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Molecular Identification of the Octopus (*Octopus cyanea*, Gray 1849) Using Mt-DNA COI from Alas Strait Waters, Indonesia

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ABSTRACT

Octopus cyanea is an important global fishery commodity that inhabits a wide range of intertidal reefs in the Indo-West Pacific region. It plays a significant role in fisheries and is classified as an economically important species due to its high nutrient content. Understanding species diversity is essential for managing octopus's resources, and effective fisheries management planning strategies are needed, particularly for octopus fisheries. In this research, the standard for species identification used as part of the DNA barcoding framework is the cytochrome c oxidase subunit I (COI) gene. The study aimed to determine octopus species based on phylogenetic analysis of COI mtDNA in the Alas Strait. Octopus sampling was conducted in July 2023 using a 10m long and 3m high octopus fishing pole, known as an octopus pocong. Samples were collected from six locations in the Alas Strait: Pringgabaya, Labuhan Haji, Tanjung Luar, Poto Tano, Labuhan Lalar, and Benete. Tentacle samples were taken by cutting approximately 5cm of the tentacles with a sterile knife, then placed in 96% ethanol and labeled. This research identified two species of octopus: *Octopus laqueus* and *Octopus cyanea*. Among the six collection locations, *Octopus cyanea* was the dominant species. Barcoding results using BLAST with primers LCO1490/HCO2198 demonstrated their suitability for octopus's identification in this study. Overall, this research highlights the feasibility of using COI sequences for species identification, providing an initial dataset for future octopus DNA barcoding, especially in the waters of the Alas Strait.

INTRODUCTION

Development of the fisheries sector is a government policy which is expected to increase fish production, increase fishermen's income, increase fish consumption and increase regional/state income. One of the waters that has great potential for development of the fisheries sector in West Nusa Tenggara (NTB) Province is the waters of the Alas Strait. This strait separate two large islands in NTB Province: Lombok Island and Sumbawa Island **(Zamuz *et al*., 2023)**.

The Alas Strait is a narrow strait, but there are a large number of fishermen who depend on it for their livelihood living on the coast of this strait. The total population in the Alas Strait coastal area is almost 20% of whom live in the area as traditional fishermen. This is indicated by the predominance of relatively small boat sizes (< 5 GT). The existence of these small boats reaches 93% of the total number of boats in the Alas Strait waters. The small size and large number of boats result in excessive fishing in coastal waters, especially for potential commodities **(Dissegna *et al*., 2023)**.

The octopus *Octopus cyanea* shares common characteristics as other merobenthic octopus e.g., high fecundity, fast growth, short lifespan, low age maturation **(Uriarte *et al*., 2014; Cripps & Harris, 2018)**. Due to these reproductive features, the *Octopus cyanea* fishery is thought to be at low vulnerability from fishing pressure (**Cripps & Harris, 2018; Willer *et al.*, 2023)**. However, concerns about overexploitation have been raised in some parts of the world, including certain areas of Indonesia **(Wiryawan *et al*., 2022; Noegroho *et al.*, 2023)**.

The condition of overfishing in the waters of the Alas Strait is highlighted by **Zamuz *et al.* (2023)**, who reported a decline in octopuses' production in this region. This situation is further supported by data from the Center for Data, Statistics and Information (2023), which indicates that octopuses' fishing has increased in the Alas Strait due to a rapid rise in the number of fishing fleets.

According to the Center for Data, Statistics and Information (2022), octopuses' production in Indonesia from 2010 to 2021 showed significant fluctuations, with both increases and decreases in annual figures. In 2010, the production value reached 546,693 tons, peaking in 2019 at 25,559,295 tons, but declining to 17,385,003 tons in 2021. Despite this decrease, the production level remains relatively high, raising concerns about the potential for excessive exploitation in the future.

Overexploitation can lead to the extinction of species **(Asada *et al.*, 2021)** and may also be driven by various factors, including parasites, pathogens, pollution, exploitation, and climate change **(Nieuwenhove *et al.*, 2019)**.

