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Identification of green mussel from Lampung Bay, Indonesia by using a morphological and molecular approach

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Green mussel widely distributed around the coastlines of Indonesia. The species spread from Sumatra, Java, Nusa Tenggara to Sulawesi island. In Lampung Bay, the green mussel can be easily found in a wooden substrate or bamboo in the intertidal zone near to the estuary areas. This research aimed to identify the species of green mussel by using both morphological and molecular analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene sequences. Genomic DNA extracted using a ZR Tissue & Insect DNA MiniPrepTM Kit (Zymo Research). The sequence had 706 base pairs (bp) in length. The phylogenetic analysis of specimen conducted by comparing the sequence with other sequences of mussels deposited in GenBank and showed that the specimens ware clustered together with species of *Perna viridis* which supported with a strong bootstrap (99%). This finding is important for taxonomic identification and further mariculture development.

Keywords: *Perna viridis*, phylogenetic, genetic, COI, Mytilidae

INTRODUCTION

Green mussel was harvested commercially as human food in Indonesia due to their dense, fast-growing, and inexpensive source of marine protein. That makes green mussel meat as an important fishery commodity.

Marine mussel of the genera *Perna* consists of green mussels and brown mussels. Morphologically, the identification based on the difference in color and shape of the shell. The taxonomic of the *Perna* divided into three recognized species, the green mussel or *P. viridis* (Linnaeus 1758), the brown mussel or *P. perna* (Linnaeus 1758), and the green-lipped mussel or *P. canaliculus* (Gmelin 1791) (Siddall, 1980; Vakily 1989).

The first two species have a wide distribution area. The green mussel can be easily found in the Indo-Pacific region, while brown mussel can be found along the coastline of the African continent and the Atlantic Ocean. The green-lipped mussel has a limited distribution which only found in New Zealand's coastal area (Vakily, 1989; Cunha et al., 2014).

According to Siddall (1980), in Indonesia, the existence of green mussel is limited to the center-western part of the Indonesian archipelago. Several well-studied populations of green mussel occurred in the Java Sea, while fewer studied of green mussel from the Indian Ocean, the Strait of Malacca and Sumatra coastline (Hunh et al., 2015). The green mussels have successfully cultivated along Jakarta Bay and Banten Bay, (Davy and Graham, 1982). The cultivation technology then spread to the other locations in Indonesia, including in the eastern region of Java

including Gresik and Surabaya coastline, and in Lampung Bay, Sumatra especially around the coastline of the Pasaran island whereas they used the floating rafts system in cultivating the green mussel (Ali et al., 2015; Noor, 2015; Noor et al., 2016).

Although green mussels have been successfully cultivated, however, there is a lack of information about the identification of green mussels originated from the western region of Indonesia. These identification methods usually obtained based on the conventional with morphologic-observation approaches. According to Zieritz et al., (2012), the only identification conducted by morphological characteristics is known to be often inconsistent, and the misidentification can cause significant losses associated with cultivation. For example, the breeding efforts will have a high level of error, so proper identification becomes a through basic knowledge of diversity both intra-species and inter-species levels (Yáñez et al., 2015).

On the other hand, the DNA markers are currently being used widely to detect differences among species. The DNA marker of mitochondrial genes provided a powerful tool in phylogenetic studies and assessed more quickly, accurately and effectively not only in analyzing genetic diversity but also in identifying species (Englbrecht et al., 2000). This molecular identification by using a standard short sequence of DNA, usually a mitochondrial DNA in the cytochrome c oxidase subunit I (COI) region with length ~650 base pairs (bp) (Hebert et al., 2003).

The COI sequences are well conserved within species. They have used in invertebrate taxonomic identification. It also performed reasonably well conserved within species and is therefore now being widely used in invertebrate taxonomy (An et al., 2005). Moreover, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene and the evolution of this gene is rapid enough to allow discrimination of closely allied species (Hebert et al., 2003; An et al., 2005).
Therefore this research was carried out by comparing the morphological data and intended to proper taxonomic identification by using a mitochondrial COI gene fragments. Current findings will surely benefit from being valuable in managing, conserving and providing the sustainability of green mussel resources in the future.

MATERIALS AND METHODS

Materials and Tools

The materials used are green mussels, caliper, ethanol, ice cubes, ZR Tissue & Insect MiniPrep™ DNA Kit, Zymo Research, KOD FX Neo (Toyobo), Zymoclean™ Gel DNA Recovery Kit (Zymo Research), and DNA sequence data of each species for phylogenetic analysis of GenBank (http://www.ncbi.nlm.nih.gov/). The research equipment includes polymerase chain reaction (PCR) device, machine sequencing device: MESQUITE v3.11 software, Molecular Evolutionary Genetic Analysis (MEGA) software v6.06 and Fig tree v1.4.3.

