DNA barcoding and phylogenetic of lollyfish, *Holothuria atra* (Jaeger, 1833) (Actinopoda: Holothuriidae), from Tablolong Beach, Kupang, East Nusa Tenggara, Indonesia

[1]Neri Kautsari, [2]Suleman, [2]Asriati Djonu, [3]Fitri S. Valen

[1] Aquatic Resources Management Department, Faculty of Animal Husbandry and Fisheries, Samawa University, Sumbawa Besar, Indonesia; [2] Aquaculture Study Program, Faculty of Animal Husbandry, Marine and Fisheries, Nusa Cendana University, East Nusa Tenggara, Indonesia; [3] Department of Aquaculture, Faculty of Agriculture Fisheries and Biology, University of Bangka Belitung, Indonesia. Corresponding author: Suleman, suleman@staf.undana.ac.id

Abstract. Sea cucumbers are an important part of the marine resource community, making important contributions to the marine ecosystem as well as the maritime economy. These organisms can be found in all of the world's oceans, and they like to live in warm shallow waters that are in close contact to corals, rocks, or seaweed. The intertidal zone of Tablolong Beach in Kupang, East Nusa Tenggara, Indonesia is distinguished by the presence of a beach that is ornamented with a substrate of white sand. In addition, there are a lot of seagrass beds in this region, which makes it an excellent environment for sea cucumbers. *Holothuria atra* is one of the 54 documented species of sea cucumbers that have been subjected to exploitation in Indonesia for numerous decades. Additionally, this species may be found near Tablolong Beach, Kupang. The population of this particular species underwent a significant reduction as a result of over-exploitation. Until now, no conservation initiatives have been undertaken for the species in question at Tablolong Beach, Kupang. Hence, the implementation of conservation and population development activities, along with other relevant studies, becomes crucial. The primary objective of this study is to undertake a thorough examination of biodiversity and employ DNA Barcoding techniques to ascertain species identification. This initial step is crucial in the realm of biodiversity and conservation research. The COI gene was subsequently selected as the standard gene for expeditious species identification of sea cucumbers. The DNA barcode of *H. atra* collected from Tablolong Beach, Kupang, was effectively sequenced, yielding a base-pair length of 710 bp. It was observed that the genetic sequence obtained from Tablolong Beach in Kupang exhibits complete similarity to that of *H. atra*, with a similarity level of approximately 99-100%.
Key Words: biodiversity, DNA barcoding, marine, phylogenetic, sea cucumbers.

Introduction. Indonesia possesses significant potential in terms of marine resources due to its extensive water coverage throughout a substantial portion of its territory (Sulardiono et al 2021; Isroni et al 2023; Nurjirana et al 2022). In addition, Indonesia has a significant degree of aquatic biodiversity encompassing marine, brackish, and freshwater biota (Hasan et al 2023a; Gani et al 2021). The Indonesian maritime region plays a significant role in the provision of sustenance for the populace of Indonesia (Mujahidah et al 2023; Hasan et al 2022). Sea cucumber is a marine resource that holds significant economic worth (Prescotta et al 2017). Tablolong, located in the Kupang district of East Nusa Tenggara, is characterized by its intertidal zone, owing to the presence of a beach adorned with a substrate of white sand (Nomleni et al 2020). Additionally, the area has abundant seagrass beds, rendering it a suitable habitat for sea cucumbers (Purcell et al 2012).

Sea cucumbers are classified within the phylum Echinodermata, indicating their possession of a spiny skin, and specifically fall within the class Holothuridea (Sulardiono et al 2021). The classification of the subject matter involved the subsequent division into three distinct subclasses, specifically Dendrochirotacea, Aspidochirotacea, and Apodacea. There exist six distinct orders within these subclasses, which are designated as

Aspidochirotida, Apodida, Dactylochirotida, Dendrochirotida, Elasipodida, and Molpadiida (Bruckner et al 2003).

Sea cucumbers are a significant component of marine resources, contributing to both ecological and economic aspects (Purcell et al 2016). These organisms are widely spread across all global oceans, typically inhabiting warm shallow waters in close proximity to corals, rocks, or seaweeds (Bordbar et al 2011). Their behaviors enhance the characteristics of the sediment, improve the chemistry of the water, and have an effect on the productivity of a wide variety of benthic creatures. Sea cucumber holds significant economic importance in Asian markets within the context of Marine culture (Purcell et al 2013). The feasibility of sea cucumber farming is substantiated by its simplicity of production, low cost, and potential for high sales income. In addition, sea cucumbers possess favorable attributes that make them very suitable for integrated multi-trophic aquaculture, primarily owing to their feeding strategy and ecological role within the food chain (Purcell et al 2016).

In addition, sea cucumbers have historically served as a dietary source and traditional medicinal ingredient. This particular species possesses a diverse array of bioactive substances, including phenols, polysaccharides, proteins (such as collagen and peptides), carotenoids, and saponins (Mazlan et al 2023). These chemicals exhibit potent antioxidant properties and other notable potentials. The marine invertebrate under consideration is known to possess a significant quantity of phenolic compounds, specifically phenolic acids and flavonoids, which are notable for their antioxidant properties (Rasyid et al 2023).

The current global count of extant sea cucumber species is approximately 1,250 (Bordbar et al 2011). *Holothuria atra* is a species that possesses a significant market value in the Asian region (Dhinakaran & Lipton 2014). *H. atra*, usually referred to as lollyfish, was identified as the prevailing black species and the most frequently found sea cucumber in Indonesia. These commonly occurring species in shallow-water habitats were infrequently observed at depths over 20 meters. They were predominantly found in inner and outer reef flats, back reefs, and shallow coastal lagoons (Hartati et al 2020).