This research is essential because the cephalopod fishery, particularly octopus, has not been extensively studied, and this is the first investigation focusing on molecular identification in the Indonesian Alas Strait **(Omar *et al*., 2020)**. Research by **Ramadhaniaty *et al.* (2022)** on the phylogenetic analysis of *O. vulgaris* indicated that not all samples of this species were accurately identified. Thus, understanding species diversity is crucial for effective management of octopus resources, and a strategic fisheries management plan for octopuses' fisheries is needed.

This study aimed to accurately identify *O. cyanea* using mitochondrial DNA cytochromes from various areas in the waters of the Alas Strait, Indonesia. **Li *et al.* (2022)** suggest using mitochondrial DNA COI as part of a DNA barcoding framework to identify species and determine species boundaries. Previous research has successfully employed mtDNA COI to identify various marine organisms, including giant clams **(Islands, 2020)**, cephalopods **(Petrić *et al.*, 2023)**, fish **(Suzuki-Ohno *et al.*, 2023)**, and copepods **(Velasquez *et al.*, 2023)**. Notably, only a small amount of tissue is required as a sample **(Subirana & Messeguer, 2023)**. In this study, COI mtDNA phylogenetic analysis was utilized to determine octopus species collected from several locations in the Alas Strait.

MATERIALS AND METHODS

Time and location

The primary goal of collecting octopus specimens is to obtain, properly label, and preserve an adequate number and size distribution of individuals for reproductive analysis. The examination of these samples aims to identify the species using molecular techniques and DNA testing.

Octopus sampling was conducted in July in the waters of the Alas Strait using an octopus pocong, which measures 10m long and 3m high, serving as an octopus fishing pole. Samples were collected from six locations in the Alas Strait: Pringgabaya (8º34’42’’S, 116º39’29’’E), Labuhan Haji (8º38’00’’S, 116º37’13’’E), Tanjung Luar (8º44’02’’S, 116º33’18’’E), Poto Tano (8º35’07’’S, 116º46’29’’E), Labuhan Lalar (8º44’01’’S, 116º45’07’’E), and Benete (8º50’58’’S, 116º43’23’’E) (Fig. 1).

Octopuses were caught by fishermen between 06:00 and 12:00 and were immediately processed. Samples were collected by cutting approximately 5cm from the tentacles using a sterile knife, then placed in 96% ethanol and labeled. All samples taken from the six locations received the same treatment.

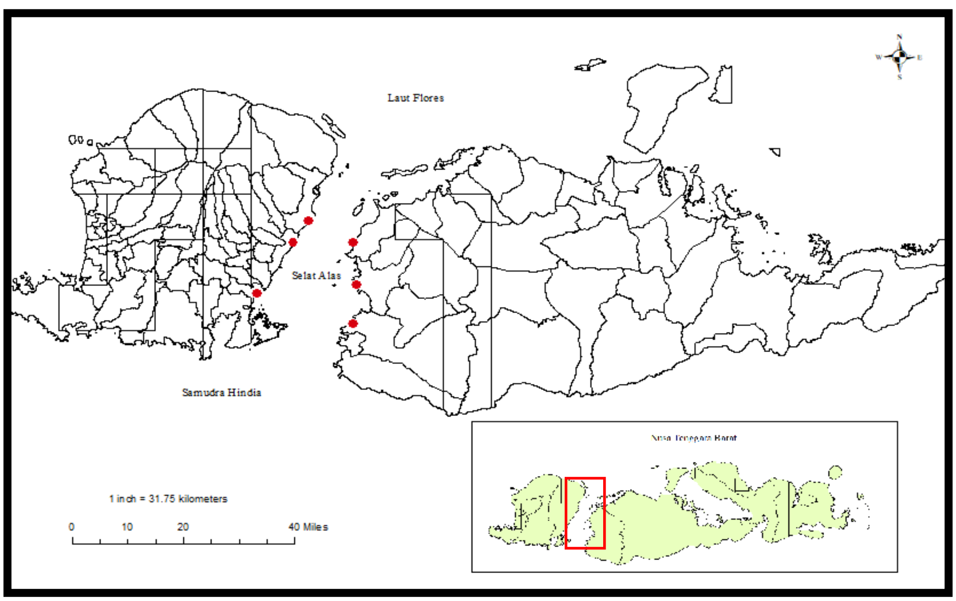

Fig. 1. Map of research locations

1. Experimental procedure

Sample extraction was performed using the DNeasy Blood & Tissue Kit. Approximately 25 mg of tissue samples were collected with tweezers and placed into a 1.5ml tube. Prior to collecting the tissue, the tweezers were dipped in 95% ethanol and sterilized using a Bunsen flame, following the protocol outlined by **Suzuki-Ohno *et al.* (2023)**.

