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Utilizing DNA barcoding approach to study the diversity of the blue swimming crab from Tuban District, East Java, Indonesia

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Abstract. *Joesidawati MI, Nursalim N, Kholilah N, Kurniasih EM, Cahyani NKD, Ambariyanto A. 2023. Utilizing DNA barcoding approach to study the diversity of the blue swimming crab from Tuban District, East Java, Indonesia. Biodiversitas 24: 4731-4737.* Blue swimming crabs are one of the economically significant fisheries resources in Indonesia. However, Indonesia's blue swimming crab fisheries still depend on wild catches that lead to a non-target species harvest, especially the cryptic swimming crabs. This study aims to investigate the genetic diversity of blue swimming crab specimens and other cryptic species using a molecular approach (DNA Barcoding). The samples were caught from the waters of Tuban District, East Java, Indonesia, using traditional foldable traps or *"bubu lipat"*. This study utilized the Polymerase Chain Reaction (PCR) method targeting the Cytochrome c Oxidase Subunit I (COI) gene. The sequences were then compared to the NCBI database to get the closest species based on the sequences. 21 out of 23 samples were identified as *Portunus pelagicus*, and two samples were identified as *Charybdis feriata* with percentage of similarity ranging from 99.68% to 100%. The phylogenetic trees were constructed using Neighbor Joining and Maximum Likelihood method with sequences from *Scylla serrata* as an outgroup. The genetic distance between *P. pelagicus* and *C. feriata* was 16%, whereas the genetic distance between *P. pelagicus* and *C. feriata* to the outgroup was 20%. The molecular method is crucial for species identification and population stock assessment. This information helps in determining stock boundaries, estimating population sizes, and understanding the genetic health of populations. Genetic data will help the government to have a better management of the fisheries activities in Indonesia by providing the diversity and population data.

Keywords: Blue swimming crabs, DNA barcoding, molecular identification, Tuban District

INTRODUCTION

Blue swimming crab is one of Indonesia's economically important fish resources and the third largest fisheries commodity after tuna and shrimp (Fauzi 2013). Demand of the crab meat from Indonesia is coming from several countries such as the United States of America (20,000 mt per year over the last decade) (Madduppa et al. 2021) and contributes to the country's foreign exchange (Zairion et al. 2019). The high selling value of blue swimming crab and significant demand have increased fishing activity among Indonesia fishermen, including fishermen from northern Java as part of WPP 712 (*Wilayah Pengelolaan Perikanan* or Fisheries Management Area) (Nugraheni 2016).

Blue swimming crabs can be found in many countries, including Japan, the Philippines, Malaysia, Brunei Darussalam, Indonesia, Australia, Fiji, and Eastern Africa (La Sara et al. 2017). They live in a vast range of habitats, from the seabed and mangrove, or swim at the surface of seawater as adults (La Sara et al. 2016). Blue swimming crabs are also found up to 50-meters in depth (La Sara et al. 2017). Data from East Java has shown that Blue swimming crabs accounted for approximately 71%, with retained bycatch ranging from 2 to 21%, and about 70% of crab landings are exported (APRI 2016). The primary harvesters in Tuban (East Java) are small-scale fishermen. Their boats are usually less than 10 m long and mostly caught with foldable traps or *"bubu lipat"*. An interview in Tuban indicated that most fishers were either directly contracted by collecting traders or acted as free agents, responding to higher prices (Personal communication, September 2022).

Blue swimming crab cannot be cultivated in Indonesia although research for cultivation started in 1998 in China (Mu et al. 1998), until now (Duan et al. 2022), resulting in high demand for intensive fishing (Kembaren et al. 2013). One fishing gear commonly used is collapsible traps or *"bubu lipat"*. This tool is a passive trap, where the target will enter and get trapped in it. "*Bubu lipat"* is also used for other targets, such as demersal fish, reef fish, and mud crabs (*Scylla serrata*). Catching the crabs using *"bubu lipat"* usually produces by-catch of several other organisms. For example, in Kolono Bay, Southeast Sulawesi, the by-catch of collapsible traps included 27 fish species, 20 crustacean species, seven mollusks species, and one echinoderm species (Hamid and Kamri 2021). Another fishing gear is called *"garok."* This tool is a modified trawl

with a fork along the opening of the net mouth and works by scraping, scratching, and filtering the substrate at the bottom of the waters. The downside of using *"garok"* is that it sometimes leads to by-catch protected animals such as sea turtles (Susilawati 2022).