The overexploitation of sea cucumbers in Indonesia poses a significant threat to their population, and *H. atra* is among the species affected by this issue. *H. atra* is among the 54 known species of sea cucumbers that have been subjected to exploitation in Indonesia for several decades (Setyastuti et al 2018). The overexploitation of *H. atra* in Indonesian seas, particularly at Tablolong Beach, Kupang, East Nusa Tenggara, Indonesia, poses a significant threat to its existence and population. Therefore, it is imperative to implement conservation and population development initiatives. An extensive study is required to substantiate the efficacy of conservation endeavors. One example is to the rapid and precise investigation of biodiversity and the identification of species (Lutfiatunnisa et al 2020; Hasan et al 2023b).

This study aimed to conduct a comprehensive investigation of biodiversity along the Tablolong shoreline in Kupang. In this particular instance, the species was determined through the utilization of the DNA Barcoding technique, which involves the examination of one or more genes inside the mitochondrial DNA. The Cytochrome C oxidase Subunit I (COI) gene, located in mitochondrial DNA, is commonly employed as a molecular marker for species identification in fish (Valen et al 2022). The COI gene has been widely used as a standardized method for molecular taxonomy and identification on a global scale (Bingpeng et al 2018). It serves as a reliable foundation for distinguishing between various animal species (Liu et al 2020). The successful application of the COI gene as a tool for species identification has been demonstrated in the context of identifying sea cucumbers in Indonesia, as documented by Madduppa et al (2017). Furthermore, the DNA Barcoding of species found in Tablolong Beach, Kupang, specifically the Sea cucumbers, will be submitted to the Genbank NCBI database. This submission will serve as a standardized method for identifying Sea cucumber species based on the COI gene. This study aimed to provide empirical evidence pertaining to the expeditious identification of species, comprehension of biodiversity, augmentation of species genetic variety, and comprehension of species' life history. Ultimately, the findings of this research endeavor will contribute to the facilitation of conservation efforts and sustainable development.

Material and Method

Sampling site, fish samples collection and water quality. The study was conducted from 9th to 30th of June 2023 in the Tablolong Beach, Kupang Regency, East Nusa Tenggara Indonesia. The collection of samples was conducted through daytime diving activities. A random sampling method was used by taking samples found at the sampling location. Water quality assessments were conducted during the collection of sea cucumber samples. Water quality observations encompass several parameters, such as temperature, salinity, pH, dissolved oxygen, and current speed. These parameters are typically measured using specific instruments, including a mercury thermometer for temperature, a refractometer for salinity, a pH meter for pH levels, a DO meter for dissolved oxygen, and a current meter for determining current speed.

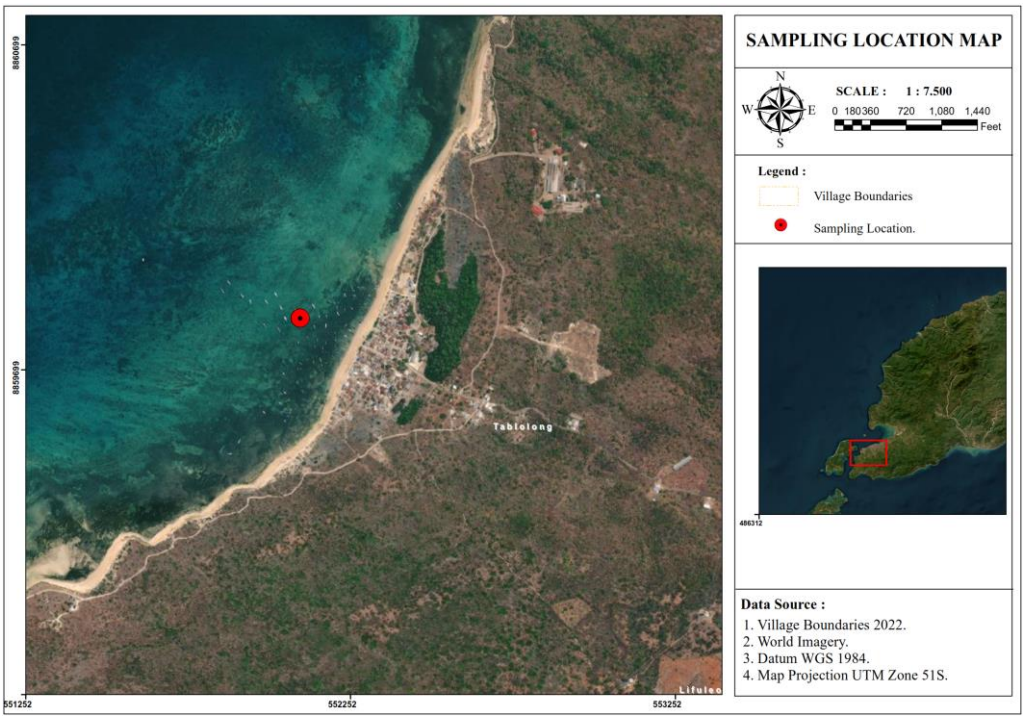

Figure 1. The location of sample collection (red circle).

Fish preservation and morphological analysis. A total of 135 specimens of *H. atra* were caught during the fieldtrip. The remaining 25 live specimens were kept as livestock at the Fish Reproduction Laboratory, University of Nusa Cendana, Indonesia for breeding and reproduction. One specimen was preserved in a 96% alcohol solution for molecular analysis. One specimen was preserved in a 7% formalin solution (Valen et al 2020) and deposited at the zoology laboratory, at the University of Nusa Cendana. The other live specimens were released to their habitat for conservation.