Next, 180µl of Buffer ATL and 20µl of proteinase K were added to the tube. The mixture was vortexed and centrifuged for about 20 seconds, then incubated in a heating block at 56°C overnight. Following this, 200µl of Buffer AL was added, and the solution was vortexed and incubated at 56°C for 10 minutes. Subsequently, 200µl of 96% ethanol was added and vortexed. The sample and reagent mixture were then transferred to a DNeasy Mini spin column and placed in a 2ml collection tube **(Li *et al.*, 2023)**.

Centrifugation was performed at 8,000rpm for 1 minute, after which the liquid in the collection tube was discarded. The spin column was then placed in a new 2ml collection tube, and 500µl of Buffer AW1 was added. Another centrifugation step at 8,000rpm for 1 minute followed, and the liquid was discarded again. The spin column was then transferred to a new collection tube, and 500µl of Buffer AW2 was added. This was centrifuged at 14,000rpm for 3 minutes, after which the liquid and collection tube were discarded.

The spin column was transferred to a new 1.5ml tube, and DNA was eluted by adding 100µl of ddH2O to the center of the membrane. This was incubated at room temperature (15-25°C), followed by centrifugation at 8,000rpm for 1 minute. This elution step was repeated with an additional 100µl of ddH2O, yielding a final volume of 200 µl. The extraction solution was then ready for amplification **(Barord *et al.*, 2023; Pirhadi *et al.*, 2023)**.

The extracted DNA was analyzed in the next stage, which involved PCR (Polymerase Chain Reaction). The primers used for amplifying the samples of octopus were LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'- TAA ACT TCA GGG TGA CCA AAA ATC A') **(Muhammad *et al.*, 2018)**. The total PCR reaction volume was 26µL, consisting of: 2µL of the DNA template from the extraction, 1.25µL of each primer (10 mM), 9µL of ddH2O, and 12.5µL of Ready Mix **(Liu *et al.*, 2023)**.

Amplification was performed using an Applied Biosystems™ 2720 Thermal Cycler. The PCR protocol consisted of the following stages: initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 58°C for 30 seconds; and extension at 72°C for 30 seconds. This cycle was repeated for 40 cycles, followed by a final extension at 72°C for 5 minutes **(Pérez *et al.*, 2023)**.

The PCR results were visualized on a 1% agarose gel stained with Nucleic Acid Gel Stain (GelRed®) **(Bogun, 2023)**. Samples displaying positive results (luminescent DNA bands) were then sent for DNA sequencing using the Sanger dideoxy method at PT. Genetic Science Jakarta.

RESULTS

The results of the BLAST analysis revealed identification percentage values ranging from 91.05 to 100%, with query coverage percentages between 97 and 100% (Table 1). These findings confirm the presence of the species *Octopus cyanea*. The alignment of nucleotide sequences and the phylogenetic tree constructed from the samples showed consistent results with the GenBank database, further validating the identification of *O. cyanea* (Table 1 & Fig. 1).

The specimens examined were fresh, displaying vibrant colors without any signs of pallor or redness. The tentacles were fully extended, and the octopuses retained their complete body structure. Additionally, the specimens emitted a fresh smell and exhibited significant mucus secretion, with visible foam present **(Petrosino *et al.*, 2022)**.