Indonesia's blue swimming crab fisheries still depend on the wild caught. This practice can impact the sustainability of blue swimming crab resources and threaten its genetic diversity, which. put blue swimming crabs into a critical status based on IUCN (Kunsook 2014; Hidayani et al. 2020). Several ways that can be used to preserve blue crabs include: limiting catches, restocking juveniles from hatcheries, and pond cultivation (Fujaya et al. 2019).

Identification of blue swimming crab can be done by observing the morphology and genetics using molecular techniques (Jung et al. 2019). Molecular identification of blue swimming crab has been carried out in several regions in Indonesia, such as Aceh, Semarang, Barru, Maumere, Raja Ampat, Orong, and Kaimana (Hidayani et al. 2020). Furthermore, research on blue swimming crab genetic diversity shows the high genetic diversity of swimming crabs in Indonesia.

This study aims to investigate the genetic diversity of blue swimming crabs specimens and its other cryptic species collected from Tuban District, East Java, Indonesia, using a molecular approach (DNA Barcoding). DNA barcoding study is the use of short DNA sequences of targeted genes to identify specific species (DeSalle and Goldstain 2019). This method is the first step to identify genetic biodiversity and will provide baseline data for further research on blue swimming crabs populations in East Java. The genetic population data is a precious source for the future conservation and management of blue swimming crabs.

MATERIALS AND METHODS

Field sampling

A total of 23 samples of blue swimming crabs were obtained from Tuban, East Java on 1-28^{t} September 2022 (Figure 1). Samples were collected using collapsible traps or *"bubu lipat"* (Figure 2), then photographed, and identified their morphological characteristics. A small tissue (around 10 g) was collected from the last swimming legs of the blue swimming crabs samples and preserved in 96% ethanol. Samples were transported to Diponegoro Marine Biodiversity Project (DMBP) Laboratory-Integrated Laboratory (Terpadu Laboratory), Diponegoro University, Semarang-Indonesia for further analysis.

Figure 2. The picture of collapsible traps or *"bubu lipat"* used to collect the blue swimming crabs (*Portunus pelagicus*) in Tuban district, East Java, Indonesia