DNA extraction and amplification. The process of sample extraction was conducted utilizing the DNeasy Blood & Tissue Kit. Tissue samples were extracted to the maximum extent feasible, with a mass of 25 mg, utilizing tweezers, and thereafter transferred into a 1.5 mL tube. Prior to and subsequent to the extraction of the tissue, the tweezers were immersed in a solution of 95% ethanol and subjected to combustion using a Bunsen burner. The 1.5 mL tube containing the tissue sample was supplemented with 180 µL of Buffer ATL and 20 µL of proteinase K. Subsequently, the sample was subjected to vortexing and centrifugation for a duration of 20 seconds, followed by overnight incubation in a heating block set at a temperature of 56°C. Subsequently, a volume of 200 µL of Buffer AL was introduced, followed by vortexing and incubation at a temperature of 56 degrees Celsius for a duration of 10 minutes. Then, 200 µL of 96% ethanol were incorporated into the mixture and perform vortexing was again performed. The combination of the sample and

reagent was put into a DNeasy Mini spin column, which was subsequently positioned within a 2 mL collecting tube. Subsequently, the sample was subjected to centrifugation at a speed of 8000 revolutions per minute for a duration of 60 seconds, then the liquid contained was disposed within the collection tube. The spin column was positioned within a fresh 2 mL collection tube, followed by the addition of 500 µL of Buffer AW1. The same sequence was repeated, but this time with the addition of 500 µL of Buffer AW2. A centrifugal force was applied at a speed of 14,000 revolutions per minute for a duration of 3 minutes and the liquid was disposed of into the collection tube. The spin column was relocated to a fresh 1.5 mL tube. The DNA elution process involved the addition of 100 µL of double-distilled water (ddH_2O) to the central region of the membrane spin column. The incubation process was conducted at the ambient temperature, specifically within the range of 15-25°C. The sample was centrifuged at a speed of 8,000 revolutions per minute for a duration of 1 minute. The final stage consisted in the addition of 100 µL of double-distilled water (ddH_2O) to achieve a final volume of 200 µL and the prepared extraction solution was available for utilization in the amplification process.

The obtained extraction results were subjected to further analysis in the subsequent stage, which involves Polymerase Chain Reaction (PCR). The polymerase chain reaction (PCR) technique employed the BIONESIA laboratory procedure. The primers employed in the amplification procedure for fish samples were COI ceF (5'-ACTGCCCACGCCCTAGTAATGATATTTTTTATGGTNATGCC-3') and reverse primer CO1 ceR (5'-TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG -3') (Hoareau & Boissin 2010). The PCR reaction was conducted using a total volume of 26 µL, including 2 µL of DNA template obtained from the extraction results, 1.25 µL of each primer with a concentration of 10 mM, 9 µL of double-distilled water (ddH_2O), and 12.5 µL of Ready mix. The reaction mixture was subsequently subjected to amplification using an Applied Biosystems™ 2720 Thermal Cycler apparatus. The temperature and time profile utilized in the PCR process is outlined as follows: the pre-denaturation step involved subjecting the sample to a temperature of 95°C for a duration of 3 minutes, followed by the denaturation stage, where the temperature is set to 94°C for a period of 45 seconds. Subsequently, the annealing stage occured, with the temperature ranging from 55°C to 60°C for a duration of 1 minute and 10 seconds. The extension stage then took place at a temperature of 72°C for 1 minute and 20 seconds. The denaturation to extension sequence was repeated for a total of 30 cycles (Robin et al 2022). The last stage involved a final extension at 72°C for 5 minutes. Subsequently, the polymerase chain reaction (PCR) outcomes were observed by employing a 1% Agarose gel supplemented with Nucleic Acid Gel Stain (GelRed®) for staining purposes (Robin et al 2023). The samples exhibiting positive characteristics, specifically luminous DNA bands, were further subjected to DNA sequencing utilizing the Sanger dideoxy (Sanger 1977) technique at PT. Genetic Science Jakarta.

Data analysis. The process of species identification involves the utilization of sequence analysis on BLASTn within the Genbank database. Furthermore, we have successfully submitted the DNA barcode sequence to Genbank, a database maintained by the National Center for Biotechnology Information (NCBI) in 1988 (National Center for Biotechnology Information 1988). The accession number assigned to our submission is OQ281707. The Genbank database can be accessed at https://www.ncbi.nlm.nih.gov/genbank/. The Neighbor-Joining method (Saitou & Nei 1987) was employed to ascertain the evolutionary history of species, accompanied by a bootstrap test consisting of 1,000 replications (Felsenstein 1985). The Maximum Composite Likelihood technique (Tamura et al 2004) was employed to compute evolutionary distances in the MEGAX software (Kumar et al 2018).

Results

DNA barcoding. DNA-barcode of *H. atra* from Tablolong Beach, Kupang, was successfully sequenced with a base-pair length of 710 bp (Table 1) using COI ceF and CO1 ceR primers (Hoareau & Boissin 2010).

Table 1

DNA barcoding of *Holothuria atra* from Belitung Island, Indonesia

DNA barcoding of *H. atra*
CCCTAGTAATGATATTTTTTATGGTTATGCCAATAATGATAGGAGGCTTCGGAAAATGATTAATAC
CACTGATGATAGGAGCCCCAGACATGGCCTTCCCTCGAATGAAAAACATGAGATTCTGATTAGTC
CCACCATCCTTCATATTACTACTAGCCTCAGCAGGAGTAGAAAGAGGGGTAGGAACAGGATGAAC
CATTTACCCCCCCTTATCAAGAAACATAGCCCACGCCGGGGGATCCGTAGACTTAGCTATTTTCTC
CCTACACCTAGCTGGGGCCTCGTCAATACTAGCATCAATAAAATTTATAACAACGATCACCAAAAT
GCGAACTCCAGGAATTACCTTCGACCGACTCCCACTATTTGTATGATCAGTTTTCATAACGGCATT
TCTCCTCTTACTTAGACTCCCCGTACTAGCAGGAGCAATCACAATGCTATTAACCGACCGAAACGT
AAAAACAACCTTCTTCGACCCTGCCGGAGGAGGAGACCCTATACTATTCCAACATCTATTCTGATT
CTTCGGACACCCAGAAGTCTACATACTTATTCTACCAGGATTCGGAATGATATCTCACGTAATAGC
CCACTATAGAGGTAAGCAAGAACCATTTGGTTACCTAGGAATGGTATACGCAATGGTTGCAATAG
GAATCCTAGGATTCCTAGTCTGAGCCCACCATATGTTCACAGTAGGAATGGAC

Molecular identification*.* The DNA barcoding of the sequence from Tablolong Beach, Kupang was analyzed and compared to the NCBI GenBank (National Center for Biotechnology Information 1988) via BLAST (Basic Local Alignment Search Tool-nucleotide) method (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BOLD system, via the Identification Engine, to find out and analyze a sequence homology. We found that the sequence from Tablolong Beach, Kupang is identical to the *H. atra*, with a level of similarity of about 99-100% (Table 2).

