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Morphological and molecular characteristics of *Donax incarnatus* (Bivalvia: Donacidae) from Badur Beach, Madura Island, Indonesia based on the *COX1* gene

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Abstract. *Wijaya CB, Ambarwati R, Rahayu DA. 2023. Morphological and molecular characteristics of* Donax incarnatus *(Bivalvia: Donacidae) from Badur Beach, Madura Island, Indonesia based on the COX1 gene. Biodiversitas 24: 2805-2813.* Donacidae, a family of marine bivalve mollusks, has a high diversity of morphological traits, including shape and color pattern variations. However, these variations often led to errors in morphological identification, necessitating the use of molecular identification methods for accurate species identification and support. This study aims to analyze the morphological and molecular characteristics of *Donax incarnatus* Gmelin, 1791 collected from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia, using the *COX1* gene as a molecular marker. Morphological characterization was conducted through qualitative and quantitative observations, while sequencing results were analyzed using bioinformatics software. The results showed that the exterior color of *D. incarnatus* has three morphological variations, namely purple-black (OQ692130.1), yellowish orange (OQ692131.1), and white (OQ692132.1). Molecular characterization indicated that the similarity value of *D. incarnatus* with these variations ranged from 96.06% to 99.61%. At the same time, the alignment results of all samples with reference species and outgroups obtained five variations of nucleotide bases. The average genetic distance between *D. incarnatus* and the ingroup was 0.01. The phylogenetic tree results obtained using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods were categorized into two subclusters: *D. incarnatus* species from Thailand and the study sample from Badur Beach, Madura Island, Indonesia, were placed in the same cluster.

Keywords: Cytochrome C Oxidase I, DNA barcode, molecular phylogenic

INTRODUCTION

The Donacidae family is a large family of approximately 100 recognized species typically found in warm waters. These clams have a habitat preference for shallow burial just below the surface of wave-swept areas of intertidal beaches (Tan and Low 2013). According to MolluscaBase Eds (2023a), the Donacidae family comprises up to five genera. In Indonesia, several studies, such as those by Ambarwati and Faizah in 2017, have reported the presence of two species of Donacidae clams. Dharma (2005) found two genera of the Donacidae family in Indonesian waters, namely *Hecuba* and *Donax*, with species such as *Donax faba* Gmelin, 1791 and *D. deltoides* Lamarck, 1818 found within the genus *Donax.*

According to a study by Sathish et al. (2020), the genus *Donax* has a broad distribution and is known as the dominant invertebrate group on sandy beaches worldwide. Approximately 75% are found in tropical waters, 22% in temperate climates, and a small number in cold climates (Ocaña 2015). Poutiers (1998) noted that *Donax* clams are found in several regions, including the Gulf of Thailand, Indo-West Pacific, and East Africa, including Madagascar, the Malay Peninsula, and Indonesia.

MolluscaBase Eds (2023b) stated that the genus *Donax* includes 79 species. Poutiers (1998) identified five species of *Donax* in the Pacific Ocean: *Donax cuneatus* Linnaeus, 1758, *D. deltoides, D. faba, D. incarnatus* Gmelin, 1791, and *D. scortum* Linnaeus, 1758. Thippeswamy and Joseph (1991) reported the presence of *D. incarnatus* at Panambur Beach, India. In addition, another publication by Tan and Low (2013) found two species of the genus *Donax* in Singapore waters, namely *D. cuneatus* and *D. faba*. Signorelli and Printrakoon (2019) discovered eight species of the genus *Donax* in Thai waters, including *D. spinosus* Gmelin, 1791, *D. semigranosus* Dunker, 1877, *D. incarnatus*, *D. scortum*, *D. cuneatus*, *D. faba*, *D. solidus* Spengler, 1798, *D. introradiatus* Reeve, 1855. Furthermore, several studies reported the presence of *Donax* clams in Indonesian waters. Ambarwati and Faizah (2017) found *D. faba* and *D. cuneatus* on Nepa Beach, Madura Island, Indonesia. Atlanta et al. (2022) reported the presence of *D. faba* on Kutang Beach, Lamongan, Indonesia.

Donacidae is known for its rich diversity in species numbers and morphological aspects, including variations in shapes and color patterns (Ambarwati and Faizah 2017). Tan and Low (2013) stated that the color variations in *D. cuneatus* in Singapore range from white, cream, brown, and gray and may exhibit radial band patterns. They further reported that *D. faba* in Singapore displays variability in shell shape and coloration patterns.

Morphological analysis of bivalve shells plays an important role in various fields, such as taxonomy and evolution (Signorelli et al. 2013). Shells are often the most noticeable and diverse features (Signorelli et al. 2013). Due to

environmental constraints or selective pressures, their variation is attributed to additive genetic variation or phenotypic plasticity (Vieira et al. 2016). The high shell variation within the genus *Donax* poses morphological identification challenges, thereby necessitating molecular identification methods. Sari et al. (2021) stated that molecular studies are needed to strengthen the accuracy of identifying a species and to support morphological identification results. DNA barcoding based on mitochondrial DNA (mtDNA) is considered one of the most effective molecular tools for species identification (Hassan et al. 2022). The genetic or molecular study, especially DNA barcoding, has played a vital role in resolving various taxonomic issues.