Sampling

Specimens were collected from Pasaran Island located in Lampung Bay, Indonesia. Samples were taken in August 2018 on a raft cultured at intertidal zone near to estuary (coordinates $05^{0}27'54.524"$S, $105^{0}15'39.468"$E) (Figure 1). After washing, the specimen was preserved in 90% ethanol and stored at 4˚C until analyzed in Aquaculture Laboratory, State Polytechnic of Lampung with deposit number of PNL.17.08.01.

Morphological identification

The morphological characteristics conducted by following Rajagopal et al*.,* (1998), were observed and measured the shell color, pallial line, posterior adductor muscle, posterior pedal retractor muscle, shell dimensions (length, width, and height) by a caliper.

Molecular identification

Molecular identification was carried out by mitochondrial DNA sequence in the cytochrome c oxidase subunit I (COI) region. The sequence compared to the data sequences of the mitochondrial COI of *Mytilidae* family obtained from GenBank represented three species of the genus Perna (*P. perna, P. viridis*, and *P. canaliculus*). Some out-groups species was added including *Mytilus edulis, M. galloprovincialis, Aulacomya atra maoriana, Modiolus areolatus*, and *Xenostrobus pulex* (Wood et al., 2007).

DNA Extraction, PCR Amplification, and sequencing

Genomic DNA was extracted from mussels meat using an automated DNA isolation procedure following company instructions (ZR Tissue & Insect MiniPrep™ DNA Kit, Zymo Research).

Figure 1. Cite location (A) of green mussel sampling in Pasaran coastal water, Lampung Bay, Indonesia (left), and green mussel raft cultivation system (right).

The mitochondrial DNA segment of the CO~ area of ~706 bp was amplified using a combination of forwarding primer LCO1490 (5 'GGTCAACAAATCATAAAGATATTGG 3') and reverse primer HCO2198 (5 'TAAACTTCAGGGTGACCAAAAACA 3') (Folmer et al., 1994). PCR amplification was performed with a total volume of 25 µl comprising: 5 µl dd H_2O, 12.5 µl 2x PCR Buffer KOD FX neo, 5 µl 2 mM dNTPs, 1 µl of each primer (10 pmol / µl), 1 µl DNA Templates as well as 0.5 µl Taq DNA polymerase (KOD FX Neo, 1.0 U / µl). The PCR was adjusted according to the following conditions: denaturation at 98^0C for 10 s, annealing at 52^0C for 30 s and extension at 68^0C for 45 s which repeated for 35 cycles

The PCR results then were examined in agarose gel on the electrophoresis machine. The PCR product then purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research) before being read by a sequence of DNA on a bi-directional sequencing machine.

DNA Data Sequence Analysis

The sequences are then aligned, edited and checked whether there is a stop codon or not manually using MESQUITE software v3.11 (Maddison and Maddison, 2017). Phylogenetic reconstruction was performed using MEGA software v6.06 (Tamura et al., 2013) with a maximum likelihood (ML) method. The substitution model used is by using Kimura 2-parameter (K2P), and evaluation of the nodes in the phylogenetic tree, non-parametric bootstraps was performed 1000 replications. Furthermore, the presentation and editing of phylogenetic trees were done by using Figtree software v.1.4.3 (Rambaut, 2009). The nucleotide sequence of the specimen registered to the DNA of Bank of Japan Data (DDBJ).

RESULTS

The specimens of green mussels can be found at Pasaran Island surrounding area, in the intertidal zone which has an estuary area of Way Belau River. They naturally settled on wood, bamboos, rocky surfaces, rope, vessel hulls, floating equipment such as artificial floating raft as a substrate when cultured.

The morphological characteristic of green mussels was closely related to the characteristic data from Rajagopal et al., (2006) (Table 1). Bivalves typically have two hinged shells closed by adductor muscles and the valve hold by a ligament at the hinge (Figure 2). The bysuss threads for anchoring to natural rope substrate tied to a floating rafts.

The PCR analysis performed on the green mussel specimen successfully amplifies a mitochondrial DNA of the COI region along ~706 base pairs (bp) (Figure 3). The alignment procedure to get the final length of the sequence for phylogenetic analysis is 620 bp. All sequences are then translated into amino acids to determine whether the sequences analyzed indicate insertion, deletion or stop codon. The translational results show that all sequences able to be converted to amino acids and they proceed for phylogenetic analysis.

The molecular identification determined by the phylogenetic pattern among the mussel of *Mytildae* family. The other data sequences from GenBank, primarily from the results of a study

conducted by Wood et al., (2007). Phylogenetic analysis showed that the genera of Perna are divided into three large clades, each containing individuals from three species namely *P. perna, P. canaliculus,* and *P. viridis*. Species *P. Perna* has sister species (the closest phylogenetic species of kinship) with *P. canaliculus*.