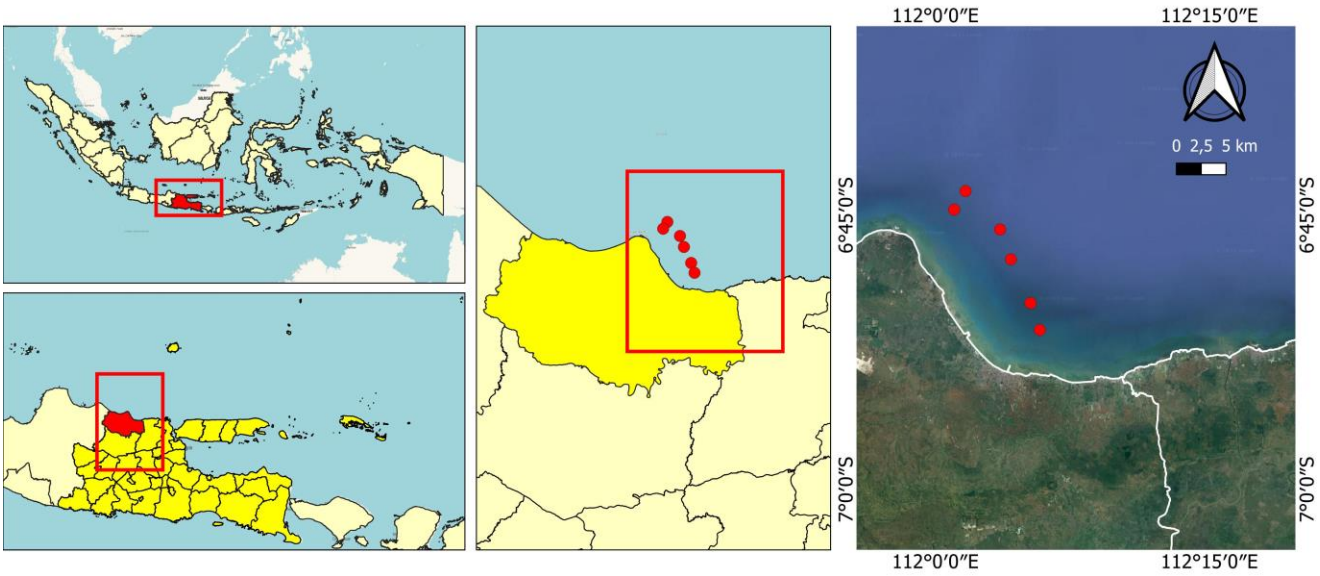

Figure 1. Sampling location in Tuban District, East Java, Indonesia

DNA extraction and amplification

DNA from the tissue samples were extracted using 10% Chelex (Walsh et al. 1991). Polymerase Chain Reaction (PCR) methods were used to amplify the DNA targeting the COI) gene. The PCR reaction was carried out in 25 µL volumes, using 1.25 µL of DNA template. Each reaction included 12.5 µL MyTeq^{TM} Red Mix (Meridian Bioscience, Ohio, United States). 1 µL of each primer and 9.25 µL ddH_2O. The COI gene was amplified using the forward primers JgLCO1490: 5'-TIT CIA CIA AYC AYA ARG AYA TTG G-3' and reverse primer JgHCO2198: 5'-TAI ACY TCI GGR TGI CCR AAR AAY CA-3' (Geller et al. 2013). The thermocycling profile included an initial denaturation of 95°C for 4 minutes, 40 cycles of 95°C for 30s, 50°C for 30s, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. The PCR reactions were checked on 1% agarose gels stained with Florosafe (1st BASE, Singapore).

Gel electrophoresis was used to visualize the results of the amplification of the COI gene fragment by PCR. This process was carried out using 1% (w/v) agarose gel, visualized with UV-transilluminator and documented with a digital camera. Amplicons were sent to the Indonesia Genetics Science laboratory for sanger sequencing method using ABI 3730xl DNA Analyzer sequence (Thermo Fisher Scientific, Massachusetts, United States).

Data analysis

Forward and reverse sequences of 23 samples were cleaned, edited, and aligned in the MEGA XI program (Kumar et al. 2018). The clean sequences were then compared to open database of NCBI (*The National Center for Biotechnology Information*; https://www.ncbi.nlm.nih.gov) using BLAST program (*Basic Local Alignment Search Tool*; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The most similar sequences from NCBI were downloaded to be aligned with the sample sequences (Table 1). Two methods were used to generate phylogenetic reconstructions: neighbor joining and maximum likelihood trees using MEGA XI (Kumar et al. 2018) with *Scylla serrata* (KC200562.1) sequence from NCBI used as an outgroup. The neighbor joining analysis and maximum likelihood analysis, based on genetic distance, using the Kimura 2-Parameter model with 1000 bootstrap were replicated to assess the clade support.

RESULTS AND DISCUSSION

The study sequenced 23 samples targeting the COI gene with a sequence length of 630 bp (base pair). COI gene has been used to identify blue swimming crabs using a molecular approach (Windsor et al. 2019; Bagheri et al. 2020; Andriyono et al. 2022). All the sequences were then submitted to NCBI with accession number OQ438120-OQ438142. The sequences were compared to the sequences in NCBI database, resulting in two species Family *Portunidae*, *Portunus pelagicus*, and *Charybdis feriata*, with a similarity range from 99.68% to 100% (Table 1). The DNA Barcoding method has been used widely to identify cryptic species including marine species (Trivedi et al. 2016). The DNA method was selected to complement the morphological identification that could be hard in cryptic organisms. On the other hand, the COI marker was selected because this gene is known to be useful in identifying many marine organisms including invertebrates (DeSalle and Goldstain 2019).