One of the molecular markers used for species identification is the Cytochrome C Oxidase subunit I (*COX1*) gene. According to Hoy (2013), DNA barcoding is a standardized method of identifying organisms based on a short fragment of genomic DNA. This technique has been developed by taxonomists, ecologists, conservation biologists, and regulatory agencies. The *COX1* gene, one of the protein-coding genes in mtDNA, has several advantages, such as fewer deletions and insertions in nucleotide base sequences and higher genetic variation than other mitochondrial genes 16s rRNA and 12s rRNA. For example, Sari et al. (2021) used the *COX1* gene to identify characteristics of *D. faba* and obtained a similarity value ranging from 72.01% to 72.12%.

Based on a preliminary survey at Badur Beach of Madura Island, Sumenep District, East Java Province, a population of *D. incarnatus* was found. However, this finding requires verification, as there have been no published reports on the presence of *D. incarnatus* on Madura Island. Therefore, this study aimed to analyze the morphological and molecular characteristics of *D. incarnatus* discovered at Badur Beach, Madura Island, Indonesia, using the *COX1* gene. Unfortunately, no information on the genetic characterization of *D. incarnatus* based on the *COX1* gene is available. Characteristics to be studied include variations in nucleotide bases, genetic distance, and their relationship. This genetic data is used to strengthen the morphological data of *D. incarnatus*.

MATERIALS AND METHODS

Study area

Samples of *D. incarnatus* were collected from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia (6°52'42"S 113°58'46"E) (Figure 1) using the free sampling method by hand picking. Badur Beach is characterized by a smooth white sand substrate, with rocks in the coastal area drained with fresh water. The samples obtained were placed in 40 mL collection bottles filled with absolute ethanol, each labeled accordingly (Sari et al. 2021). Three variations of *D. incarnatus* were sampled, with one variation for each individual. These variations' exterior shell color characteristics include blackish-purple, yellowish-orange, and white.

Procedures

Morphological identification

Morphological identification of the samples was conducted based on the references provided by Poutiers (1998) and Signorelli and Printrakoon (2019). Both qualitative and quantitative characteristics were observed to determine the characteristics of each variation. Qualitative and quantitative characteristics were obtained through visual observations and measuring shell dimensions using a caliper. The quantitative characteristics include Shell Length (SL), Shell Width (SW), Dorsal Height (DH), Shell Height (SH), and Umbo Margin Line (UML) (Figure 2), where the morphometric data was measured. Meanwhile, the qualitative characteristics observed included shell shape, shell color, shell carving, ligament color, muscle attachment marks, umbo location, umbo color, pallial lines, pallial sinuses, and the number of cardinal and lateral teeth (Ambarwati and Faizah 2017).