Phylogenetic analysis exhibits that the green mussel specimen from the Pasaran Island were in the same clade with *P. viridis* species originating in Philippine, Thailand, Vietnamese and Indian waters (Figure 4). The phylogenetic position placement of the specimen analyzed supported by a very high bootstrap value (99%). In addition, a valid species of *P. picta* collected from the Mediterranean, and Moroccan waters are the same clade with *P. perna* (Wood et al., 2007).

Table 1. **Diagnostic characters of green mussel from Lampung Bay, Indonesia**

Characteristic	Features
Shell colour	Green, bluish green
Shell type	Thick, equivalve, inequilateral, elongate and oval
Shell dimensions	56.5; 24.2; 15.7 mm (length, width, height)
Anterior	Pointed, like a beak and bottom-twisted
Ventral	Concave
Dorsal ligamental	Curved
Byssus apparatus	Large and at the posterior of the foot
Inner shell	Smooth and bluish-green

Table 2. Species, species origin and nucleotide codes in the Gen Bank

Species	**Species Origin**	**Gen Bank Code**
Perna viridis	Pulau Pasaran, Lampung Bay, Indonesia	LC360888*
	Chennai, India	DQ917612
	Nha Trang, Vietnam	DQ917583
	Philippines	DQ917599
	Thailand	DQ917589
Perna perna	Santa Catarina, Brazil	DQ917594
	Sao Paulo, Brazil	DQ917592
	Cumaná, Venezuela	DQ917588
	Umhlanga, South Africa	DQ917618
Perna picta	Temara, Morocco	DQ917603
	Cansado, Mauritania	DQ917597
Perna canaliculus	Houhora, New Zealand	DQ917607
Mytilus edulis	Swansea, Wales	DQ917606
Mytilus galloprovincialis	Wellington, New Zealand	DQ917605
Xenostrobus pulex	Mt Maunganui, New Zealand	DQ917582
Modiolus aerolatus	Wellington, New Zealand	DQ917604
Aulacomya atra maoriana	Wellington, New Zealand	DQ917614

* current research

Figure 2. Morphological characteristic of green mussel (six months ages)

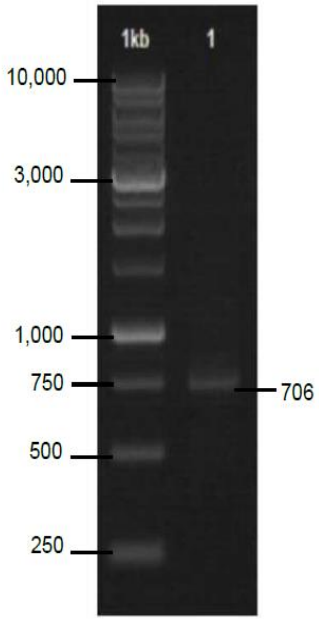

Figure 3. PCR amplification of mitochondrial DNA of the COI region of green mussels after examination with agarose gel exhibits a fragment of 706 bp.