Table 1. Barcode results using BLAST from samples using LCO1490 / HCO2198 primers

Field ID	sample ID	Species	Bp	Accession number	Query cover (%)	Identity (%)
1.	K01_Pringgabaya	*O. cyanea*	683	MW556238.1	100	100
2.	K02_Pringgabaya	*O. cyanea*	683	MW556237.1	100	100
3.	LH01_LabuhanHaji	*O. cyanea*	683	MW556236.1	100	100
4.	LH02_LabuhanHaji	*O. cyanea*	683	MW556235.1	100	100
5.	TL01_TanjungLuar	*O. cyanea*	683	MW556234.1	100	100
6.	TL02_TanjungLuar	*O. cyanea*	683	MW556233.1	100	100
7.	P01_PotoTano	*O. cyanea*	683	MW556232.1	100	100
8.	P02_PotoTano	*O. cyanea*	683	MW556231.1	100	100
9.	T01_LabuhanLalar	*O. laqueus*	683	MW556230.1	91.05	97
10.	T02_LabuhanLalar	*O. laqueus*	683	MW556229.1	91.05	97
11.	J01_Benete	*O. cyanea*	683	MW556228.1	100	100
12.	J02_Benete	*O. cyanea*	683	MW556227.1	100	100

The day octopus, *Octopus cyanea*, is an important global fishery commodity found in a variety of intertidal reefs throughout the Indo-West Pacific region **(Willer *et al.*, 2023)**. While this species is not commercially exploited in Australia, it is the primary octopus species targeted in Indonesia **(Willer *et al.*, 2023)**. *O. cyanea* can inhabit shallow waters and is also found at depths of 4 to 5 kilometers **(Ainsworth *et al.*, 2023; Imran *et al.*, 2023)**.

Typically, octopuses are social and can be seen swimming together in large groups. They are benthic creatures that often seek shelter in the crevices of coral, rocks, and seaweed in coastal waters **(Roura *et al.*, 2023)**. Their preferred habitats include rocky areas with holes where they can hide. Octopuses navigate their environment by crawling on the rocky or sandy seafloor, using their eight arms, which are connected at the base by a thin, strong sheet of skin **(Macchi *et al.*, 2022)**.

Fig. 2. Documentation of the octopus species studied

The octopus's main behavior is that it changes color quickly in case of enemy attack. Apart from that, if the octopus is frightened, it will emit water through the siphon so that the octopus can move forward or run away **(Battaglia *et al*., 2023)**. Some octopus species that inhabit deep-sea environments possess webbed arms resembling umbrellas, allowing them to swim similarly to jellyfish. Unlike many other marine creatures, octopuses do not have physical weapons to defend themselves against predators. Instead, their primary defense mechanism is to evade threats. When attacked, they often flee and seek refuge by hiding in coral, rocks, or even burrowing into sand for protection **(Andrade *et al.*, 2023; Park *et al.*, 2023**). This ability to camouflage and conceal themselves is essential for their survival in the diverse and often perilous habitats they occupy.

Octopus is a species known for its rapid growth and short life cycle and does not have a high tolerance for changes in environmental conditions **(Galosi *et al.*, 2023)**. Octopus is a biota that is classified as having low adaptation to changes in the aquatic environment, because this animal prefers dimly lit habitats and feels threatened by the presence of light **(Acuña-Ramírez *et al*., 2023)**. Octopus is a marine animal biota that has low tolerance to changes in low salinity, so it is very rarely found in Baltic Sea waters because it has low salinity levels **(Noegroho *et al*., 2023)**. This animal also has a very sensitive, shy, timid and easily stressed nature, and thus it tends to like habitats with hiding places **(Wicaksono *et al*., 2023)**.

