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DNA barcoding *Clithon* sp. (Gastropoda: Neritidae) from Badur Beach, Madura, Indonesia, based on COI gene molecular marker

INTAN SALSABILA DJOEMHARSJAH, RENI AMBARWATI, DWI ANGGOROWATI RAHAYU♥
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya. Jl. Ketintang, Surabaya 60231, East Java, Indonesia.
Tel./fax.: +62-31-8296427, ♥email: dwirahayu@unesa.ac.id

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Abstract. *Djoemharsjah IS, Ambarwati R, Rahayu DA. 2023. DNA barcoding* Clithon *sp. (Gastropoda: Neritidae) from Badur Beach, Madura, Indonesia, based on COI gene molecular marker. Biodiversitas 24: 2779-2787.* Neritidae is polymorphic with various shell colors and patterns; therefore, they were doubts about the identifying species from the genus *Clithon*, namely *Clithon* sp. from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia. In addition, DNA barcoding could provide genetic information using short DNA sequences to quickly and precisely identify species. This study aimed to identify the genus *Clithon* sp. from Badur Beach, based on Cytochrome Oxidase subunit I (COI) genes and analysis of phylogenetic relationships. The research methods include sampling, sample preservation, morphological identification of species, DNA isolation, amplification, electrophoresis, and sequencing using the Sanger method with genetic analysis using bioinformatics software. The results of COI barcode identification obtained a DNA sequence length of 490 bp with a similarity value of the three *Clithon* sp. samples between 96.75 to 98.97%. The identification with the Barcode of Life Data System consisted of three variations of nucleotide bases, and the average value of the genetic distance with the in-group was 1.74% as *Clithon* sp. The Phylogenetic tree *Clithon* sp. from Badur Beach, was in the same clade as *Clithon sowerbianum* Récluz 1843 and *Clithon mertonianum* Récluz 1843 with the Neighbor-Joining Tree and Maximum Likelihood methods with bootstrap values between 96-100. Therefore, the COI barcode DNA markers analysis successfully identified *Clithon* sp. from Badur Beach, Madura, Indonesia as *C. sowerbianum.*

Keywords: DNA barcode, molecular identification, phylogenetic, similarity

INTRODUCTION

Neritidae can live in marine, brackish, and freshwater habitats (Marković et al. 2014). Park et al. (2016) stated that Neritid gastropods are generally attached to hard substrates (e.g., rocks, woods, and artificial materials), so it's unusual for neritids to attach to algae (except for *Smaragdia* species living on seagrasses). Instead, neritids feed primarily on small algae on hard substrates, i.e., large algae enough to attach on. The shape of the shell identifies Neritidae snails with large body whorl, coiled, and short whorl units (Killburn 2000). Members of Neritidae are often polymorphic, meaning that one species can have various shell colors and patterns (Tan and Clements 2008).

Members of the Neritidae are divided into 13 genera within the present classification, including the validity of three genera, namely *Neritodryas*, *Smaragdia*, and *Septaria* (Eichhorst 2016). Killburn (2000) also states that members of the Neritidae are divided into three genera: *Clithon*, *Nerita*, and *Neritina* (Killburn 2000). Based on research conducted by Hylleberg (2000), in Vietnam and Cambodia, members of the Neritidae are found, namely three species from the genus *Clithon*, nine species from the genus *Nerita* and two species from the genus *Neritina*. Dharma (2005) also reported that in Indonesia, the members of the Neritidae found in freshwater consist of 17 species, and 21 live in the sea, some of which are the genera *Clithon*, *Nerita*, and *Neritina*. One member of the Neritidae that is abundant in Java is *Clithon*, a group of snails that are small to medium in size and live in freshwater, estuary, and marine waters (Tan and Clements 2008).

Djoemharsjah et al. (2023) reported a population of Neritidae snails from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia, which consists of six species from the genus *Clithon*: *Clithon* sp., *Clithon diadema* Récluz 1841, *Clithon oualaniense* Lesson 1831, *Clithon faba* G.B.Sowerby I 1836, *Clithon bicolor* Récluz 1843, and *Clithon tritonense* Le Guillou 1841. However, this study expected difficulties in morphological identification. Thus, morphological identification must be strengthened to obtain more information about the species studied. Furthermore, several methods can be used to obtain accurate information in molecular identification. Therefore, Juniar et al. (2021) stated that molecular-based identification could strengthen identification based on morphological characteristics.

Molecular species identification can be used as molecular markers; DNA barcoding is often used. Furthermore, the DNA barcode technique was fast and simplified; it is easier to identify an organism using standardized DNA sequences (Antil et al. 2022). In addition, the DNA barcoding technique can identify a species in various taxa that may be difficult to distinguish morphologically (Bingpeng et al. 2018). DNA barcoding targets for animals use markers from mitochondrial DNA, namely the Cytochrome Oxidase subunit I (COI) gene (Juniar et al. 2021).

The COI gene could be used to determine the *Clithon* species genus with one of the protein-coding gene sequences

in mitochondrial DNA (mtDNA). Similar research has been conducted by Juniar et al. (2021) regarding the molecular identification of the COI gene in specimens from the northern coast, Madura, Indonesia which shows a species similarity level of *C. oualaniense* at 98.26-100%. In addition, molecular identification using the COI gene in Neritidae members has been carried out by Rabi et al. (2020) on the Mediterranean coast, Israel, to a high level of similarity of species from *Nerita sanguinolenta* Menke 1829 at 99.04-99.36%.