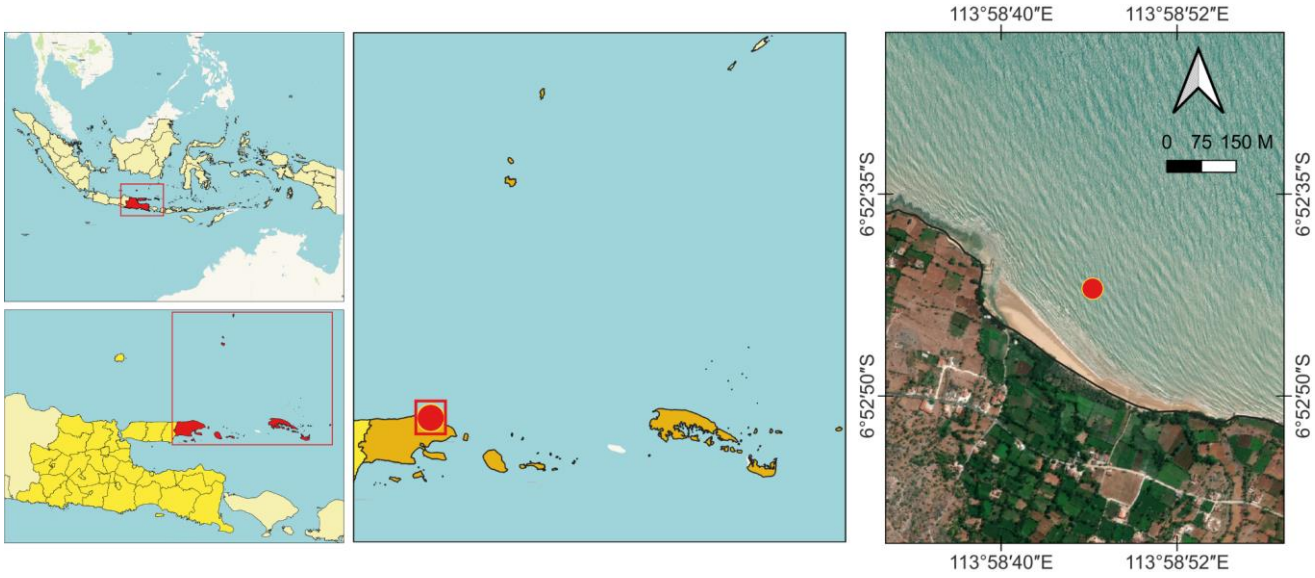

Figure 1. Study area on Badur Beach, Madura Island, Indonesia

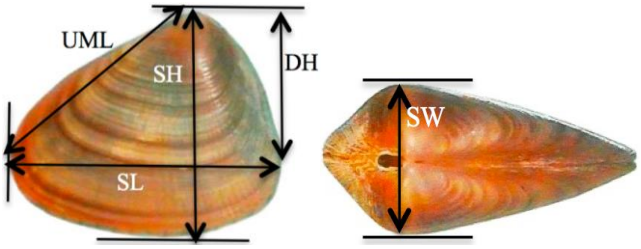

Figure 2. Morphometric measurements. SL: Shell Length, SW: Shell Width, SH: Shell Height, DH: Dorsal Height, UML: Umbo Margin Line

DNA isolation

DNA extraction process from *D. incarnatus* tissue samples using the NEXprep DNA Mini Kit involves several steps. Initially, 0.05 g of muscle tissue is ground in a mortar pestle until it becomes homogeneous, intending to disrupt the cell membrane to release DNA from the cell nucleus. The resulting sample is transferred to a collection tube using a spatula spoon. Subsequently, 200 µL of Buffer GT1 is added to the tube, and the mixture is homogenized using a vortex machine. Then, 200 µL of Buffer GT2 and 200 µL of Proteinase K are mixed using a vortex machine. The mixture is then incubated at 56°C for 10 minutes. After the incubation, 200 µL of absolute ethanol is added, and the sample is remixed using a vortex machine. The sample is then transferred to a spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through is discarded, and the spin column is reassembled. The purification process begins by adding 500 µL of Buffer W1 to the spin column and centrifuging it at 13,000 rpm for 1 minute. The flow-through is discarded, and the spin column is reassembled. Next, 700 µL of Buffer W2 is added to the spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through is discarded again, and the spin column is reassembled and centrifuged for 2 minutes at 13,000 rpm. Next, DNA bound to the column is eluted with 100 µL of Elution Buffer preheated to 70°C and centrifuged at 13,000 rpm for 1 minute to remove contaminants. The resulting DNA is then stored at -20°C until it is needed for subsequent experiment stages (Juniar et al. 2021).

Amplification of the COX1 gene

The PCR technique was employed to amplify the *COX1* gene, using a pair of primers, namely LCO: (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO: (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') that targeted the *COX1* gene (Folmer et al. 1994). The PCR reaction was performed in a volume of 30 µL, adjusted based on the amount of total DNA obtained, and underwent 40 cycles of amplification. The amplification process included initial denaturation, denaturation, annelation, extension, and final extension at 94°C, 94°C, 45°C, 72°C and 72°C for 1 minute, 45 seconds, 45 seconds, 1 minute 30 seconds, and 10 minutes, respectively (Juniar et al. 2021).

Electrophoresis

Furthermore, 0.2 g of agarose was mixed with 20 mL of 0.5x TBE Buffer to prepare the electrophoretic medium to make a 1% agarose gel. The mixture was stirred using a magnetic stirrer for 5 minutes, poured into an agarose gel slab, and left to solidify for 20 minutes. Next, 3 µL of sample amplicon was mixed with 1 µL of loading dye and 2 µL of distilled water to make a total volume of 6 µL, and the mixture was loaded into the agarose well. Electrophoresis was performed at 48 V and 0.5 A for 20 minutes, and DNA base strand length was determined by comparison with a 100bp low mass ladder loaded in the first well. A 1.5% agarose gel electrophoresis medium was prepared for the next stage by mixing 0.3 g agarose with 30 mL of 0.5x TBE Buffer with a magnetic stirrer for 5 minutes. The mixture was poured into the agarose wells and left to solidify for 20 minutes. Next, the template DNA was added with 2 µL of NEXview Nucleic Acid Stain. Then, 3 µL of PCR results were mixed with 1 µL of loading dye and 2 µL of distilled water to make a total volume of 6 µL, and the mixture was loaded into the agarose well. Again, electrophoresis was performed, and the resulting bands were visualized using a UV transilluminator. Finally, the sample's single and thick band characteristics were produced (Juniar et al. 2021).