Table 2

Species identification and similarity of *Holothuria atra* from Tablolong Beach, Kupang

Field ID	*Lab ID*	*Species*	*bp*	*Gene*	*Accession number*	*Query cover (%)*	*Identity (%)*
TBL 02	BIOSUB224.002	*H. atra*	710	COI	OP458809	99%	99
		H. atra	710	COI	MZ823511.1	80%	99,54%

Classification. Based on Blast DNA applied to the DNA barcode of *H. atra* from theTablolong Beach, Kupang Regency, East Nusa Tenggara Indonesia, the sample was classified as follows:

Kingdom: Animalia
Phylum: Echinodermata
Class: Holothuroidea
Order: Holothuriida
Family: Holothuriidae
Genus: *Holothuria*
Species: *H. atra*
Popular name: Lollyfish

Identification and grouping of species into categories or classifications are very important to conclude on the results of species identification in a more complete manner. Classification was carried out based on cytochrome c oxidase sub unit I (COI) gene. The research results will form the basis for the species conservation and domestication process.

Water quality. Optimal water quality parameters are indicative of a favorable carrying capacity for the sustenance of sea cucumbers' life cycle. Under favorable water quality circumstances, sea cucumber populations exhibit robust growth and reproductive capabilities. The spatial distribution of sea cucumber species is influenced by many aquatic environmental variables. The temperature of the surrounding environment plays a crucial role in the survival and development of aquatic creatures. The factors that are measured

in the aquatic environment include temperature, salinity, pH, dissolved oxygen (DO), and current speed (Meirinawati et al 2020) (Table 3).

Table 3

Water quality from Tablolong Sea, Kupang, Indonesia

Water quality parameters	*Water quality measurement results*
Temperature	29-30°C
Salinity	32 ppt
pH	7.5
Dissolved oxygen	7 ppm
Current speed	0.3 m sec^{-1}

Discussion. DNA barcoding has been employed as a valuable tool for species identification, as demonstrated by Guo et al (2022). The provided material holds significant value in enhancing scientific understanding, particularly in taxonomy (Tadmor-Levi 2022) and advancing knowledge in the biotechnology field. The DNA barcode of *H. atra* originating from Tablolong Beach in Kupang represents the initial barcode entry recorded in the Genbank database. The examination of the BLAST DNA sequence obtained from Tablolong Beach, Kupang revealed that the identified species was *H. atra*, with a similarity percentage ranging from 99 to 100%. According to Hebert et al (2003), it has been posited that species exhibiting similarity levels ranging from 97 to 100% can be considered identical. Consequently, any variations in species over a 3% threshold, as determined by the COI genes, are indicative of the emergence of new species. Additionally, the sequence data obtained from this study has been deposited in the Genbank database (National Center for Biotechnology Information 1988) under the Accession Number OR166098. In subsequent periods, this particular sequence will function as a point of reference for the purpose of identifying species through the use of DNA barcoding and the COI gene markers, as indicated by Hebert et al (2003). The provided information holds significant value in enhancing scientific understanding, particularly in the realms of taxonomy (Hasan et al 2021) and biotechnology advancement. The purpose of registering this sequence is to provide a comprehensive account of eukaryotic biodiversity in order to contribute to the Barcode of Life (BOL) project. Genetic samples of species are valuable in elucidating the population structure. The total length of the DNA barcode of *H. atra* collected from Tablolong Beach, Kupang, is 710 base pairs. Guo et al (2022) have posited that the utilization of COI gene sequences exceeding 658 base pairs can serve as a benchmark for discerning various animal species. According to Hebert et al (2003), the mitochondrial DNA cytochrome oxidase subunit I (COI) gene, when consisting of a minimum of 658 base pairs, possesses the potential to function as a universal identifier for all animal species. Furthermore, the *H. atra* DNA barcode obtained from Tablolong Beach, Kupang exhibits a significantly elevated amount of adenine and thymine, hence belonging to the A-T rich category, constituting around 60% of the overall nucleotide composition. The A-T hydrogen bond is composed of two hydrogen bonds, exhibiting lesser strength compared to the G-C hydrogen bond which has three hydrogen bonds. Consequently, the likelihood of species mutation is elevated (Insani et al 2022; Valen et al 2021). The nucleotide composition of *H. atra* from Tablolong Beach, Kupang, was determined to be as follows: thymine (T) accounted for 24.9% of the total nucleotides, cytosine (C) accounted for 25.9%, adenine (A) accounted for 30.5%, and guanine (G) accounted for 18.7%. In this study, the phylogenetic tree is also generated by utilizing the Neighbor-Joining technique, which is based on the Tamura-Nei model (Tamura et al 2004), with the MEGA X (Kumar et al 2018), and with 1000 replicates, in order to demonstrate the evolutionary tree of the species (Figure 1). In order to finish the phylogenetic tree that was based on the COI gene, the sequences of the seven species that belong to the genus *H. atra* and the other 17 species that belong to the genus *Holothuria* were collected from Genbank.