Based on previous studies, scientific information regarding identifying *Clithon* sp. found at Badur Beach, Madura, must be observed. That identification could use molecular markers, especially the COI gene, to reinforce the morphological data needs to be done. In addition, analyzing phylogenetics to determine the relationships between *Clithon* sp. concerning Genbank NCBI (National Center for Biotechnology Information) is also very important. Therefore, the study aimed to identify *Clithon* sp. from Badur Beach, using a molecular marker, namely the COI gene, to characterize their biodiversity and conservation.

MATERIALS AND METHODS

Study area

This research is a qualitative descriptive study using the observation method. This research was conducted from October 2022 to January 2023. Samples were collected from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia (6°52'42"S 113°58'46" E) using a purposive sampling method according to the *Clithon* sp. type; only taking the basic motif is orange, it has grooves with black dots, white subepidermal, and black reticulate patterns and the operculum is yellow to gray (Djoemharsjah et al. 2023) (Figure 1).

Procedures

DNA isolation

Moreover, DNA isolation using NEXprep Cell/Tissue DNA Mini Kit. First, foot tissue *Clithon* sp. 0.05 g was chopped until it was smooth using a mortar pestle. Next, the smooth foot tissue was taken using a spatula and put into a collection tube; then added with 200 µL Buffer GT1 and homogenized using a vortex. Then, the lysis stage was carried out by adding 200 µL Buffer GT2 and 20 µL Proteinase K into the sample and mixing it using a vortex. Next, the incubation was carried out in a water bath at 56ºC for 10 minutes. Next, the binding stage was conducted by adding 200 µL of absolute ethanol to the sample and mixing it with a vortex. Then, the sample was put into the spin column and centrifuged at 13,000 rpm for 1 minute. Then, the flow-through is discarded, and the high filter tube is reattached to the collection tube. Next, the purification or washing stage was carried out by adding 500 µL of Buffer W1 to the spin column and centrifuging at 13,000 rpm for 1 minute. Then, the flow through is removed, and the high filter tube is reattached to the collection tube. Next, 700 µL of Buffer W2 was added to the spin column and centrifuge at 13,000 rpm for 1 minute. Then the flow through was discarded, and the high filter tube was attached back to the collection tube and centrifuge at 13,000 rpm for 2 minutes. Finally, the elution stage was carried out by adding 100 µL of elution buffer, and the DNA sample was incubated simultaneously at 70°C for 1 minute and then centrifuged at 13,000 rpm for 1 minute. The resulting DNA can be stored at -20ºC for the next stage.

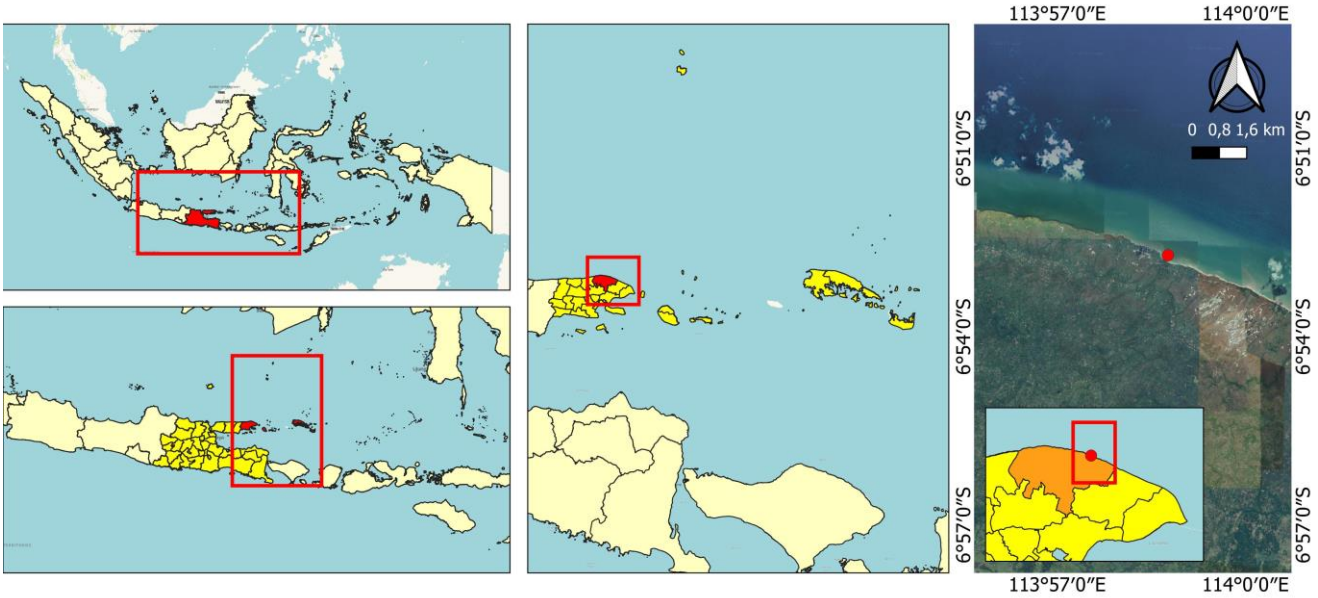

Figure 1. Sampling Locations on Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia

Amplification of the COI gene

DNA amplification was conducted with a Thermal Cycler using a pair of primers with the COI gene target "LCO1490" and "HCO2198" (Folmer et al. 1994). PCR method by Thermal Cycler used is the hot-start method using PCR master mix (2x Mytaq HS Red Mix). A repeating principle starts with denaturation, annealing, and extension, which is carried out with an amplification cycle of 40 cycles to get a suitable PCR quality without a smear. Each cycle consisted of a double thread attachment process (pre-denaturation) at 94°C for 1 minute, a denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and an extension at 72°C for 1 minute 30 seconds. Then it proceeded further, with a final extension at 72°C for 10 minutes. The results of the good amplification show the number of identifiable DNA bands.