Table 1. The list of blue swimming crabs samples used in this study is based on NCBI sequences

Sample ID	Accession number	Similarity	Species
DBP012101	OQ438120	100%	*Portunus pelagicus* (MN635713.1)
DBP012102	OQ438121	100%	*Portunus pelagicus* (KP976341.1)
DBP012103	OQ438122	100%	*Portunus pelagicus* (KP976341.1)
DBP012104	OQ438123	99.68%	*Portunus pelagicus* (KP976341.1)
DBP012105	OQ438124	100%	*Portunus pelagicus* (MN635713.1)
DBP012106	OQ438125	99.84%	*Portunus pelagicus* (KP976341.1)
DBP012107	OQ438126	99.84%	*Portunus pelagicus* (KP976341.1)
DBP012108	OQ438127	99.84%	*Portunus pelagicus* (MN635713.1)
DBP012109	OQ438128	99.52%	*Portunus pelagicus* (MN635713.1)
DBP012110	OQ438129	100%	*Portunus pelagicus* (KP976341.1)
DBP012111	OQ438130	99.84%	*Portunus pelagicus* (MN635713.1)
DBP012112	OQ438131	99.68%	*Portunus pelagicus* (MN635713.1)
DBP012113	OQ438132	100%	*Portunus pelagicus* (MN635713.1)
DBP012114	OQ438133	100%	*Portunus pelagicus* (KP976341.1)
DBP012115	OQ438134	99.68%	*Portunus pelagicus* (KP976341.1)
DBP012116	OQ438135	100%	*Portunus pelagicus* (KP976341.1)
DBP012118	OQ438136	100%	*Portunus pelagicus* (KP976341.1)
DBP012119	OQ438137	100%	*Portunus pelagicus* (KP976341.1)
DBP012120	OQ438138	100%	*Portunus pelagicus* (MN635713.1)
DBP012121	OQ438139	99.84%	*Portunus pelagicus* (KP976341.1)
DBP012122	OQ438140	99.84%	*Portunus pelagicus* (KP976341.1)
DBP012123	OQ438141	100%	*Charybdis feriata (*MK091830.1)
DBP012124	OQ438142	100%	*Charybdis feriata* (MK091830.1)

Phylogenetic trees were constructed using Neighbor-Joining and Maximum Likelihood methods, and including sequences of *P. pelagicus* (KP976341.1 and MN635713.1) and *C. feriata* (KM091830.1, KM977709.1 and KP976186.1) obtained from NCBI and several sequences of other species such as *P. trituberculatus* (KT321526.1, GU321243.1 and GU321243.1), *P. armatus* (EF661902.1 and EF661901.1), *P. sanguinolentus* (KM5281299.1, KM528128.1 and MK091822), *P. segnis* (EF661958.1, EF661957.1 and EF661955.1) and *P. reticulatus* (EF661975.1, EF661976.1 and EF661974.1) (Figure 3). The 23 samples were grouped into two prominent clades and identified as two different species, *P. pelagicus* and *C. feriata*. Most of the samples (21 out of 23 individual samples) were identified as *P. pelagicus*. The genetic distance between *P. pelagicus* and *C. feriata* is 16%, and between those two species with the outgroup is 20% (Table 2).

The genetic distance between species ranges from 8.5%-89.7%. The lowest was the distance between *Portunus reticulatus* and *Portunus segnis* species and the highest was between *Prtunus segnis* and *Charybdis feriata* sepsis. The genetic distance within the same species ranges from 0%-0.8%. The lowest value was *C. feriata* and the highest was *P. sanguinolentus*.

Discussion

Blue swimming crab or *rajungan* (*Portunus pelagicus*) fishing in Indonesia is one of the high economic potential of marine fisheries commodities. Blue swimming crabs export from Indonesia reaches Rp 4,6 trillion per year. However, Indonesia's blue swimming crab fisheries still depend on wild catches that lead to a non-target species harvest, especially the cryptic swimming crabs. By using molecular approaches, this study was able to differentiate two different species from 23 samples from Tuban, East Java, Indonesia. Both species are *P. pelagicus* and *Charybdis feriata*.

P. pelagicus (Linnaeus, 1758) has a very distinct morphology and is distributed in different geographies with different morphological patterns (Lai et al. 2010; Hidayani et al. 2020). In Tuban, blue swimming crab is mainly caught with collapsible traps (*bubu lipat*) set by fishermen. A recent report studied bycatch form using traps, trammel nets, and other non-specific gear in several sites in Java (APRI 2016). The proportion of blue swimming crabs with small sizes caught has increased in the last decades and indicates this species is under over-exploitation.