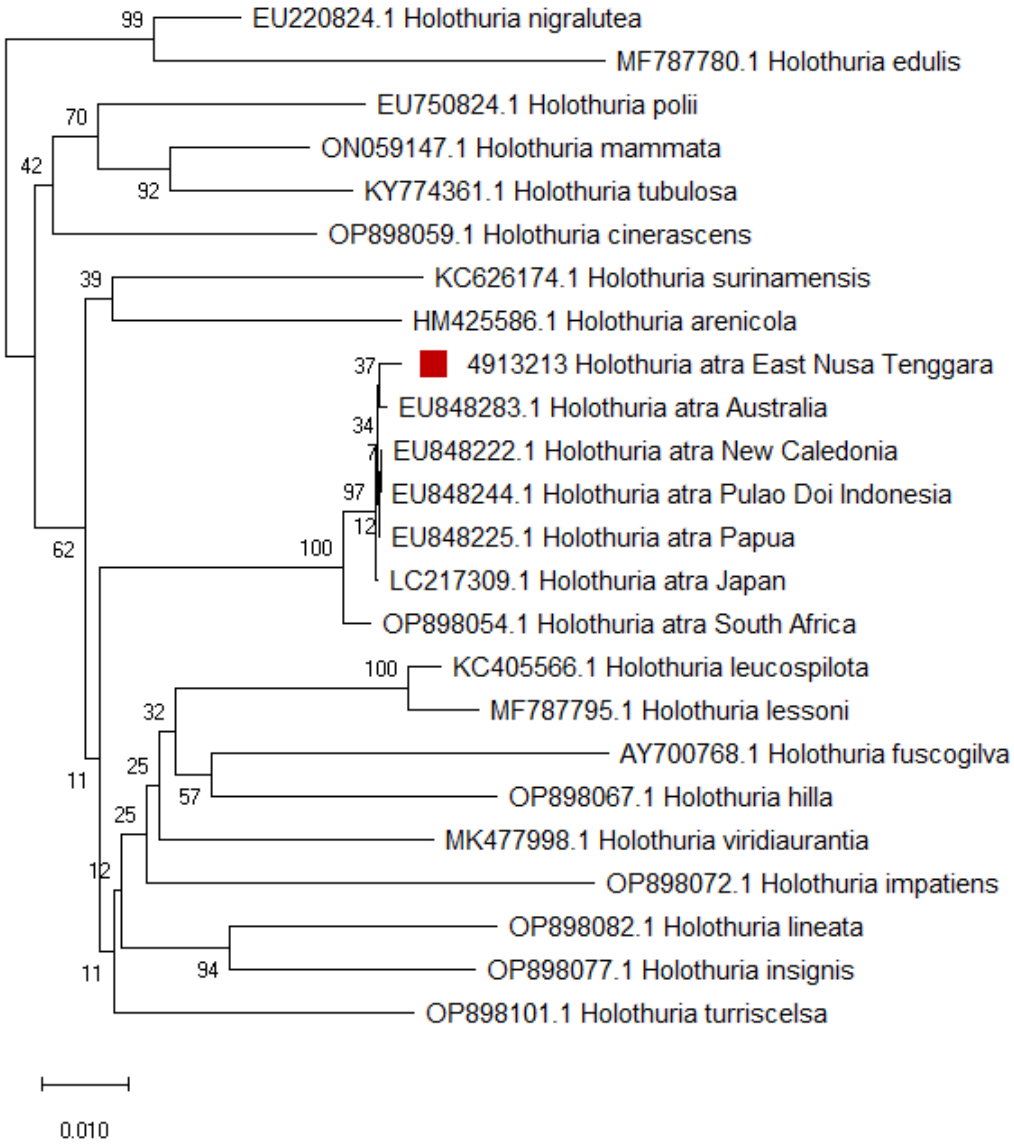

Figure 1. The evolutionary tree of *Holothuria atra* (red square) based on COI gene.

Based on the findings of the phylogenetic analysis, it can be observed that H. atra specimens collected from Tablolong Beach, Kupang have a close evolutionary relationship with *H. atra* populations from various geographic locations, including Australia, New Caledonia, Pulao Doi Indonesia, Papua, Japan, and South Africa. Nevertheless, it is worth noting that *H. atra* specimens collected from Tablolong Beach, Kupang have a remarkably similar genetic kinship to those collected from Pulao Doi, Indonesia. This is evidenced by a genetic distance of 0.006, indicating that only 6 out of every 1,000 DNA bases exhibit variation in their base sequences (Table 4). From a geographical perspective, it can be observed that these two regions possess bodies of water that intermingle due to the effect of various currents and oceanographic elements.

The distribution of biodiversity and gene flow in marine waters is influenced by several oceanographic conditions, either directly or indirectly. The hydrodynamic patterns of ocean currents exert a significant impact on the geographical distribution of marine nutrients (Perassoli et al 2020), as well as on the biodiversity and gene flow dynamics, operating over extensive spatial scales and prolonged temporal durations (Rodrigues et al 2015). The water flow originating from the South Pacific exhibits an indirect movement as it traverses the Lifamatola Strait towards the Banda Sea. This flow possesses a notably higher saline level and greater density. The concentration of this water mass in the Banda

Sea is facilitated by the interaction of currents originating from both the eastern and western directions. The mixing of water masses is influenced by various factors, including tidal effects, Ekman spirals, and the interaction between warm fresh water on the ocean surface and variations in sea surface temperature. Water movement originating from the Banda Sea will flow outwards through the Timor Sea, Ombai Strait, and Lombok Strait in a southerly direction towards the Indian Ocean. The similarity in biodiversity, especially the genetic similarity of various species in this aquatic region, can be attributed to passive distribution patterns and to the impact of global water mass movements. Despite H. atra not being a migratory species, it exhibits planktonic behavior during the egg to larva stages, allowing it to be transported by ocean currents.

The most distant intraspecies relationship is observed between *H. atra* specimens collected from Tablolong Beach, Kupang, and those collected from South Africa. This phenomenon arises as a result of limited genetic exchange caused by the considerable geographical separation and the influence of oceanic variables. A limited level of gene flow results in the absence of gene exchange between populations, hence exacerbating the genetic divergence due to varying environmental stressors. The genetic dissimilarity amongst the aforementioned groups is quantified as 0.022, indicating that among a total of 1000 DNA base pairs, there exist 22 distinct DNA base pairs (Valen et al 2023).