Electrophoresis

Electrophoresis medium for the results of DNA isolation was prepared with a 1% agarose gel composition (0.2 g agarose and 20 mL 0.5x TBE) mixed using a magnetic stirrer for 5 minutes. Then the medium was put into the gel slab for 20 minutes. The result of the gel slab was then put into the electrophoresis chamber, add 10x TBE into the electrophoresis chamber until it reached a height of 1 mm above the gel. Next, a 3 µL DNA template is mixed with 2 µL loading dye NEXview Nucleic Acid Stain and 1 µL Solution Distilled Water (SDW) on parafilm paper to balance the reaction with a total of 6 µL, and the mixture is then put into the agarose well. The length of the DNA base strands was compared using a 4µL bp Lowmass ladder of 100 bp inserted into the agarose. Next, the machine performed the electrophoresis with a voltage of 48 V and a current of 0.5 A for 20 minutes. Next, the electrophoretic medium for the PCR results was prepared with a 1.5% agarose gel composition (0.3 g agarose and 30 mL 0.5x TBE) mixed using a magnetic stirrer for 5 minutes. Then put it in the gel mold and wait for 20 minutes. The result of the gel mold was put into the electrophoresis chamber, add 10x TBE until it reached a height of 1 mm above the gel. The next step was to mix 3 µL of the amplified DNA with 2 µL of loading dye NEXview Nucleic Acid Stain and 1 µL of Solution Distilled Water (SDW) on parafilm paper to balance the reaction with a total of 6 µL. This mixture was then put into the agarose wells. The length of the DNA base strands can be compared using a 4 µL bp Lowmass ladder of 100bp. The sample band is produced by having the characteristics of a single and thick band.

DNA sequencing

DNA sequencing in the target area of the COI gene was carried out by First Base, Malaysia, using the Sanger method (1977). This DNA sequenced was a pair of primers target "LCO1490" and "HCO2198". The result of sequencing data is base sequences in ABI chromatogram format.

Bioinformatic analysis

The sample DNA sequencing results were then processed by conducting the main analysis, namely the chromatogram, using Finch TV and translating proteins online via the Expasy web (https://web.expasy.org/translate). The Barcode of Life Data System online via the BOLD System web (https://barcodinglife.org) was used to confirm the COI gene sequence results to show the relationship of the sample with the BOLD data. Sequence alignment was conducted between the study sample and the reference sequence from Genbank NCBI using Clustal X version 2.1. The final alignment using Bioedit version 7.0.5.3 to analyze nucleotide base composition on research samples and variations of nucleotide bases between the samples with data from Genbank NCBI. Compilation of phylogenetic tree reconstructed using MEGA 6.0 software to obtain genetic distances and phylogenetic tree. The genetic distance was calculated using the Kimura-2 parameter (K2P) model to obtain the matrix calculation. Phylogenetic tree reconstruction was analyzed using the Neighbor-Joining Tree (NJ) and Maximum Likelihood (ML) methods with a *bootstrap* value of 1,000 replications. Finally, the similarity values were calculated: similarity percentage = (1-Genetic distance) x 100%.

RESULTS AND DISCUSSION

Systematics and description

Based on the identification results of Neritidae snails found on Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia there are *Clithon* sp. with the following morphological description according to research (Djoemharsjah et al. 2023).

Collection number: C1; C2; C3

Shell description. Shell is smooth and shiny; low or conical spire appears increasingly conical in larger shells; the motif on the shell, namely the basic motif is orange; It has grooves with black dots, white subepidermal, and black reticulate patterns. The surface of the columella is convex, has serrations on the columella, the grooves on the columella are small, and the operculum is yellow to gray (Figure 2) (e.g., Eichhorst 2016; Ng et al. 2016, Figure 1-24). Shell length 11.7±0.13 mm, shell width 8.4±0.20 mm, columella width 8.1±0.06 mm, aperture width 6.3±0.27 mm, and aperture height 7.1±0.15mm. Habitat attaches to rocks close to streams.

Visualization of DNA

COI gene target DNA amplification was performed using universal primers, namely target "LCO1490" and "HCO2198". The COI gene targets DNA amplification results were then electrophoresed using 1.5% agarose gel and visualized with a UV-transilluminator. A well-amplified COI gene target was indicated by the presence of thick DNA bands and no smears, with DNA visualization results obtained at 680 bp (Figure 3).

Figure 2. *Clithon* sp. shells on Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia. Note: C1: *Clithon* sp.; C2: *Clithon* sp.; and C3: *Clithon* sp., scale bar: 10 mm)

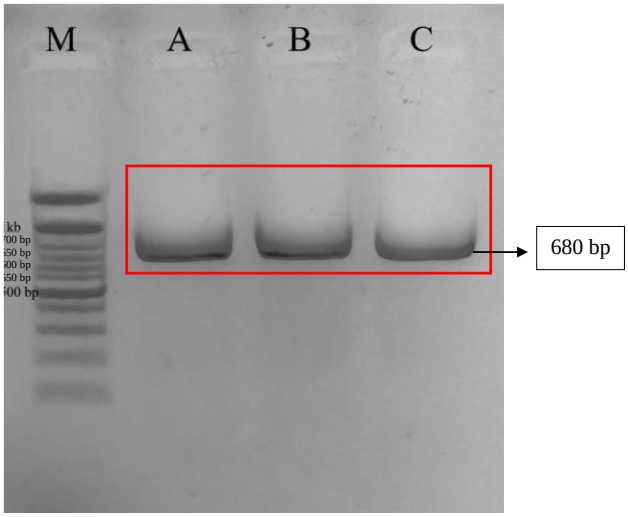

Figure 3. Visualization of DNA specimens of *Clithon* sp. from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia, in a 1.5% agarose gel with a 100bp DNA ladder. Note: M: Marker, A: *Clithon* sp. C1, B: *Clithon* sp. C2, C: *Clithon* sp. C3