Sequencing

The Sanger method (1977) was used for DNA sequencing, performed by a sequencing company with the PCR products sent to First Base, Malaysia. The resulting chromatograms were analyzed using bioinformatics software, including Finch TV (Bhat et al. 2019), DNA Baser Assembler v5.15.0, Clustal X Version 2.1 (Ferrari and Patrizio 2021), BioEdit Version 7.0.5.3, and MEGA 6.0 (Tamura et al. 2013). DNA sequences obtained were used to determine genetic variation, nucleotide and amino acid composition, genetic distance, and relationships within the *COX1* gene.

Data analysis

Morphological character analysis

The morphological character analysis is a method that utilizes descriptive data analysis techniques to provide a clear and distinct differentiation of obtained study data. This technique involves the comparison of descriptions, distributions, and pictures with identification books to identify morphological of a specimen. The analysis of morphological characters yields descriptive paragraphs that include the specimen's collection number, description, and habitat profile.

Molecular character analysis

Data were collected in the form of the *COX1* gene sequences from *D. incarnatus* clams and descriptively analyzed using the FinchTV program (Bhat et al. 2019), which produced good nucleotide base sequences. The good chromatogram results were then copied using the DNA Baser Assembler program. Subsequently, the nucleotide bases were compared to the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/) (Ferrari and Patrizio 2021), which yielded information regarding the nucleotide identity and similarity between the query and the hit. The nucleotide identity was then translated into an amino acid sequence using Expasy (https://web.expasy.org/translate/), resulting

in the *D. incarnatus COX1* gene protein sequence. The amino acid sequences were then analyzed with the BOLD System (https://www.boldsystems.org/) to check for homology, using GenBank to compare with related species. Next, the sequence results were aligned with ClustalX (Ferrari and Patrizio 2021) to create multiple alignments between the *COX1* gene samples and the closely related *D. incarnatus* database collected from Badur Beach, Madura Island, Indonesia. Finally, the alignment sequences were analyzed with BioEdit, producing DNA sequences in fasta format. A phylogenetic tree was then obtained using MEGA 6.0.

RESULTS AND DISCUSSION

Morphological character

The sampling results conducted on Badur Beach, Madura Island, Indonesia, revealed that 68 individuals of *D. incarnatus* variation had blackish purple exterior shell color, and 19 of the same species had yellowish-orange exterior shell color. In contrast, four had white exterior shell color (Figure 3).

Donax incarnatus OQ692130.1 (*Gmelin, 1791*) (*Figure 2A*)

Collection number. D1

Description. The shell is characterized by its triangular shape and blackish-purple color. It has finely carved concentric radials and measures approximately 15.4±0.45 mm, 12.6±0.39 mm, and 6.4±0.25 mm in length, height, and width, respectively. The dorsal height is 9.5±0.30 mm. The umbo margin line is about 14.5±0.32 mm. The umbo is opisthogyrate, prominent, and blue. The antero-dorsal margin is long and straight, while the anterior end is rounded. The ventral and postero-dorsal margins are slightly convex and serrated. The shell has a short ligament that is blackish-brown in color and visible pallial lines. There is a deep pallial indentation, and the shell has two lateral and cardinal teeth.

Habitats. These varieties were uncovered in the intertidal zone, buried in a fine sandy substrate at Badur Beach, Madura Island, Indonesia.

Donax incarnatus OQ692131.1 (*Gmelin, 1791*) (*Figure 2B*)

Collection number. D2

Description. The shell displays a triangular shape and exhibits a yellowish-orange color. Its finely carved concentric radials are evident, and it measures approximately 12.8±0.15 mm, 10.4±0.26, and 5.9±0.15 mm in length, height, and width, respectively. The dorsal height measures relatively 5.7±0.09 mm. The umbo margin line is about 12.2±0.17 mm. The umbo is opisthogyrate, prominent, and orange in color. The antero-dorsal margin is long and straight, with a rounded anterior end. The ventral and postero-dorsal margins are slightly convex and serrated. The shell has a short ligament that is blackish-brown in color and visible pallial lines. There is a deep pallial indentation, and the shell has two lateral and cardinal teeth.

Habitats. These varieties were buried in the intertidal zone on a fine sandy substrate at Badur Beach, Madura Island, Indonesia.

Donax incarnatus OQ692132.1 (*Gmelin, 1791*) (*Figure 2C*)

Collection number. D3

Description. The shell exhibits a triangular shape and displays a white color. It has finely carved concentric radials, with approximately 14.2±0.9 mm, 7.7±0.5 mm, and 4.1±0.05 mm in length, height, and width, respectively. The dorsal height measures around 5.7±0.9 mm. The umbo margin line is approximately 8.2±0.2 mm. The umbo is opisthogyrate, prominent, and white. The antero-dorsal margin is long and straight, with a rounded anterior end, while the ventral and postero-dorsal margins are slightly convex and serrated. The shell has a short ligament that is blackish-brown in color, with visible pallial lines. In addition, there is a deep pallial indentation with two lateral and cardinal teeth.