In addition, it is established that *H. atra*, found in Tablolong Beach, Kupang, exhibits a more proximate association with *H. turriscels,* as observed by the inter-species connections within the genus *Holothuria*. In order to enhance genetic variety and generate high-quality seeds, interspecies mating is occasionally required within breeding and hybridization systems (Soliman et al 2016). This measure is implemented with the objective of augmenting the quantity of cultivated seed production. Based on the findings of this study, it is possible to draw numerous recommendations by examining the relationships within and between species. In the context of interspecies breeding, it is advisable to consider the crossbreeding of *H. atra* with *H. turriscelsa* and *H. nigralutea* as potential options. This particular combination is deemed to be safe due to its tight relationship, while also exhibiting a substantial genetic diversity, as indicated by a genetic distance of 0.168 and 0.179. During the initial phases of the experiment, it is imperative to ensure that the species under investigation exhibit a tight evolutionary relationship, while simultaneously possessing a sufficiently high level of diversity. This is crucial in order to facilitate effective hybridization during the early stages of the experiment.

Table 4

Estimates of evolutionary divergence between *Holothuria atra* based on COI gene

		1	2	3	4	5	6	7	8	9
1	*H. atra* East Nusa Tenggara									
2	*H. atra* New Caledonia	0.009								
3	*H. atra* Japan	0.009	0.002							
4	*H. atra* Australia	0.009	0.003	0.005						
5	*H. atra* Pulao Doi Indonesia	0.006	0.000	0.002	0.003					
6	*H. atra* South Africa	0.022	0.017	0.019	0.021	0.018				
7	*H. atra* Papua	0.011	0.002	0.002	0.003	0.000	0.017			
8	*H. leucospilota*	0.179	0.179	0.177	0.186	0.179	0.177	0.179		
9	*H. nigralutea*	0.168	0.168	0.166	0.167	0.164	0.172	0.168	0.204	
10	*H. turriscelsa*	0.184	0.171	0.168	0.172	0.173	0.174	0.171	0.192	0.210

Moreover, according to the findings derived from temperature measurements conducted in Tablolong waters, the recorded temperature is at 29-30 degrees Celsius. Hartati et al (2020) also reported that sea cucumbers typically exhibit adaptability within a temperature range of 29-30°C. Moreover, the salinity readings obtained from the

Tablolong coastline waters indicated a value of 32 parts per thousand (ppt). According to Aulia et al (2021), sea cucumbers have been seen to thrive within a salinity range of 30-31%. The pH measurements obtained from the waters of Tablolong beach showed a value of 7.5. The pH level, which represents the degree of acidity, is an environmental factor that has a significant impact on the growth of sand sea cucumbers. According to Hartati et al (2020), the pH of the surrounding waters plays a crucial role in influencing the growth of sand sea cucumbers. It has been observed that sea cucumbers thrive within a pH range of 6.50-7.50 in productive waters, while in highly productive waters, the optimal pH range for their growth is 7.50-8.50 (Aulia et al 2021). The field observations of dissolved oxygen (DO) yielded a measurement of 7 ppm. The presence of dissolved oxygen in aquatic environments is primarily attributed to two processes: air diffusion and photosynthesis by aquatic plants, encompassing microorganisms such as phytoplankton, as well as microorganisms like seagrass, macroalgae, and mangroves. The presence of dissolved oxygen is essential for the respiratory processes of some aquatic creatures, such as sand sea cucumbers (Jørgensen et al 2022). In the present study site, the observed velocity was recorded as 0.3 m sec^{-1}. Sea cucumbers have been observed to thrive in environments characterized by calm water conditions.

Conclusions. This study discovered and documented the identification of *H. atra* based on COI gene from Tablolong Beach, Kupang, East Nusa Tenggara, Indonesia. We additionally elucidate the interconnections and evolutionary patterns within the genus *Holothuria*, utilizing the COI gene as a molecular marker. The DNA-barcode of *H. atra* was successfully sequenced with a base-pair length of 710 bp using COI ceF and CO1 ceR primers. The DNA sequence obtained from Tablolong Beach was analyzed using the Basic Local Alignment Search Tool (BLAST), which identified it as belonging to the species *H. atra.* The match rate between the obtained sequence and the reference sequence was found to be between 99% and 100%. In the context of the phylogenetic tree, it is observed that *H. atra* specimens originating from Tablolong Beach, Kupang have a significant evolutionary affinity with *H. atra* populations found worldwide. The genetic distance observed ranges from 0.006 to 0.009 among the populations, indicating that out of every 1000 DNA bases, there are approximately 6 to 9 variations in the DNA bases.

Acknowledgements. The authors express their gratitude to the Directorate of Research, Technology and Community Services (DRTPM) of the Ministry of Education, Culture, Research and Technology, Republic of Indonesia, for their facilitating the research and publication process through the research grant "Penelitian Kerja Sama Dalam Negeri", with Contract Number: 184/E5/PG.02.00.PL/2023 and 3538/LL8/AL.04/2023 for the financial year 2023.

Conflict of interest. The authors declare no conflict of interest.

References

Aulia E. D., Muzaki F. K., Saptarini D., Setiawan E., Setiamarga D., Lutvianti I. D., Rosyidah S. K., Muhammad N. A., 2021 Diversity of sea cucumber from intertidal area of Pacitan and Bangkalan, East Java, Indonesia. Biodiversitas 22:2136-2141.

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(6):e0198109.

Bordbar S., Anwar F., Saari N., 2011 High-value components and bioactives from sea cucumbers for functional foods—A review. Marine Drugs 1761-1805

Bruckner A. W., Johnson K. A., Field J. D., 2003 Conservation strategies for sea cucumbers: Can a CITES Appendix II listing promote sustainable international trade? SPC Beche-de-mer Information Bulletin 18:24–33.

Dhinakaran D. I., Lipton A. P., 2014 Bioactive compounds from *H. atra* of Indian ocean. Springerplus 3:673.