Identification using the BOLD System

Clithon sp. samples' nucleotide base has been identified from Badur Beach, Madura, using a 680 bp sequencing length. Next, these sequencing results were processed using Expasy through translation into protein until no stop codon was found in the middle of the nucleotide base. Then the results of Expasy were analyzed using the BOLD System (Table 1) to determine the highest degree of relationship of the research samples. The analysis showed similarities between the three *Clithon* sp. samples with *C. sowerbianum*, namely *Clithon* sp. C3 with very high BOLD System data between 98.77-98.97%, *Clithon* sp. C1 with BOLD System data between 98.56-98.77%, and *Clithon* sp. C2 with BOLD System data between 96.75-97.36%.

The results of the three *Clithon* sp. samples compared to the BOLD System data, sequence similarity with an average high similarity value between 96.75-98.97% (Table 1). This similarity value indicates that these three samples have been identified at the species level. Therefore, species similarities exist between the three *Clithon* sp. samples with *C. sowerbianum.* Bhattacharjee et al. (2012) stated that the similarity value in the BOLD System with a value range of 96-100% is a sequence similarity at the species level, or it can be stated that it is the same species. Therefore, research on molecular identification of *Clithon* sp. could add data on marine biota, especially gastropods of the Neritidae family in Indonesia. It also could add to the DNA barcoding library collection (BOLD System) directly by following the requirements flow from the Genbank NCBI. Those requirements are (i) nucleotide sequences that are ±500 bp long and originate from the COI gene barcoding site, (ii) the amplification carried out refers to the primers that the consortium has determined, (iii) trace files that are accessible and open, and (iv) the naming of species that have been approved refers to documents that have been certified (Zein 2013).

Composition of nucleotide bases

Based on the alignment stages of all samples (research samples with Genbank data), a 490 bp sequencing length was obtained from the COI *Clithon* sp. gene barcode sequence data. Among the three samples, the average G+C nucleotide base composition was 38.5%, while the average A+T base composition was 61.5% (Table 2). Based on these average results, the composition of the nucleotide bases of G+C was lower than the composition of the nucleotide bases of A+T.

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

Name sample	Identification of BOLD (highest 3)	Similarity (%)	Status	ACC number BOLD system	References
Clithon sp. C1	*Clithon sowerbianum*	98.77	Published	ADB1545	Ng et al. (2016)
	Clithon sowerbianum	98.56	Published		
	Clithon sowerbianum	98.56	Published		
Clithon sp. C2	*Clithon sowerbianum*	97.36	Published	ADB1545	Ng et al. (2016)
	Clithon sowerbianum	96.75	Published		
	Clithon sowerbianum	96.75	Published		
Clithon sp. C3	*Clithon sowerbianum*	98.97	Published	ADB1545	Ng et al. (2016)
	Clithon sowerbianum	98.77	Published		
	Clithon sowerbianum	98.77	Published		

Table 2. The nucleotide base composition of *Clithon* sp. from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia

Specimen	A (%)	C (%)	G (%)	T (%)	G+C (%)	A+T (%)
Clithon sp. OQ692133.1 (*This study*)	22.7	18.1	20.6	38.5	38.7	61.3
Clithon sp. OQ692134.1 (*This study*)	22.5	17.4	20.9	39.2	38.3	61.7
Clithon sp. OQ692135.1 (*This study*)	22.7	17.9	20.6	38.7	38.5	61.5
Rata-rata	22.7	17.8	20.7	38.8	38.5	61.5

Note: A: Adenine; C: Cytosine; G: Guanine; T: Thymine

The nucleotide base composition of the DNA sequence of *Clithon* sp. from Badur Beach, Madura, Indonesia shows the value of the nucleotide base composition of G+C was between 38.3-38.7%, and the value of the nucleotide base composition A+T was between 61.3-61.7% (Table 2). The value of the nucleotide base composition of the A+T result is higher than that of the nucleotide base composition at G+C. On the other hand, the A+T content was higher than the G+C content, which is consistent with the characteristics of the mitochondrial base composition (Cen et al. 2023). In addition, the differences in the composition of the nucleotide bases in each sequence indicate the existence of genetic variation between species (Saleky et al. 2020).

Variation of nucleotide bases

On the variation of nucleotide bases, there are three patterns of nucleotide bases, transition. Table 3 shows three transition nucleotide base substitutions. The transition on nucleotide base substitutions was found in nucleotide base number 127; *C. sowerbianum* shows base T (thymine), while *Clithon* sp. shows base C (cytosine). Likewise, in nucleotide base number 175, *Clithon sowerbianum* shows base T (thymine), while *Clithon* sp. shows base C (cytosine). Finally, in nucleotide base number 235, *C. sowerbianum* shows base C (cytosine), while *Clithon* sp. shows base T (thymine).

These results show two unique automorphic nucleotide bases were found as markers of the identification of the characteristics, such as base: nucleotide number 127 (cytosine) and 175 (cytosine), which is not owned by other species.