This study also identifies one different species of swimming crab, *C. feriata*. Both species were grouped as swimming crabs or Portunidae. *C. feriata* was characterized by some granular transverse lines on its carapace with cream and brown colors (Stephenson 1972). Meanwhile, *P. pelagicus* has a blue marking in the carapace with white spots (Anbarasu et al. 2019). *C. feriata* is usually found in muddy to sandy, and rocky bottoms in depths of approximately 10-100 m (Kumar et al. 2019). Meanwhile, *P. pelagicus* is commonly found in rocky coastal areas, coral reefs, sandy-muddy bottoms, and macroalgae areas. Environmental and genetic factors influence differences in color and pattern on the carapace. With increasing size and molting, there was a color change, but there was no difference in pattern (Anbarasu et al. 2018).

The molecular diversity of blue swimming crabs samples can be identified by genetic distance where the same species usually has a genetic distance of less than 3% (Bucklin et al. 2011; Insafitri et al. 2023). This is in accordance with the results of research, where *P. pelagicus* and *C. feriata* have a 16% genetic distance, and so do other species with a value of 8.5%-89.7% (Table 2). Not only the genetic distance between species can be calculated, but within species it can also be done. Genetic distances within the same sepsis reflect inter-individual nucleotide variation (diCenzo and Finan 2017). In this study the genetic distance of *C. feriata* was 0%, this indicates no nucleotide variation. While in *P. pelagicus* 0.5%, these results illustrate that there are nucleotide differences. As well as the genetic distance of other species ranging from 0.2-0.8% (Table 3).

Table 3. Genetic distance Within *Portunus pelagicus, Charybdis feriata, Portunus trituberculatus, Portunus sanguinolentus, Portunus reticulatus, Portunus segnis* and *Portunus armatus* clades obtained from MEGA XI program Kimura 2 parameter

Species	Genetic distance
P. pelagicus	0.005
C. feriata	0.000
P. trituberculatus	0.002
P. sanguinolentus	0.008
P. reticulatus	0.006
P. segnis	0.006
P. armatus	0.004

Table 2. Genetic distance between *Portunus pelagicus, Charybdis feriata, Portunus trituberculatus, Portunus sanguinolentus, Portunus reticulatus, Prtunus segnis* and *Portunus armatus* clades obtained from MEGA XI program Kimura 2 parameter

Species	*P. pelagicus*	*C. feriata*	*P. tritubeculatus*	*P. reticulatus*	*P. segnis*
P. pelagicus					
C. feriata	0.154				
P. tritubeculatus	0.151	0.177			
P. reticulatus	0.739	0.846	0.841		
P. segnis	0.759	0.897	0.849	0.085	