Felsenstein J., 1985 Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.
Gani A., Nurjirana Bakri A. A., Adriany D. T., Wuniarto E., Khartiono L. D., Satria D. H., Hasan V., Herjayanto M., Burhanuddin A. I., Moore A. M., Kobayashi H., 2021 First record of *Stiphodon annieae* Keith & Hadiaty, 2015 (Teleostei, oxudercidae) from Sulawesi Island, Indonesia. Check List 17(1):261-267.
Guo M., Yuan C., Tao L., Cai Y., Zhang W., 2022 Life barcoded by DNA barcodes. Conservation Genetics Resources 14:351–365.
Hartati R., Zainuri M., Ambariyanto A., Widianingsih W., 2020 Feeding selectivity of *H. atra* in different microhabitat in Panjang Island, Jepara (Java, Indonesia). Biodiversitas 21:2233-2239.
Hasan V., Andraini N. E., Isroni W., Sari L. A., Nafisyah A. L., Dewi N. N., Putri D. N. A., Prasasti T. A. B., Ramadhani A. A., Daniel K., South J., Vieira L. O., Ottoni F. P., Maftuch, Faqih A. R., Wirabuana P. Y. A. P., Tamam M. B., Valen F. S., 2023a Fish diversity of the Bengawan Solo River estuary, East Java, Indonesia. Biodiversitas 24(4):2207-2216.
Hasan V., Vieira L. O., South J., Ottoni F. P., 2023b First record of *Mastacembelus notophthalmus* (Actinopterygii: Synbranchiformes: Mastacembelidae) for Belitung Island, Indonesia. Acta Ichthyologica et Piscatoria 53:123-127.
Hasan V., South J., Katz A. M., Ottoni F. P., 2022 Premier signalement du lotier aux petits yeux *Prionobutis microps* (Weber, 1907) (Teleostei: Eleotridae: Butinae) à Java, Indonésie. Cybium 46(1):49-51.
Hasan V., Valen F. S., Islamy R. A., Widodo M. S., Saptadjaja A. M., Islam I., 2021 Short communication: Presence of the vulnerable freshwater goby *sicyopus auxilimentus* (gobiidae, sicydiinae) on Sangihe Island, Indonesia. Biodiversitas 22(2):571-579.
Hebert P. D. N., Cywinska A., Ball S. L., deWaard J. R., 2003 Biological identifications through DNA barcodes. Proceedings of the Royal Society of London 270:313–321
Hoareau T. B., Boissin E., 2010 DNA barcoding design of phylum-speciûc hybrid primers for DNA barcoding: addressing the need for efûcient COI ampliûcation in the Echinodermata. Molecular Ecology Resources 10:960-967.
Insani L., Jatayu D., Valen F. S., Widodo M. S., Hasan V., 2022 Comparing genetic *Mystacoleucus marginatus* and *Mystacoleucus padangensis* (Cypriniformes: Cyprinidae) based on Cythochrome C Oxidase sub unit I (COI) gene. Iranian Journal of Ichthyology 9(4):195–203.
Isroni W., Sari P. D. W., Sari L. A., Daniel K., South J., Islamy R. A., Wirabuana P. Y. A. P., Hasan V., 2023 Checklist of mangrove snails (Gastropoda: Mollusca) on the coast of Lamongan District, East Java, Indonesia. Biodiversitas 24(3):1676-1685.
Jørgensen B. B., Wenzhöfer F., Egger M., Glud R. N., 2022 Sediment oxygen consumption: Role in the global marine carbon cycle. Earth-Science Reviews 223:103987.
Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018 MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.
Liu K., Zhao S., Yu Z., Zhou Y., Yang J., Zhao R., Yang C., Ma W., Wang X., Feng M., Tang Y., Li K., Zhou C., 2020 Application of DNA barcoding in fish identification of supermarkets in Henan province, China: More and longer COI gene sequences were obtained by designing new primers. Food Research International 136:109516.
Lutfiatunnisa, Widodo M. S., Faqih A. R., Sari L. D., Valen F. S., 2020 Molecular approach for identification of Asian seabass (*Lates calcarifer* Bloch 1790) based on COI gene sequence from Java, Indonesia. AACL Bioflux 13(5):2828-2834.
Madduppa H., Taurusman A. A., Subhan B., Anggraini N. P., Fadillah R., Tarman K., 2017 DNA barcoding reveals vulnerable and not evaluated species of sea cucumbers (*Holothuroidea* and *Stichopodidae*) from Kepulauan Seribu reefs, Indonesia. Biodiversitas 18:893-898.
Mazlan N. B., Abd Rahman N. N. B., Shukhairi S. S. B., Nazahuddin M. N. A. B., 2023 Sea cucumbers: Source of nutritional, medicinal, and cosmeceutical products. In: Marine biotechnology: Applications in food, drugs and energy. Shah M. D., Ransangan J., Venmathi Maran B. A. (eds), pp. 171–188, Springer, Singapore.