Nucleotide base variations are base changes that occur in the form of transitions and transversions. Transition is the change between purine bases, i.e., A (adenine) and G (guanine), or between pyrimidine bases, i.e., C (cytosine) and T (thymine). At the same time, transversion is a change between purine and pyrimidine bases and vice versa (Aziz et al. 2022). The results of the variations in nucleotide bases indicated that two automorphic nucleotide base patterns were only present in the *Clithon* sp. samples. These variations were found in nucleotide base numbers 127 (cytosine) and 175 (cytosine). Automorphic nucleotide bases are only owned by *Clithon* sp. from Badur Beach, Madura, Indonesia compared to other species (Table 3). Jannah and Rahayu (2019) stated that certain species only possess the character of automorphic nucleotide bases as a marker or distinguishing feature between the species and the species being compared.

Genetic distance

The final alignment results were processed using MEGA 6.0 software to determine the genetic distance of *Clithon* sp. with their relatives using the Kimura-2 parameter model calculation. As a result, the genetic distance matrix results are obtained, and shown in Table 4 is the average genetic distance of the *Clithon* sp., and the in-group was 1.74%.

Table 3. Clithon sp. nucleotide base variations, based on the COI gene

Specimen	Variation of nucleotide bases		
	127	175	235
Clithon sowerbianum MF687989.1	T	T	C
Clithon sowerbianum MF687990.1	●	●	●
Clithon sowerbianum MT230542.1	●	●	●
Clithon sowerbianum MZ831915.1	●	●	●
Clithon sowerbianum MZ831916.1	●	●	●
Clithon sowerbianum MZ831917.1	●	●	●
Clithon sowerbianum MZ831918.1	●	●	●
Clithon sowerbianum MZ831919.1	●	●	●
Clithon sp. OQ692133.1 (This study)	C	C	T
Clithon sp. OQ692134.1 (This study)	C	C	T
Clithon sp. OQ692135.1 (This study)	C	C	T
Clithon mertonianum KU318334.1	●	●	●
Clithon oualaniense MN389016.1	●	●	T
Clithon oualaniense MN389017.1	●	●	T
Clithon oualaniense MN389018.1	●	●	T
Clithon oualaniense EU732364.1	●	●	T
Clithon corona KU318331.1	●	●	T
Clithon corona EU732362.1	●	●	T
Cliton reticulare MF958991.1	●	●	T
Cliton reticulare MF958992.1	●	●	T
Cliton diadema KU318332.1	●	●	●
Clithon lentiginosum KU318333.1	●	●	T
Cliton chlorostoma EU732363.1	●	●	T
Nerita helicinoides EU732251.1	●	●	G
Nerita helicinoides EU732252.1	●	●	G

Note: (●) is a conserved nucleotide base

Table 4. The genetic distance of *Clithon* sp. is based on COI gene sequences using Kimura-2 parameter model (percentage) calculations

Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
C. sowerbianum MF687989.1																						
C. sowerbianum MF687990.1	2.38																					
C. sowerbianum MT230542.1	0.23	2.13																				
C. sowerbianum MZ831915.1	0.23	2.13	0.00	2.13																		
C. sowerbianum MZ831916.1	1.41	1.89	1.17	1.17	1.89																	
C. sowerbianum MZ831917.1	0.23	2.13	0.00	0.00	1.17	2.13																
C. sowerbianum MZ831918.1	0.23	2.13	0.00	0.00	1.17	0.00	2.13															
C. sowerbianum MZ831919.1	0.23	2.13	0.00	0.00	1.17	0.00	0.00	2.13														
Clithon sp. OQ692133.1 (*This study*)	1.41	1.89	1.17	1.17	0.94	1.17	1.17	1.17														
Clithon sp. OQ692134.1 (*This study*)	2.86	1.89	2.62	2.62	1.89	2.62	2.62	2.62	2.38													
Clithon sp.OQ692135.1 (*This study*)	1.65	2.13	1.41	1.41	1.17	1.1	1.41	1.41	0.23	2.62												
C. mertonianum KU318334.1	1.17	1.65	0.94	0.94	0.70	0.94	0.94	0.94	0.70	2.13	0.94											
C. oualaniense MN389016.1	20.28	21.27	19.95	19.95	19.95	19.95	19.95	19,95	20,61	19.95	20.28	20.28										
C. oualaniense MN389017.1	20.28	21.27	19.95	19.95	19.95	19.95	19.95	19,95	20,61	19.95	20.28	20.28	0.00									
C. oualaniense MN389018.1	21.61	21.27	21.27	21.27	20.61	21.27	21.27	21.27	21.27	19.95	20.94	20.94	1.65	1.65								
C. oualaniense EU732364.1	18.98	19.95	18.66	18.66	18.66	18.66	18.66	18.66	19.30	18.66	18.98	18.98	2.13	2.13	286							
C. corona KU318331.1	12.32	12.32	12.03	12.03	11.74	12.03	12.03	12.03	12.90	12.90	12.61	12.61	21.20	21.20	20.54	19.88						
C. corona EU732362.1	13.14	14.33	12.85	12.85	13.14	12.85	12.85	12.85	13.73	13.73	13.44	13.44	16.74	16.74	16.74	16.74	9.33					
C. reticulare MF958991.1	11.45	12.90	11.17	11.17	11.17	11.17	11.17	11.17	11.74	11.74	12.03	11.45	17.97	17.97	19.88	18.60	10.69	11.21				
C. reticulare MF958992.1	14.06	14.06	13.76	13.76	14.06	13.76	13.76	13.76	14.66	13.46	14.36	13.76	16.50	16.50	18.37	17.43	13.81	13.22	11.48			
C. diadema KU318332.1	13.46	13.46	13.17	13.17	13.46	13.17	13.17	13.17	14.06	12.87	13.76	13.17	16.19	16.19	18.05	17.11	14.42	13.22	12.05	0.94		
C. lentiginosum KU318333.1	10.07	10.63	9.79	9.79	10.07	9.79	9.79	9.79	10.63	10.63	10.35	10.35	18.95	18.95	18.31	18.31	4.60	8.25	9.87	12.36	11.78	
C. chlorostoma EU732363.1	13.06	13.64	12.77	12.77	13.06	12.77	12.77	12.77	13.06	14.23	12.77	12.77	17.04	17.04	17.04	18.29	12.36	10.33	11.17	15.76	15.15	11.52