Habitats. The varieties above were found buried on a fine sandy substrate within the intertidal zone of Badur Beach, situated on Madura Island, Indonesia.

Donax incarnatus clam exhibits a triangular shell shape with finely carved concentric radials. Additionally, it has a prominent umbo that is opisthogyrate, a long and straight antero-dorsal margin, and ventral and postero-dorsal margins that are slightly convex and serrated. The clam also possesses a rounded anterior end, short ligament, and visible pallial lines. There is a deep pallial dent with two lateral and cardinal teeth. The morphological characteristics of the *D. incarnatus* sample align with a previous study (Poutiers 1998; Signorelli and Printrakoon 2019).

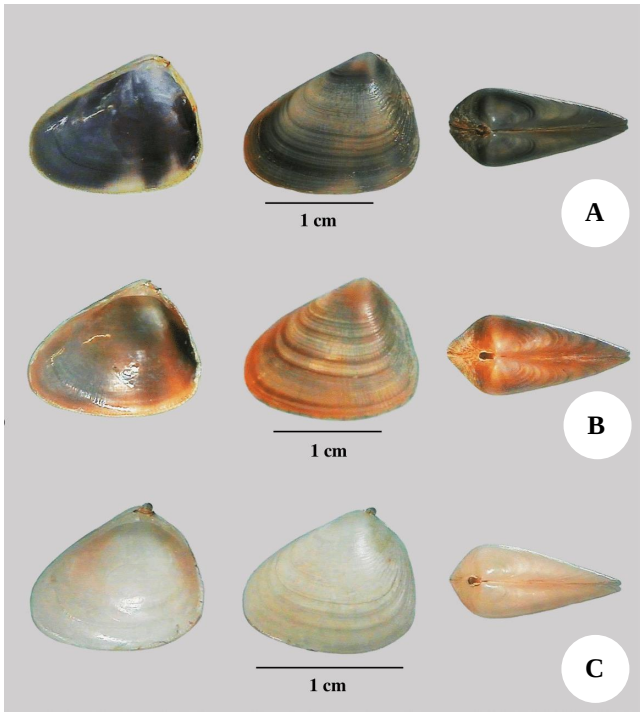

Figure 3. Variation of *Donax incarnatus* from Badur Beach, Madura Island, Indonesia

The coloration pattern of the shell is secreted by the outer layer of the mantle and is determined by the pigmentation of the neurosecretory. Three main shell pigment classes are melanins, tetrapyrroles, and carotenoids (Williams 2016). Many factors control the shell color, such as genetic, biotic, and abiotic factors like food, temperature, salinity, or pH. Despite recent efforts, a knowledge gap exists in understanding the genetic underlying shell color in mollusks (Williams et al. 2017). Cen et al. (2023) recently analyzed the differences in shell color morphology in *Charonia tritonis* Linnaeus, 1758 (Mollusca: Gastropoda). It was reported that no genetic differences were detected in the various shell color patterns. Therefore, the dissimilarities in shell color in *C. tritonis* are likely due to phenotypic plasticity caused by environmental factors or the presence of genetic differences without forming a separate related structure

The sampling results suggest that the habitat of *D. incarnatus* is in the intertidal zone buried in fine sand substrates. This is consistent with the study conducted by Signorelli and Printrakoon (2019) that these species prefer such habitats. Wagey et al. (2018) stated that both biotic and abiotic factors influence the abundance and diversity of mollusks. One critical factor is the substrate, which provides a place for mollusks to live. Zhang et al. (2022) further emphasized the importance of substrate in determining the growth, survival, population, and distribution of mollusks dwelling in mudflat environments.

Identification BOLD system

However, using Expasy online, 486 nucleotide bases among the three sequenced samples were successfully translated into protein without a stop codon in the middle of the sequence, constituting a pseudogene (Duvaud et al. 2021). The resulting protein contained 162 amino acids without any insertions or deletions. The nucleotide bases were further analyzed through the BOLD System for online identification (Table 1), using the Kimura 2 parameter model to calculate the sequence similarities. The similarities of *D. incarnatus* OQ692130.1, *D. incarnatus* OQ692131.1, and *D. incarnatus* OQ692132.1 to the BOLD data of *D. incarnatus* were categorized as extremely high, ranging from 96.06% to 99.61%. The absolute similarity between *D. incarnatus* from Badur Beach, Madura Island, Indonesia, was attributed to the *COX1* barcode sequence high homology with the BOLD System database.

The BOLD system was used to genetically identify *D. incarnatus* based on the *COX1* gene, and a high similarity value was obtained. The results of the three samples collected from Badur Beach showed a similar percentage of 96.06 to 99.61, as shown in Table 1. According to Juniar et al. (2021), a similarity range of 89 to 100% indicates that the species are identical or similar. Therefore, based on the high similarity values obtained from the three study samples, it was concluded that they belong to the *D. incarnatus* species.