Meirinawati H., Prayitno H. B., Indriana L. F., Firdaus M., Wahyudi A. J., 2020 Water quality assessment and monitoring of closed rearing system of the sea cucumber *H. scabra*. ASEAN Journal on Science and Technology for Development 37(2):73-80.
Mujahidah K., Ramadan A., Hasan V., Yanti S., Islam I., Iqrar I., 2023 Zeolite-microfragmenting media: A potential strategy to accelerate coral growth. E3S Web of Conferences 374:00020.
Nomleni A., Widodo M. S., Kilawati Y., Valen F. S., 2020 Contemporary records of sea urchin *Tripneustes gratilla* (Echinodermata: Echinoidea) in Timor Island, Indonesia. AACL Bioflux 13(4):1899-1905.
Nurjirana, Burhanuddin A. I., Keith P., Haris A., Moore A. M., Afrisal M., Gani A., Hasan V., Wuniarto E., Bakri A. A., Adriany D. T., 2022 Additional records of *Sicyopus discordipinnis* (Watson, 1995) (Oxudercidae: Sicydiinae) in Central Sulawesi, Indonesia. Cybium 46(1):41-43.
Perassoli F., Ghisolfi R. D., Lemos A. T., 2020 Spatial distribution of nutrients associated with water masses in the Tubarão Bight (20°S–22°S), Brazil. Journal of Marine Systems 212:103425
Prescotta J., Riwub J., Prasetyoc A. P., Staceyd N., 2017 The money side of livelihoods: Economics of an unregulated small-scale Indonesian sea cucumber fishery in the Timor Sea. Marine Policy 82:197-205.
Purcell S. W., Conand C., Uthicke S., Byrne M., 2016 Ecological roles of exploited sea cucumbers. Oceanography and Marine Biology: An Annual Review 54:367-386.
Purcell S. W., Hair C. A., Mills D. J., 2012 Sea cucumber culture, farming and sea ranching in the tropics: Progress, problems and opportunities. Aquaculture 368-368:68-81.
Purcell S. W., Mercier A., Conand C., Hamel J. F., Toral-Granda M. V., Lovatelli A., Uthicke S., 2013 Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. Fish and Fisheries 14:34-59.
Rasyid A., Putra M. Y., Yasman, 2023 Antibacterial and antioxidant activity of sea cucumber extracts collected from Lampung waters, Indonesia. Kuwait Journal of Science 50(4):615-621.
Rodrigues F., Valente S., González-Wanguemert M., 2015 Genetic diversity across geographical scales in marine coastal ecosystems: *Holothuria arguinensis* a model species, Journal of Experimental Marine Biology and Ecology 463:158-167.
Robin, Insani L., Swarlanda., Prananda M., Valen F S., 2022 Range extension of Spanner barbs, *Barbodes lateristriga* (Valenciennes, 1842) (Cypriniformes: Cyprinidae) to Bangka Island, Indonesia. Iranian Journal of Ichthyology 9(3):149–157.
Robin, Valen F. S., Ramadhanu D., Nomleni A., Turnip G., Insani L., 2023 A new distributional record of flying barb, *Esomus metallicus* (Actinopterygii: Cyprinidae), from Kapalo Banda River, West Sumatra, Indonesia. International Journal of Aquatic Biology 11(1):59–68.
Saitou N., Nei M., 1987 The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.
Sanger F., Nicklen S., Coulson A. R., 1977 DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences (PNAS)74(12):5463–5467.
Soliman T., Fernandez-Silva I., Reimer J. D., 2016 Genetic population structure and low genetic diversity in the over-exploited sea cucumber *Holothuria edulis* Lesson, 1830 (Echinodermata: Holothuroidea) in Okinawa Island. Conservation Genetics 17(4):811-821.
Sulardiono B., Hartoko A., Aini A. N., Wulandari D., Budiharjo A., 2021 Genetic diversity of commercial sea cucumbers *Stichopus* (Echinoderm: Stichopodidae) based on DNA Barcoding in Karimunjawa, Indonesia. Biodiversitas 23:922-927.
Tadmor-Levi R., Borovski T., Marcos-Hadad E., Shapiro J., Hulata G., Golani D., David L., 2022 Establishing and using a genetic database for resolving the identification of fish species in the Sea of Galilee, Israel. PLoS One 17(5):e0267021.
Tamura K., Nei M., Kumar S., 2004 Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030-11035.

Valen F. S., Notonegoro H., Pamungkas A., Swarlanda, Hasan V., 2023 Revolutionary breakthrough: Unveiling the first DNA barcoding of the endemic wild *Betta burdigala* (Kottelat and Ng 1994) (Anabantiformes: Osphronemidae): A critically endangered wild Betta from Bangka Island, Indonesia. International Journal of Agriculture and Biology 30(4):269-275.
Valen F. S., Sambah A. B., Wicaksono K. P., Widodo M. S., Soemarno, Hasan V., 2021 Genetic diversity of yellow finnedbarb *Mystacoleucus marginatus* (Valenciennes, 1842) (Teleostei, Cyprinidea) in Brantas basin Upstream, Indonesia. Ecology, Environment and Conservations 27(2):695-699.
Valen F. S., Soemarno, Widodo M. S., Wiadnya D. G. R., Hasan V., 2020 Contemporary distribution records of yellow finned barb *Mystacoleucus marginatus* (Valenciennes, 1842) in brantas basin, Indonesia. Ecology, Environment and Conservation 26:S40-S43.
Valen F. S., Widodo M. S., Islamy R. A., Wicaksono K. P., Soemarno, Insani L., Hasan V., 2022 Molecular phylogenetic of silver barb *Barbonymus gonionotus* (Bleeker, 1849) (Cypriniformes: Cyprinidae) in Java, Indonesia. IOP Conference Series: Earth and Environmental Science 1036(1):012011.
*** National Center for Biotechnology Information, NCBI, 1988 Bethesda (MD): National Library of Medicine, US, https://blast.ncbi.nlm.nih.gov/Blast.cgi

Received: 30 October 2023. Accepted: 14 December 2023. Published online: 03 January 2024.
Authors:
Neri Kautsari, Aquatic Resources Management Department, Faculty of Animal Husbandry and Fisheries, Samawa University, Sumbawa Besar, Indonesia, e-mail: nerikautsari040185@gmail.com
Suleman, Aquaculture Study Program, Faculty of Animal Husbandry, Marine and Fisheries, Nusa Cendana University, East Nusa Tenggara, Indonesia, e-mail: suleman@staf.undana.ac.id
Asriati Djonu, Aquaculture Study Program, Faculty of Animal Husbandry, Marine and Fisheries, Nusa Cendana University, East Nusa Tenggara, Indonesia, e-mail: asriati@staf.undana.ac.id
Fitri Sil Valen, Aquaculture Department, Faculty of Agriculture Fisheries and Biology, University of Bangka Belitung, Jl Kampus terpadu UBB, Balunijuk, Bangka Belitung 33127 Indonesia, e-mail: fitrisilvalen@ubb.ac.id

How to cite this article:
Kautsari N., Suleman, Djonu A., Valen F. S., 2024 DNA barcoding and phylogenetic of lollyfish, *Holothuria atra* (Jaeger, 1833) (Actinopoda: Holothuriidae), from Tablolong Beach, Kupang, East Nusa Tenggara, Indonesia. AACL Bioflux 17(1):1-12.