Note: The highlight section shows the shortest genetic distance between the study sample and the in-group

Genetic distance values were calculated using the Kimura-2 parameter model calculation to determine the close genetic relationship between individuals. Furthermore, the parameter model analysis uses the Kimura-2 calculations to consider the mutation's transition and transversion substitution point (Jannah and Rahayu 2019). The value of the genetic distance is affected by the number of nucleotide base variations (Sari et al. 2021). Zein (2013) stated that the distance is determined by the number of bases that experience changes; the more differences, the more frequent the mutation process occurs, which indicates the farther distance. The genetic distance of the *Clithon* sp. samples from Badur Beach, Madura, Indonesia was compared with Genbank NCBI data to calculate the genetic distance values matrix to show each sample's degree of kinship with data from the Genbank NCBI (Table 4). Based on the compared genetic distance results, the average genetic distance value between *Clithon* sp. compared to Clithon sowerbianum is 1.74%. Cai et al. (2016) stated that a genetic distance value with a percentage of less than 2% indicates that the group is the same species, while a genetic distance value with a percentage of more than 2% indicates that the group is a different species from members of the group. This could indicate that the sample of *Clithon* sp. from Badur Beach, was identified as one species with *C. sowerbianum*. In addition, according to Chiu et al. (2013), the diversity of genetic distances can be influenced by environmental factors, excessive exploitation, and geographical location. On the other hand, specific environmental factors on a species could also change the morphology and phylogenetic populations.

Reconstruction of phylogenetic trees

Phylogenetic tree reconstruction was performed using the Neighbor-Joining Tree (NJT) and Maximum Likelihood (ML) methods for the sample *Clithon* sp. from Badur Beach, Madura, with data from Genbank NCBI (Figure 4). The results are as follows: cluster in-group I consisted of *Clithon* sp. from Badur Beach, *C. sowerbianum* from China and Hongkong, *C. mertonianum* from Singapore, and *C. diadema* from Singapore, cluster in-group II consisted of *Clithon reticulare* from Myanmar, cluster in-group III consisted of *C. corona* from petshop aquarium at Singapore and Fiji, *C. lentiginosum* from petshop aquarium at Singapore, and *C. chlorostoma* from Japan, and cluster in-group IV consisted of *C. oualaniense* from China and Australia. Finally, the out-group cluster consisted of *Nerita helicinoides Reeve* 1855 from Japan. Furthermore, Figure 4 shows that the *Clithon* sp. sample from Badur Beach, forms the same clade as *C. sowerbianum* and *C. mertonianum* with a bootstrap value of 96-100.