Composition of nucleotide bases

The average G+C and A+T nucleotide base compositions of the three samples were 34.91%, and 65.09%, respectively. These results demonstrate that the A+T nucleotide base composition is higher than the G+C, as shown in Table 2.

Table 1. The three species with the highest match from the identification through the BOLD System with the representation of similarity values

Sample	Phylum	Class	Order	Family	Genus	Identification BOLD	Similarity (%)	Status	ACC number
Donax incarnatus	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	99.17	Published	ACQ5978
OQ692130.1	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	99.01	Published	ACQ5978
(*this study*)	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	98.68	Published	ACQ5978
Donax incarnatus	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	99.61	Published	ACQ5978
OQ692131.1	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	99.42	Published	ACQ5978
(*this study*)	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	99.03	Published	ACQ5978
Donax incarnatus	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	96.58	Published	ACQ5978
OQ692132.1	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	96.40	Published	ACQ5978
(*this study*)	Mollusca	Bivalvia	Cardiida	Donacidae	*Donax*	*incarnatus*	96.06	Published	ACQ5978

Table 2. Composition of nucleotide bases of *Donax incarnatus* at Badur Beach, Madura, Indonesia

Sample	A (%)	C (%)	G (%)	T (%)	G+C (%)	A+T (%)
Donax incarnatus OQ692130.1 (*this study*)	24.49	16.46	18.52	40.53	34.98	65.02
Donax incarnatus OQ692131.1 (*this study*)	24.69	16.26	18.72	40.33	34.98	65.02
Donax incarnatus OQ692132.1 (*this study*)	24.69	16.05	18.72	40.53	34.77	65.23
Average	24.62	16.26	18.65	40.46	34.91	65.09

Note: A: Adenin, C: Sitosin, G: Guanin, T: Timin

DNA sequence analysis of *D. incarnatus* samples obtained from Badur Beach, Madura, indicated that the three samples' nucleotide base composition was similar, suggesting they belonged to the same species. The average nucleotide base composition of the *D. incarnatus* sample revealed a lower percentage of G+C nucleotide bases than the A+T, as shown in Table 2. These findings are consistent with the mitochondrial base composition characteristics (Cen et al. 2023).

Variation of nucleotide bases

The alignment stage of all samples (including the study sample with GenBank NCBI) revealed 486 bp with five nucleotide base variations and 481 bp of conserved nucleotide bases. Nucleotide base variations were found in sequence numbers 2, 62, 143, 170, and 222. These variations occurred due to changes in nucleotide bases, either transition or transversion. Nucleotide base variations were compared between the ingroup *D. incarnatus* from Thailand and the study sample from Badur Beach, Madura. Specifically, *D. incarnatus* OQ692131.1 exhibited a transitional change at nucleotide base number 2, where thymine (T) was replaced by cytosine (C). *Donax incarnatus* OQ692130.1 showed a transversion change at nucleotide base number 62, where adenine (A) was replaced by thymine (T). Additionally, *D. incarnatus* OQ692130.1 exhibited a transition change at nucleotide base number 143, where thymine (T) was replaced by cytosine (C). *Donax incarnatus* OQ692132.1 had a transversion change at nucleotide base number 170, where thymine (T) was replaced by guanine (G). Lastly, *D. incarnatus* OQ692130.1 displayed a transversion change at nucleotide base number 222, where guanine (G) was replaced by cytosine (C), as shown in Table 3.

The alignment results (Table 3) showed that 486 bp of nucleotide bases were obtained, and five nucleotide base variations were observed at positions 2, 62, 143, 170, and 222. Nucleotide base variations can occur through two types of mutations: transitions and transversions (Sari et al. 2021). A transition mutation occurs when another purine or pyrimidine base substitutes a purine or pyrimidine base. In contrast, in the case of a transversion, purine is replaced by a pyrimidine or vice versa (Sari et al. 2021). The obtained *COX1* gene sequences were free from pseudogenes, indicating that the sequences have the potential to serve as standard barcode identification for *D. incarnatus* in Indonesian waters. Ambarwati et al. (2021) stated that diagnostic nucleotide bases, along with simple diagnostic nucleotide characters, are essential for species identification using DNA barcoding. Additionally, the *COX1* gene showed high genetic variation within species.

Genetic distance

The range of genetic distance values can be used to differentiate between interspecies and intraspecies relationships. The Kimura 2 parameter model was used to calculate the genetic distance between samples of *D. incarnatus* and its close relatives in the Donacidae family. The genetic distance value for *D. incarnatus* OQ692130.1 was 0.010, the closest distance to *D. incarnatus* MT334593.1. The genetic distance value for D. incarnatus OQ692131.1 was 0.006, the closest distance to *D. incarnatus* MT334593.1. The genetic distance value for *D. incarnatus* OQ692132.1 was 0.006, the closest distance to *D. incarnatus* MT334592.1. The average genetic distance among *D. incarnatus* individuals from Badur Beach, Madura, was 0.005 (Table 4). The average genetic distance between the study sample and the ingroup was 0.01, while between the study sample and the outgroup was 0.427, as shown in Table 4.