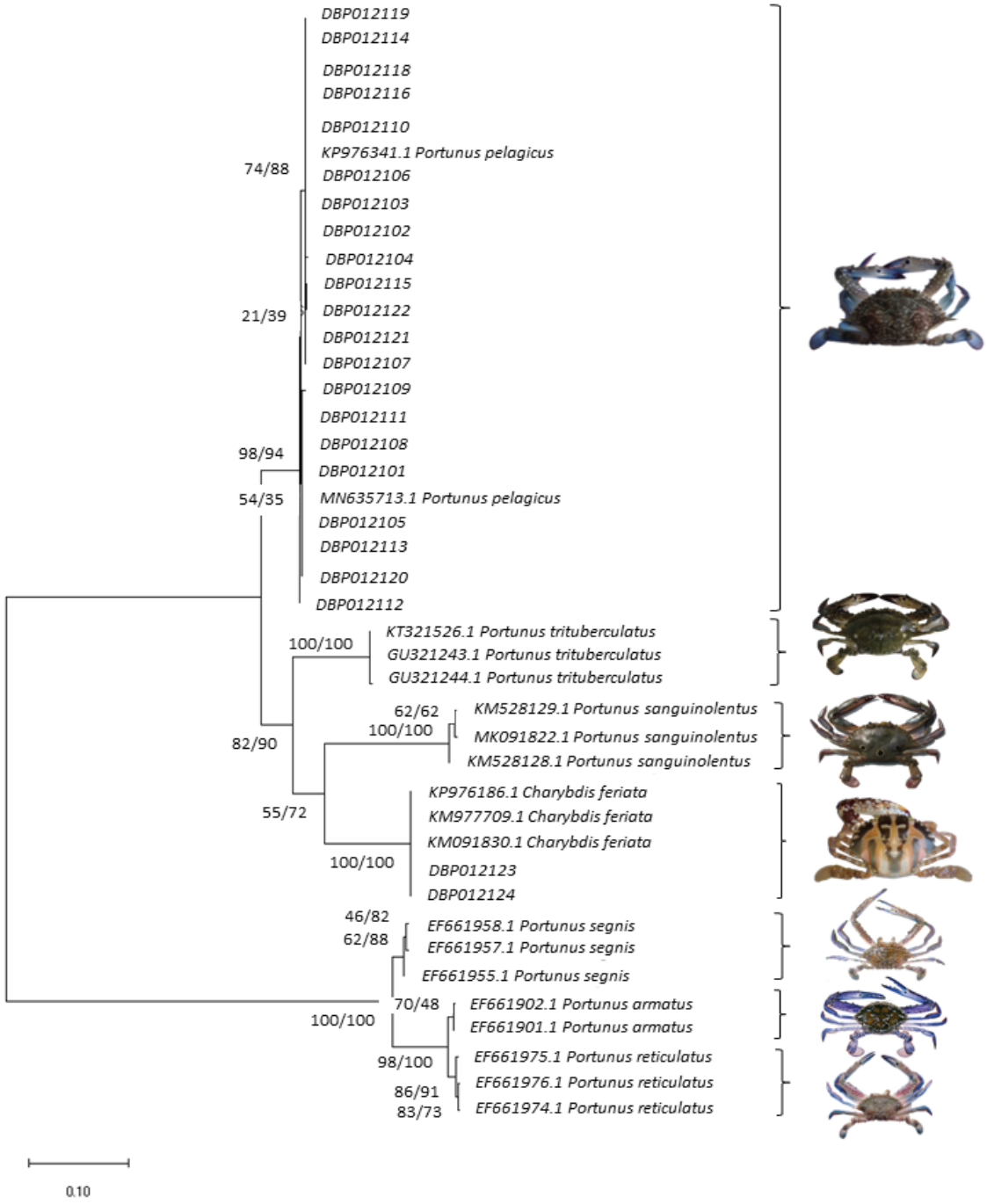

Figure 3. Phylogenetic tree with Neighbor-Joining and Maximum Likelihood topology generated from 630 bp of mtDNA COI gene. Numbers below the major nodes indicate bootstrap support for 1000 replicates using neighbor-joining and maximum likelihood, respectively

The blue swimming crab needs more genetic information for the management and knowledge observed by the fisherman in terms of the biology of the crabs (Ernawati et al. 2017). There was a dearth of information regarding Indonesia Blue swimming crab stock status, the fishery's impacts on bycatch and habitat, and its fisheries regulation, however the management of fisheries under national policy or guidance have been implemented by the local government (FPIK-UNDIP and APRI 2014). Since the blue swimming crab has acquired high commercial value and is increasingly in demand, we need to understand the genetic diversity of the blue swimming crabs in Indonesia. Minimizing the ecological impact of the blue swimming crab will be an obligatory step in its management of fishery resources.

Molecular identification techniques (DNA barcoding) are widely used to identify cryptic species or avoid mislabeling some wild catch products (Kholilah et al. 2021; Afiati et al. 2022; Nursalim et al. 2022). However, mislabeling can threaten the sustainability of a species due to the possibility of unregulated or unmanaged overfishing (Hu et al. 2018). Mislabeling can also be detrimental to consumers, considering that the price and quality consumers get are not following the costs incurred (Donlan and Luque 2019; Munguia-Vega et al. 2022). Illegal, unregulated, and undocumented (IUU) fishing is a global phenomenon that has negative effects on the economy, social, and environment (Tickler et al. 2018; Spijkers et al. 2019; Sumaila et al. 2020). In Asia, combating IUU fishing significantly weakens the rising national income (Hosch and Macfadyen 2022). In addition, the molecular method is now widely used for population stock assessment of several organisms that depend on wild catches, such as blue swimming crabs and several types of shrimp (Khamnamtong et al. 2021; Madduppa et al. 2021; Hardianto et al. 2022; Li et al. 2022; Rumisha and Kochzius 2022). Adding genetic methods to fishing management activities will help the government carry out better fisheries management.

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