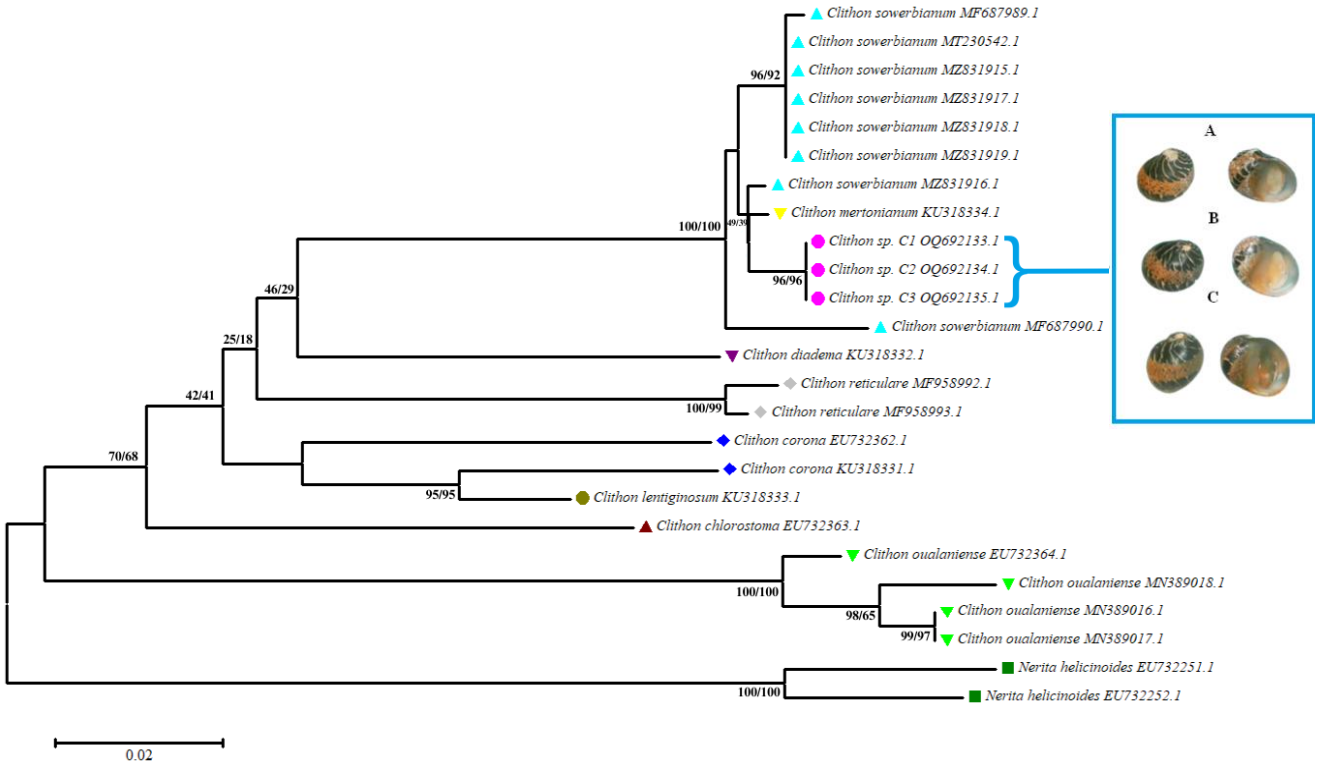

Figure 4. The phylogenetic topology of *Clithon* sp. from Badur Beach of Madura Island, Sumenep District, East Java Province, Indonesia, concerning the COI gene from Genbank NCBI using the Neighbor-Joining Tree (NJT) and Maximum Likelihood (ML) method with a bootstrap of 1,000 replications. (Caption: A: *Clithon* sp. C1, B: *Clithon* sp. C2, C: *Clithon* sp. C3)

The Neighbor-Joining Tree (NJT) method can construct a phylogenetic tree based on the genetic distance between pairs of individuals, namely sequences with the closest genetic distance combined with being in the same cluster (Limpiti et al. 2014). Furthermore, the Neighbor-Joining Tree (NJT) method is most suitable for predicting phylogenetic trees correctly using the branch lengths of trees whose topology is known to change by stimulating the degree of variation of evolutionary changes (Morrison 2016). Moreover, the Maximum Likelihood (ML) method is a characteristics-based statistical method that compares all sequences in the alignment to calculate the closeness value of each tree (Yang and Rannala 2012). In addition, this method considers all possible numbers of changes/mutations in the sequences for each tree. Therefore, it is suitable for constructing phylogenetic sequences with few samples.

Phylogenetics is a method of classifying taxonomy to determine diversity by reconstructing genetic distance (Juniar et al. 2021). The final alignment data were further analyzed using MEGA6 to examine the research sample's relationship with Genbank NCBI data. The results of the bootstrap values can indicate the stability of the obtained phylogenetic tree branches (Dominova and Zhukov 2022). In addition, bootstrap analysis is used to test the validity of sequence data for the arrangement of branches in a phylogenetic tree (Kapli et al. 2020). The phylogenetic topology using Neighbor-Joining Tree (NJT) and Maximum Likelihood (ML) revealed that *Clithon* sp. forms the same clade as *C. sowerbianum* and *C. mertonianum* with a bootstrap value of 96-100. A higher bootstrap value of the branch can indicate stronger branching in the phylogenetic tree (Saleky et al. 2020). In addition, Hesterberg (2015) states that bootstrap at 1000 repetitions with a percentage value above 80% on branching shows a very good result because this value strongly supports that the samples in one branch are correct or in the same species.

C. mertonianum is in the same clade as *Clithon* sp. and *C. sowerbianum* but differs in the operculum, in which *Clithon* sp. has a yellow-to-gray one. Eichhorst (2016) described the operculum as greenish gray with a dark brownish orange in *C. mertonianum* and white, beige, or grey, with fine axial growth lines, and the tan to yellow in *Clithon sowerbianum*. Based on their characters and COI barcode, the DNA markers analysis could identify *Clithon* sp. from Badur Beach, Madura, Indonesia as *C. sowerbianum*. Cryptic or species similarity based on morphology generally occurs in invertebrates, so the identification by molecular approach is conventional and important to get the accuracy of species identification (Juniar et al. 2021). This genetic identification result on *Clithon* sp. adds data on marine biota, especially gastropods of the Neritidae family at Badur Beach. This result could be sent in DNA barcoding libraries BOLD System collection directly by following the requirements flow according to the database GenBank, as previously stated (Jannah and Rahayu 2019).

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REFERENCES

Aziz R, Sen P, Beura PK, Das S, Tula D, Dash M, Ray SK. 2022. Incorporation of transition to transversion ratio and nonsense mutations improves the estimation of the number of synonymous and non-synonymous sites in codons. DNA Res 29 (4): dsac023. DOI: 10.1093/dnares/dsac023.

Antil S, Abraham JS, Sripoorna S, Maurya S, Dagar J, Makhija S, Toteja R. 2022. DNA barcoding an effective tool for species identification: A review. Mol Biol Rep 50: 761-775. DOI: 10.1007/s11033-022-08015-7.

Bingpeng X, Heshan L, Zhilan Z, Chunguang W, Yanguo W, Jianjun W. 2018. DNA barcoding for identification of fish species in the Taiwan Strait. PloS ONE 13: e0198109. DOI: 10.1371/journal.pone.0198109.

Bhattacharjee MJ, Laskar BA, Dhar B, Ghosh SK. 2012. Identification and re-evaluation of freshwater catfishes through DNA barcoding. PLoS ONE 7 (11): e0049950. DOI: 10.1371/journal.pone.0049950.

Cen X, Zhang G, Liu H, Yao G, Xiong P, He M, Liu W. 2023. Analysis of genetic diversity in two different shell colors of the giant triton snail (*Charonia tritonis*) based on mitochondrial COI sequences. Front Mar Sci 9: 1066750. DOI: 10.3389/fmars.2022.1066750.

Chiu YW, Bor H, Tan MS, Lin HD, Jean CT. 2013. Phylogeography and genetic differentiation among populations of the moon turban snail *Lunella granulata* Gmelin 1791 (Gastropoda: Turbinidae). Intl J Mol Sci 14 (5): 9062-9079. DOI: 10.3390/ijms14059062.