The degree of relatedness between the samples and the NCBI GenBank data can be determined by values of genetic distances shown in Table 4. Intragroup genetic diversity tends to affect the average genetic distance, with a value of less than 2% indicating the same species. However, when the value exceeds 2%, it indicates a different species (Ambarwati et al. 2021). High genetic distance helps in identification at the family or genus level (Ran et al. 2020). Environmental factors and over-exploitation can influence the diversity of genetic distances (Chiu et al. 2013). Geographic location and environmental conditions can also lead to changes in morphological and phylogenetic populations (Twindiko et al. 2013).

Table 3. Variation of nucleotide bases from *Donax incarnatus* based on the *COX1* gene

Sample	2	62	143	170	222
Donax incarnatus MT334591.1	T	A	T	T	G
Donax incarnatus MT334592.1	●	●	●	●	●
Donax incarnatus MT334593.1	●	●	●	●	●
Donax incarnatus OQ692130.1 (*this study*)	●	T	C	●	C
Donax incarnatus OQ692131.1 (*this study*)	C	●	●	●	●
Donax incarnatus OQ692132.1 (*this study*)	●	●	●	G	●
Donax obesulus MH194540.1	●	●	●	A	●
Donax obesulus MH194549.1	●	●	●	A	●
Donax fossor MW628259.1	●	●	●	G	●
Donax fossor MW628283.1	●	●	●	G	●
Donax faba MT334598.1	●	G	A	●	●
Donax faba MT334599.1	●	G	A	●	●
Donax cuneatus MT334594.1	●	G	●	●	●
Donax cuneatus MT334595.1	C	G	●	●	●
Arctica islandica KR084734.1	●	●	G	●	T
Arctica islandica KR084887.1	●	●	G	●	T

Note: (●) is a conserved nucleotide base

Table 4. Genetic distance between groups (OTU) of bivalves of the Donacidae family based on the *COX1* gene barcode sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Donax incarnatus MT334591.1															
Donax incarnatus MT334592.1	0.008														
Donax incarnatus MT334593.1	0.006	0.002													
Donax incarnatus OQ692130.1 (*this study*)	0.017	0.012	**0.010**												
Donax incarnatus OQ692131.1 (*this study*)	0.012	0.008	**0.006**	0.008											
Donax incarnatus OQ692132.1 (*this study*)	0.012	**0.006**	0.008	0.004	0.002										
Donax obesulus MH194540.1	0.237	0.234	0.234	0.248	0.243	0.240									
Donax obesulus MH194549.1	0.237	0.234	0.234	0.242	0.237	0.234	0.004								
Donax fossor MW628259.1	0.230	0.224	0.224	0.236	0.230	0.224	0.197	0.195							
Donax fossor MW628283.1	0.227	0.222	0.222	0.233	0.227	0.222	0.195	0.192	0.006						
Donax faba MT334598.1	0.234	0.222	0.225	0.234	0.234	0.234	0.254	0.254	0.269	0.278					
Donax faba MT334599.1	0.234	0.222	0.225	0.234	0.234	0.234	0.254	0.254	0.269	0.278	0.000				
Donax cuneatus MT334594.1	0.214	0.211	0.214	0.219	0.217	0.216	0.251	0.251	0.268	0.268	0.204	0.204			
Donax cuneatus MT334595.1	0.198	0.201	0.198	0.203	0.195	0.201	0.247	0.241	0.239	0.233	0.198	0.198	0.158		
Arctica islandica KR084734.1	0.419	0.416	0.416	0.427	0.427	0.426	0.473	0.473	0.491	0.499	0.511	0.511	0.480	0.493	
Arctica islandica KR084887.1	0.419	0.416	0.416	0.427	0.427	0.426	0.473	0.473	0.491	0.499	0.511	0.511	0.480	0.493	0.00

Note: The highlight section shows the closest genetic distance between the study sample and the ingroup

Phylogenetic tree

Phylogenetic trees were constructed using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods with the Kimura 2 parameter model. The resulting phylogenetic topology for *D. incarnatus* was based on forming two subclusters. The first cluster included *D. obesulus*, *D. faba*, *D. fossor*, and *D. cuneatus* from Peru, Thailand, the USA, and Thailand, respectively. The second cluster consisted of *D. incarnatus* from Thailand and Badur Beach, Madura, respectively, with a bootstrap value of 100/99 (Figure 4).

Phylogeny explores the evolutionary relatedness among groups of organisms and provides a hypothesis about their history and relationships (Mittal et al. 2013; Kanojia et al. 2019). A phylogenetic tree visually represents the estimated relationship among taxa (or sequences) and their hypothetical common ancestors (Hall 2013). Therefore, bootstrap analysis is employed to assess the homogeneity of clusters in a phylogenetic tree, and it is also used for cluster validation of branch arrangement (Studer 2021). In addition, determining the number of repetitions in sequence alignment using the bootstrap method improves the accuracy of identifying possible species relationships in a phylogenetic tree (Hikam et al. 2021).