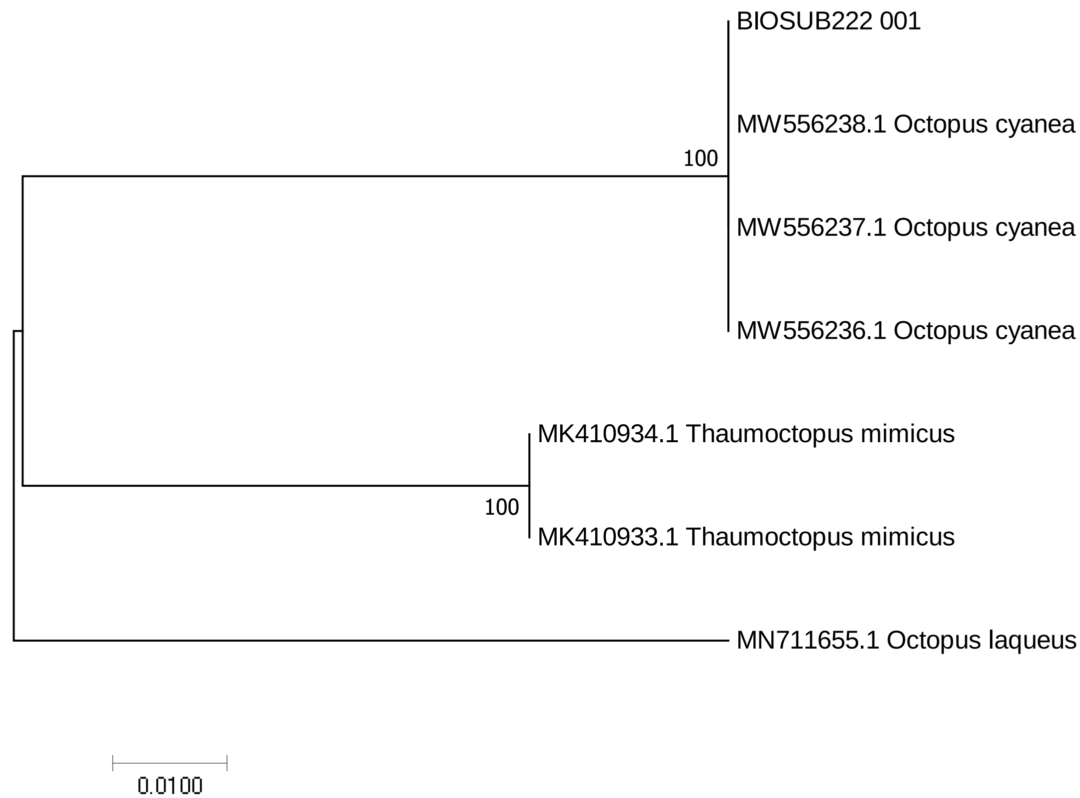

Fig. 3. Phylogenetic tree of octopus samples from six locations including Pringgabaya (8º34'42''S, 116º39'29''E), Labuhan Haji (8º38'00''S, 116º37'13''E), Tanjung Luar (8º44'02''S, 116º33'18''E), Poto Tano (8º35'07''S, 116º46'29''E), Labuhan Lalar (8º44'01''S, 116º45'07''E), Benete (8º50'58''S, 116º43'23''E)

Following the research by **Wiryawan *et al.* (2022)**, which utilized the COI marker, this study produced COI mt-DNA data with an average nucleotide sequence of 683bp from 12 individuals collected at six locations: Pringgabaya, Labuhan Haji, Tanjung Luar, Poto Tano, Labuhan Lalar, and Benete. The findings confirmed the presence of *Octopus cyanea*, with BLAST results showing identification percentage values ranging from 91.05 to 100% and query coverage between 97 and 100% (Table 1). Phylogenetic analysis corroborated these results, matching the samples to the GenBank database and confirming the species **(Kholilah *et al.*, 2021; Macchi *et al.*, 2022; Golikov *et al.*, 2023)**.

For *O. cyanea*, the average pairwise distance within the species is only 0.3%, indicating a consistent genetic makeup across various regions, including Anambas, Buton, Wakatobi, and Lombok **(Trieu *et al.*, 2023)**. The Alas Strait area, as identified by **Mulyani *et al.* (2023)**, is a suitable habitat for octopuses, making it highly likely to encounter *O. cyanea* there. Commonly known as the rock octopus, this species is widely distributed throughout the Indo-Pacific in shallow waters.

O. cyanea exhibits nocturnal activity, remaining in its den during the day **(Gonza *et***

***al.*, 2023; Wicaksono *et al.*, 2023)** It is noted for its resilience to climate change among marine species. Although heavily fished, *O. cyanea* can produce between 160,000 and 600,000 eggs **(Hakim *et al.*, 2020)**. The planktonic larvae of *O. cyanea* can drift in the water column for less than three months before settling, aided by currents that facilitate their dispersal over hundreds of kilometers **(Macchi *et al.*, 2022; Vidal & Shea, 2023)**. This adaptability likely contributes to the wide geographic distribution of *O. cyanea* throughout the waters of the Alas Strait and beyond in Indonesia.