Cai Y, Zhang L, Wang Y, Liu Q, Shui Q, Yue B, Zhang Z, Li J. 2016. Identification of deer species (Cervidae, Cetartiodactyla) in China using mitochondrial cytochrome c oxidase subunit I (mtDNA COI). Mitochondrial DNA Part A DNA Mapping. Seq Anal 27 (6): 4240-4243. DOI: 10.3109/19401736.2014.1003919.

Dharma B. 2005. Recent and Fossil Indonesian Shells. Conchbooks, Hackenheium.

Djoemharsjah SI, Isnaningsih RN, Ambarwati R. 2023. Variasi morfologi dan kekerabatan keong Neritidae di Badur Beach, Madura. Berita Biologi. (In press). [Indonesian]

Dominova IN, Zhukov VV. 2022. Mollusc crystallins: Physical and chemical properties and phylogenetic analysis. Diversity 14 (10): 827. DOI: 10.3390/d14100827.

Eichhorst TE. 2016. Neritidae of The World. Volume 1. ConchBooks, Harxheim.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3 (5): 294-299. DOI: 10.1371/journal.pone.0013102.

Hesterberg T. 2015. Bootstrap Methods and Permutation Tests (Issue February). W. H. Freeman and Company, New York. DOI: 10.1080/00031305.2015.1089789.

Hylleberg J. 2000. Molluscs collected by in Vietnam and Cambodia. Phuket Mar Biol Center Special Pub 21 (1): 519-573.

Jannah M, Rahayu DA. 2019. Indonesian Animal and Plant DNA Barcodes. Yayasan Inspirasi Ide Berdaya, Jakarta Selatan. DOI: 10.17605/OSF.IO/TY7E5. [Indonesian]

Juniar AE, Ambarwati R, Rahayu DA. 2021. Genetic identification of *Clithon oualaniense* (Gastropoda: Neritidae) from Madura, Indonesia. AACL Bioflux 14 (2): 1046-1056.

Killburn R. 2000. Shallow Water Archaeogastropods of South East Asia: An Introduction. Phuket Marine Biological Center Special Publication 21 (3): 595-601. DOI: 123456789/14101.

Kapli P, Yang Z, Telford MJ. 2020. Phylogenetic tree building in the genomic age. Nat Rev Genet 21 (7): 428-444. DOI: 10.1038/s41576-020-0233-0.

Limpiti T, Amornbunchornvej C, Intarapanich A, Assawamakin A, Tongsima S. 2014. iNJclust: Iterative neighbor-joining tree clustering framework for inferring population structure. IEEE/ACM Trans Comput Biol Bioinform 11 (5): 903-914. DOI: 10.1109/TCBB.2014.2322372.

Marković V, Tomović J, Ilić M, Kračun-Kolarević M, Novaković B, Paunović M, Nikolić V. 2014. Distribution of the species of Theodoxus Montfort, 1810 (Gastropoda: Neritidae) in Serbia: An overview. Acta Zool Bulgarica 66 (4): 477-484. DOI: 10.11646/zootaxa.5003.1.1.

Morrison DA. 2016. Genealogies: Pedigrees and phylogenies are reticulating networks not just divergent trees. Evol Biol 43: 456-473. DOI: 10.1007/s11692-016-9376-5.

Ng TH, Tan SK, Wong WH, Meier R, Chan SY, Tan HH, Yeo DC. 2016. Molluscs for sale: Assessment of freshwater gastropods and bivalves in the ornamental pet trade. PLoS ONE 11 (8): e0161130. DOI: 10.1371/journal.pone.0161130.

Park SY, Patnaik BB, Kang SW, Hwang HJ, Chung JM, Song DK, Lee YS. 2016. Transcriptomic analysis of the endangered Neritid species *Clithon retropictus*. De novo assembly, functional annotation, and marker discovery. Genes 7 (7): 35. DOI: 10.3390/genes7070035.

Rabi C, Rilov G, Morov AR, Guy Haim T. 2020. First record of the red sea gastropod *Nerita sanguinolenta* Menke, 1829 (Gastropoda: Cycloneritida: Neritidae) from the Israeli Mediterranean coast. BioInvasions Records 9 (3): 496-503. DOI: 10.3391/bir.2020.9.3.06.

Saleky DPO, Leatemia SF, Pattiasina T, Isma I, D Pangaribuan R, Welliken MA, Melmambessy E HP, Dailami M. 2020. Analisis pola pertumbuhan dan pendekatan DNA barcoding untuk identifikasi *Turbo stenogyrus* P. Fischer 1873 (Mollusca: Gastropoda). Biotropika Jurnal Tropical Biologi 8 (2): 79-86. DOI: 10.21776/ub.biotropika.2020.008.02.03.

Sari SYP, Ambarwati R, Rahayu DA. 2021. Molecular characteristics of *Donax faba* (Bivalvia: Donacidae) from Nepa Beach, Madura, based on Cytochrome Oxidase Subunit I gene sequences. AACL Bioflux 14 (4): 2416-2426.

Tan SK, Clements R. 2008. Taxonomy and distribution of the Neritidae (Mollusca: Gastropoda) in Singapore. Zool Stud 47 (4): 481-494.

Yang Z, Rannala B. 2012. Molecular phylogenetics: Principles and practice. Nat Rev Genet 13 (5): 303-314. DOI: 10.1038/nrg3186.

Zein MSA, Prawiradilaga DM. 2013. Indonesian Fauna Barcode DNA. Prenada Media, Jakarta. [Indonesian]