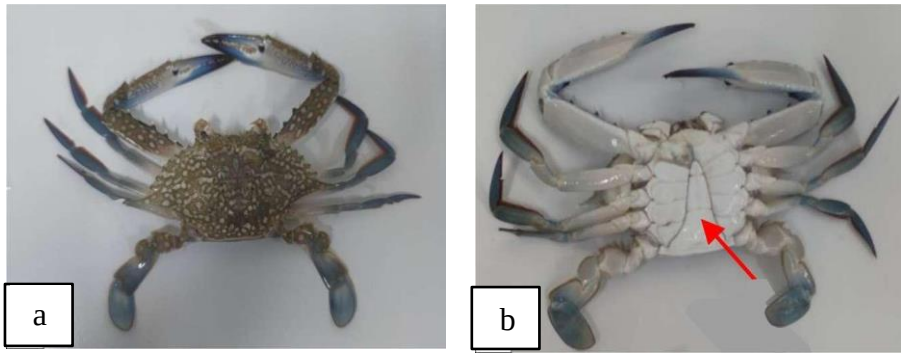

Figure 1. The differentiation color of claws and carapace between male (TAB) and female (KM) of blue swimming crab; dorsal view of male crab (a), ventral view of male crab (b); dorsal view of female crab (c), and ventral view of female crab (d). The shape of the abdominal flap of a male crab is narrower ("V" shape), and then the female crab is wider ("U" shape) indicated with a red arrow

Molecular Identification

DNA Extraction

The DNA extraction method is a series of important initial stages in molecular processes. This is an important stage for separating DNA from other cell components so if this process does not follow the procedures, the process will fail and be repeated from the beginning. DNA Extraction Method for these crab mitochondria uses a protocol according to the Genomic DNA Mini Kit (Tissue) from Geneaid™ DNA isolation kit. The procedure included (1) sample preparation: 30 mg of muscle tissue from crab claws were collected and placed in an Eppendorf tube, (2) destruction of cell membranes and mitochondria: this involves two steps: (a) using GT buffer for homogenization followed by incubation, and (b) using GBT buffer to freeze the sample, then centrifuging it. The supernatant containing DNA was transferred to a new tube, (3) separation: DNA is isolated from remaining compounds or enzymes by adding absolute ethanol to a GS column, homogenization, and centrifugation. The mixture in the collection tube was discarded and the DNA in the GS column was purified, (4) purification: the process was repeated twice using wash buffer to eliminate any residual compounds or enzymes, followed by centrifugation. The DNA sample was centrifuged once more without adding any liquid, (5) final isolation: elution buffer, previously incubated on a heat block at 60 °C, was added to a new collection tube, followed by centrifugation. The purified DNA was stored in the freezer for the amplification process.

DNA Amplification

The COI gene segment from the mitochondrial genome was amplified using a universal pair of DNA Barcoding primers, including the forward primer LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and the reverse primer HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The PCR amplification process is by adjusting the temperature of the PCR machine such as pre-denaturation temperature of 94 °C for five minutes; 35 cycles with each denaturing at 94 °C for 1 minute, annealing at 56 °C for 1 minute; and extension at 72 °C for one minute 90 seconds; and ending with post extension at 72 °C for five minutes. Visualization of PCR results using electrophoresis on a 1% agar gel under a UV transilluminator.

DNA Visualization

DNA visualization is useful for checking whether there is DNA after the amplification process by electrophoresis gel. Samples that are not visible when carried out visualization are expected to be redone from scratch. This is because there is something wrong, either in the amplification or extraction process. Gel electrophoresis was conducted by dissolving 0.4 g of agarose powder in 40

mL of TBE buffer solution, heating the solution to 280 °C for 10 minutes, and pouring it into an agarose mold to solidify. The agarose gel was then placed in an electrophoresis chamber containing a TBE buffer solution. Subsequently, a PCR product containing blue swimming crab's DNA was injected into the wells, and electrophoresis was conducted at 100 V for 30 minutes. The gel was then immersed in a 1% ethidium bromide solution for 30 minutes and visualized under a UV-transilluminator to confirm successful DNA amplification. The presence of DNA bands detected under a UV lamp confirms the successful amplification step.