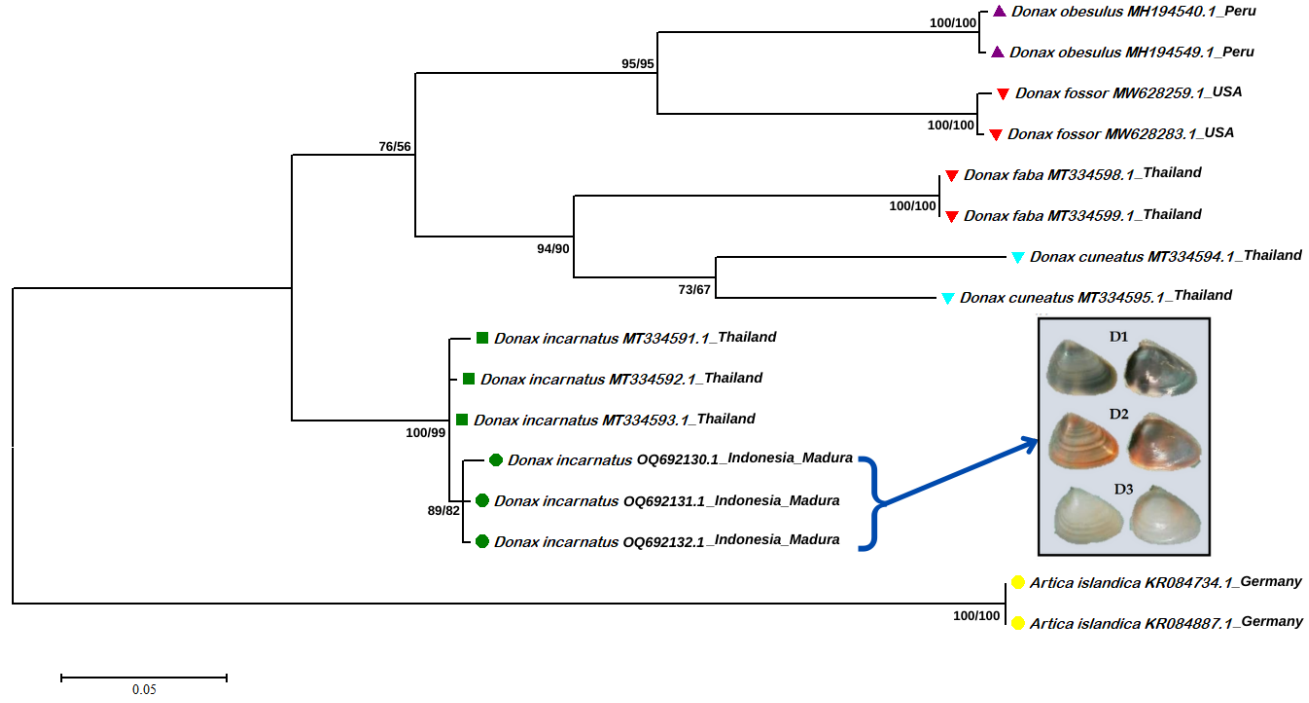

Figure 4. Phylogenetic tree of Donacidae family from Pantai Badur, Sumenep, Madura, Indonesia regarding the *COX1* gene from GenBank NCBI (NJ and ML with 1000 bootstrap replications)

NJ method is a popular tool for constructing phylogenetic trees, relying on genetic distance between species as a basis (Kanojia et al. 2019; Hong et al. 2021). According to Hong et al. (2021), this method is widely used due to its few assumptions, fast operations, and high accuracy. In addition, the method is based on the distance between taxa. For example, using the NJ method to construct a phylogenetic tree (Figure 4) revealed that the sample obtained from Badur Beach was in the same clade as *D. incarnatus* from Thailand with a bootstrap value of 100. A bootstrap value of ≥70 is usually considered well-supported or reliable and corresponds to the real clade with an extremely high probability (Jackson et al. 2021; Khan et al. 2022).

ML method is used to investigate the sequence variations that occur due to mutations. This method attempts to maximize the probability that the observed data will fit onto a tree under a given evolutionary model. However, it requires specific assumptions about sequence evolution, such as mutation rates (Bawono and Heringa 2014). The phylogenetic tree constructed using the ML method (Figure 4) shows that the study sample obtained from Badur Beach and Thailand belongs to the same clade with a bootstrap value of 99. A bootstrap value of 70 or more is considered reliable for grouping species (Khan et al. 2022).

The study presented various genetic characterizations and molecular taxonomy of *D. incarnatus* from Badur Beach, Madura, which can serve as crucial information and helps future investigations.

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