Table 2. Nucleotide sequence of the sample

Sample Code	Sequence result
BIOSUB222.001	ATAAAGATATTGGTACTCTATATTTCATTTTCGGAATTTGATCAGGA CTTTTAGGTACTTCCCTAAGCTTAATAATTCGAACAGAATTAGGACA ACCAGGATCTCTCCTCAATGATGATCAATTATATAATGTAATTGTAA CAGCCCATGCATTTGTAATAATTTTTTTTTTAGTAATACCTGTTATAA TTGGAGGATTTGGAAATTGACTAGTCCCATTAATATTAGGAGCCCCT GATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTATTACC CCCTTCTCTAACATTGCTATTATCTTCAGCCGCAGTCGAAAGAGGTG TTGGAACTGGATGAACTGTATATCCTCCCCTTTCAAGAAATTTAGCC CATATAGGACCATCTGTTGACCTAGCTATTTTTTCTCTTCATTTAGCA GGAATTTCGTCAATTCTAGGAGCTATTAATTTTATTACTACCATTATT AATATACGATGAGAAGGTATACTAATAGAACGACTTCCTCTATTTGT ATGATCTGTATTAATTACTGCAGTTCTCTTACTACTATCCCTCCCAGT TCTTGCAGGCGCAATTACTATATTATTAACCGACCGAAATTTTAATA CTACATTCTTTGACCCAAGAGGTGGAGGAGACCCAATCTTATATCAA CATCTATTCTGATTTTTTGGT

The morphology of *Octopus cyanea* in the waters of the Alas Strait does not significantly differ from that of this species in other regions. Key morphometric measurements include mantle length, arm length, the diameter of both enlarged and normal arm suckers, sucker count, calamus length, eye orifice diameter, funnel length, head length, head width, ligule length, mantle width, pallial aperture extent, total length, and web depth.

However, notable differences exist when comparing *O. cyanea* to other species. Research by **Nateethatana (1997)**, cited in **Paruntu *et al.* (2009)**, highlights that the ocellus of *O. cyanea* is similar to that of *O. exannulatus* and *O. ocellatus*. Specifically, *O. exannulatus* features a flat oval ocellus with black spots and no rings, while *O. ocellatus* has an ocellus adorned with a pink, dark blue, or purple ring. In contrast, the ocellus of *O. cyanea* is characterized by an oval black spot surrounded by a pale inner ring and a dark outer ring.

Morphologically, the most significant difference observable in the field is the octopus's ability to camouflage itself, blending seamlessly with its environment. When captured, *O. cyanea* typically displays a color pattern that mimics its surroundings, a strategy that enhances its survival against predators. During the research in the Alas Strait, the captured octopus was noted to swim freely and to release ink as a defensive mechanism **(Ramadhaniaty *et al.*, 2022)**. This species employs camouflage not only for protection but also to assist in hunting, utilizing a variety of color patterns and skin textures that replicate the marine substrate. The octopus's complex nervous system enables rapid changes in skin color and texture, allowing it to adapt instantly to different surfaces such as sand, coral, and debris, effectively evading predators and enhancing its predatory skills.

(a) (b) (c)

Fig. 4. (a) *O. Cyanea* found in the waters of Aceh (the westernmost area of Indonesia) **(Balansada, 2019)**; **(b)** *O. Cyanea* found in Papuan waters (the easternmost area of Indonesia) **(Ramadhaniaty *et al.*, 2022)**; **(c)** *O. cyanea* found in the Alas Strait research area

CONCLUSION

In conclusion, this study identified two octopuses' species: *Octopus laqueus* and *Octopus cyanea*. The sampling locations—Pringgabaya, Labuhan Haji, Tanjung Luar, Poto Tano, Labuhan Lalar, and Benete—fall within the geographic distribution of *O. cyanea*. The results from barcoding using BLAST with the primers LCO1490/HCO2198 confirmed that the samples analyzed are indeed *O. cyanea*, highlighting the suitability of these primers for this octopus species. This research contributes valuable data for the management and conservation of octopus resources in the Alas Strait.

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