Data Analysis

Data analysis was performed using SnapGene and MEGA 11 software. Crab species were identified by conducting Basic Local Alignment Search Tool (BLAST) searches in the NCBI (National Center for Biotechnology Information) database. The phylogenetic tree was constructed using MEGA 11 software, comparing it with mitochondrial DNA data from the genera Portunus and Callinectes in the NCBI database. The method used for sequence alignment was Clustal W, while the method for constructing the phylogenetic tree was the Maximum Likelihood bootstrap method with 1,000 replicates and the Kimura-2-parameter model.

RESULTS

The results of mitochondrial DNA amplification show that KB, KM, TAB, and TAM samples can be identified on the UV tool transilluminator. The base pair length represents the four samples against the DNA ladder + loading dye, namely having parallel lengths. Based on the amplification results, samples KB, KM, TAB, and TAM are long base pairs of approximately 700 bp. The results of the amplification of the four samples can be seen in Figure 2.

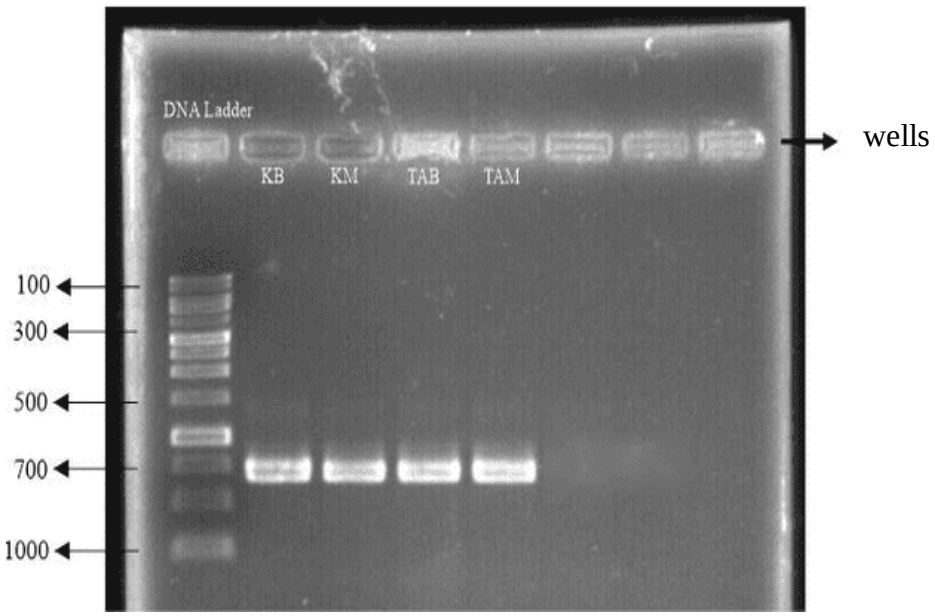

Figure 2. Results of COI gene amplification using DNA barcode universal primer LCO 1490 and HCO 2198

Table 1 displays the sequence data obtained from the sequencing facility before processing with MEGA 11. The sequences were aligned between the forward and reverse primers, with the removal of any N elements, and then merged. The electropherogram displayed numerous ambiguous peaks, denoted as 'N', indicating suboptimal sequencing results. The combined sequences of the forward and reverse primers were processed with MEGA 11 consisting of the information of their nucleotide composition between males and females in Table 1. The composition of nitrogen bases in all samples shows similarity. The result of the BLAST program on the NCBI website, utilizing GenBank data, is presented in Table 2, ranging from 97.02–99.42% similarity. The phylogenetic tree resulting from the processing using MEGA 11 is shown in Figure 3.

Table 1. Results of forward and reverse sequences from crab samples

Sample code	Primer	Sequence length (bp)	Number of N	T(U) (%)	C (%)	A (%)	G (%)	Total sequence after alignment (bp)
TAB	*Forward*	679	0	36.8	19.9	26.1	17.2	717
	Reverse	686	0					
TAM	*Forward*	680	1	37.2	20.2	25.3	17.3	643
	Reverse	697	0					
KB	*Forward*	677	1	36.5	20.1	25.6	17.8	715
	Reverse	685	0					
KM	*Forward*	682	0	36.9	20.3	25.6	17.2	715
	Reverse	686	0					

Note: N represents unreadable bases/ multiple peaks in the electropherogram of Sanger sequencing; nucleotide/ nitrogen bases consist of T(U)= thymine (uracil); C cytosine; A= adenine; G= guanine

Table 2. BLAST results of crab DNA sequences with COI primers

Sample code	Species identification result (BLAST)	Reference no.	Sequence length (bp)	Identity percentage
TAB	*Portunus pelagicus*	MN336964.1	717	99.13%
TAM	*Portunus pelagicus*	MN337020.1	643	97.02%
KB	*Portunus pelagicus*	MN635713.1	715	99.14%
KM	*Portunus pelagicus*	MN336866.1	715	99.42%

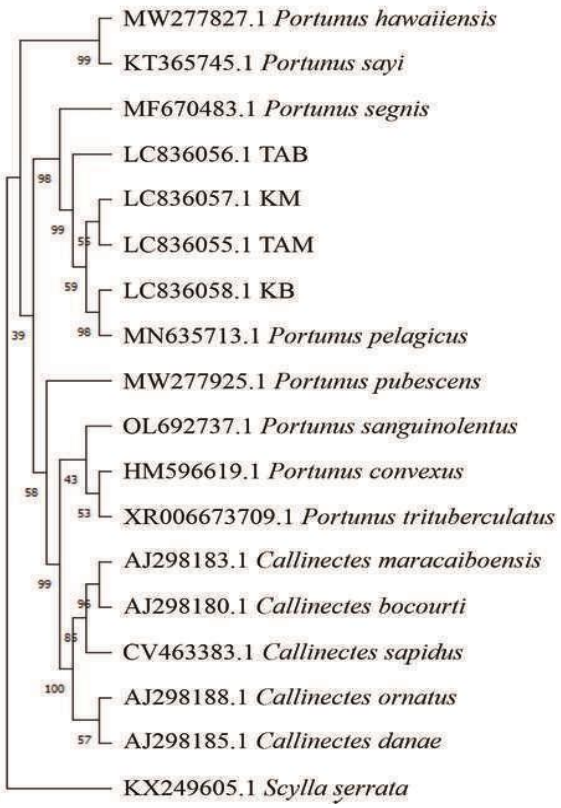

Figure 3. Phylogenetic tree of crabs based on genetic distance from COI gene DNA sequences using maximum likelihood method with 1000× Bootstrap Kimura-2-parameter model

DISCUSSION

Based on the research findings, the blue swimming crab samples are coded according to the color of their claws and where they were obtained. The full form of each sample code for blue swimming crabs in the sequence is KB (crab obtained at Kartini Beach with blue claws), KM (crab obtained at Kartini Beach with red claws), TAB (crab obtained at Awur Bay with blue claws), and TAM (crab obtained at Awur Bay with red claws). Sequences were processed by arranging them

using MEGA 11 software. The sequences were then input into the BLAST program on the NCBI website to determine their species. NCBI is a website that provides online resources for biological data, such as nucleotide acid sequences on GenBank, using the BLAST program (Sayers et al., 2019). The success of DNA amplification was proven by the presence of DNA bands, visible in the visualization of sample bands (KB, KM, TAB, and TAM) which were formed at a size of approximately 700 to 750 bp, indicating intact DNA results. This sequence comes from an amplification process using COI primers, ensuring alignment with the target gene. As highlighted by Novitasari et al. (2014), intact DNA is marked with a thick and clear band without any staining. The appearance of thin or thick electrophoresis bands can be influenced by various factors, including the conditions of the DNA sample used, as stated by Yilmaz et al. (2012). The main determining factors for the appearance of amplification results include the DNA isolation process, primer selection, and potential contaminant factors. The alignment sequences revealed that all samples do not show different nucleotide compositions, even though their length of sequence is varied (Table 1). This finding also relates to a study from Karohmatullah et al. (2024) comparing male and female crab nucleotide composition in *Charybdis anisodon*. However, the proportion of Guanine and Cytosine (G + C) in this study was much higher than G + C in *Charybdis anisodon* at ±2% on average. Kudla et al. (2006) stated that high GC content is responsible for gene expression.

On the other hand, the BLAST program is useful for identifying the species of a sample by comparing it with sequences of similar species available in GenBank. Some functions of BLAST include efficiently finding the desired DNA, deducing the function of a gene, and identifying sequences (Anwar et al., 2022). Based on the BLAST program on the NCBI website (Table 2), the TAB sample shows a similarity level of 99.13% with the species *Portunus pelagicus* MN336964.1. The GenBank accession number for the TAB sample is LC836056.1 with a sequence length of 717 base pairs (https://www.ncbi.nlm.nih.gov/nuccore/LC836056.1). The TAM sample shows a similarity level of 97.02% with the species *Portunus pelagicus* MN337020.1. The GenBank accession number for the TAM sample is LC836055.1 with a sequence length of 643 base pairs (https://www.ncbi.nlm.nih.gov/nuccore/LC836055.1). The KB sample shows a similarity level of 99.14% with the species *Portunus pelagicus* MN635713.1. The GenBank accession number for the KB sample is LC836058.1 with a sequence length of 715 base pairs (https://www.ncbi.nlm.nih.gov/nuccore/LC836058.1). The first sample, KM shows a similarity level of 99.42% with the species *Portunus pelagicus* MN336866.1. The GenBank accession number for the KM sample is LC836057.1 with a sequence length of 715 base pairs (https://www.ncbi.nlm.nih.gov/nuccore/LC836057.1). The identity or similarity results not reaching 100% in the BLAST results are due to differences in some nucleotide pairs that were aligned (Budiarsa et al., 2022). The sequencing results for the TAM crab sample have the lowest similarity value, which is 97.02%. A similarity level of a sample greater than 97% indicates a high homology between the two sequences, suggesting that the species is identical to the compared sample. Similarity values between 93% and 97% suggest that the sample belongs to the same genus but is a different species (Kusuma et al., 2021).

Based on Figure 2, the resulting phylogenetic tree is divided into three clades and one outgroup. The genus *Callinectes* was selected as a reference in the construction of the phylogenetic tree because, in the Pasuruan area of East Java, there is one species of blue swimming crab from the genus *Callinectes*, namely *Callinectes sapidus. Callinectes sapidus* or blue crab, has significant potential to become an export commodity (Primyastanto et al., 2023). The species *Scylla serrata* is used as the outgroup because crabs from this species are commonly found in Indonesian waters and still belong to the same *Portunidae* family as the blue swimming crab. Based on the length of the branches produced by the phylogenetic tree, the longest branch is possessed by the species *Callinectes danae*, a member of clade III. This indicates that the species *Callinectes danae* has a long evolutionary history or can be considered more distantly related to the ancestors of the TAM, TAB, KM, and TAB samples. Most species of the *Callinectes* genus are found in the Pacific and Atlantic waters of America. The distribution of the *Callinectes danae* species occurs due to temperature changes, making it now commonly found in the waters around the Southwest Atlantic (Herrera & Costa, 2022).

One species of the *Callinectes* genus, *Callinectes sapidus*, originated from the western Atlantic (Mancinelli et al., 2021). Sequences from KM, TAM, KB, and TAM show a close relationship with the *Portunus pelagicus* species. This is evidenced by the shorter branch lengths on the phylogenetic tree. These short branches indicate that these four samples are considered descendants of the ancestor *Portunus pelagicus.* A phylogenetic tree with monophyletic characteristics, high bootstrap values, dichotomous branching, and consistently formed clades are features of a phylogenetic tree considered good and acceptable in systematics (Lestari et al., 2018).

CONCLUSION

Based on the PCR results using DNA Barcoding, it indicates that the crab species caught in the Northern Waters of Jepara is only one species, which is *Portunus pelagicus*. The similarity levels of the TAB, TAM, KB, and KM samples with the *Portunus pelagicus* species, based on BLAST results in sequence, are sequentially 99.13%, 97.02%, 99.14%, and 99.42% and the DNA sequences already deposited to GenBank with accession number LC836055.1-LC836058.1. The KM, TAM, KB, and TAM samples show a close relationship with the *Portunus pelagicus* species. This is evident from the shorter branches of the phylogenetic tree.

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