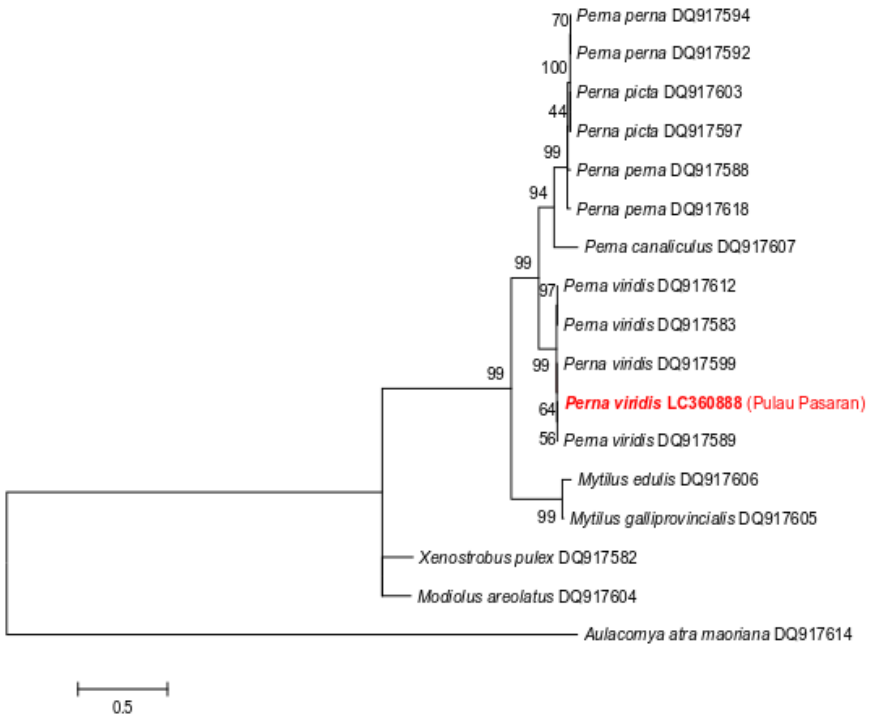

Figure 4. Phylogenetic trees are reconstructed using the Mitochondrial DNA of the COI region. *Aulacomya atra maoriana* is a species of freshwater shells and used as out group on phylogenetic analysis.

DISCUSSION

Marine mussels are highly economical and nutritional values of shellfish. One of the most attracting potential mussels for commercial cultivation in Asian countries is green mussel or Asian green mussel (*Perna viridis*). It was extensively cultured because of their value as a cheap source of animal protein for human consumption (Monirith et al., 2003).

The native habitat of the Asian green mussel is in the Indo-Pacific region, which encompasses regions between Japan to New Guinea and from the Persian Gulf to the South Pacific Islands (FAO, 2006). According to Spencer (2002), green mussel has cultivated in India, Indonesia, the Philippines, Singapore, Thailand, and Malaysia. FAO (2006), reported that globally the leading mussel producing countries of the world include: Canada, United States of America, South American countries, Korea and Japan, China, Spain, Italy, Thailand, New Zealand, France, Ireland, and the Netherlands.

In Indonesia, the species distributed along the coastline of the center to the western region of Indonesian archipelagoes. They are easily forming high densities and biomass naturally attached to the substrates. Some report indicated an increased demand for green mussel meat especially for culinary and raw material for seafood restaurants. They are filled either through manual capture by collecting from wild shellfish or by harvesting from cultivation sites in several locations in Indonesia (Evans et al., 1995; Sudharyanto et al., 2005; Ali et al., 2015).

Based on the morphological inspection to the specimen it has a closely related to the Asian green mussel (*Perna viridis*) describe by the Rajagopal et al., (2006). Mytilidae family including *P. viridis* are coastal bivalves, generally inhabits marine intertidal, subtidal and estuarine environments, which have high salinity and receive more nutrients from land run-off (Rajagopal et al., 2006).

The morphological characteristics are mainly carried out regarding the color and shape of the shell (Siddall, 1980). *P. viridis* had an outside colour of shell whitish under a bright periostracum which is dark brownish green anteriorly and olive-green to bright green posteriorly (Pouters, 1998). They typically had two hinged shells which connected with a posterior adductor muscle, and a strong ligament holds the two valves together at the hinge, has equivalve shell (equally convex) with a byssal gape (Pouters, 1998). According to Siddal (1980), the attachment through proteinaceous byssal threads during the early

stage of larvae.

In addition, Noor et al., (2019), states morphological characteristics of green mussel originated from coastal of Pasaran Island, Indonesia was shell length of males and females mussels were 55.7 and 57.3 mm at the age of 6 months while weighing were 10.38 and 9.82 g respectively. The body condition index (BCI) value of female is double compared to males, after six months of cultivation (1.63 and 0.84), and first-time gonads found at the age of 3 months after cultivation on a raft system.

The topology of the phylogenetic resulted consistent with the previous study, i.e., the *Perna* genera consist of three major groups representing three different species: *P. perna, P. canaliculus*, and *P. viridis*. Shafee et al., (1989), identified a Mediterranean green mussel species as *P. picta* (Born 1780). However, the presumed *P. picta* species from the Mediterranean originating from Morocco and Mauritania coast have the same clade with a South American *P. Perna* clade. It suggests that *P. picta* is most likely a junior synonym of *P. perna* (Thankakkon and Edward, 2013; Vallejo et al., 2016) (Table 2).

Another species identified as *P. indica*. It was only found in a restricted distribution along south Indian and north Sri Lanka coast (Appukuttan et al., 2001; Ramachandran et al., 1998). However lately, though there is no proof exists, the species might also be described actually as *P. perna* (Vakily, 1989; Hicks et al., 2001). It was considered an inaccurate method due to the high potential of differentiating and errors in the result. This confusion leads to misidentifying between *P. indica* and *P. perna* where both are the same species (Divya et al., 2009). The molecular analysis also exhibited that the specimens were *P. viridis*, and the phylogenetic position combined into one clade with *P. viridis* from another geographic location (Table 2).

The cultivation of *Perna* introduced to several cites of the world. In Japan, *P. viridis* cultivation was pioneered in 1967 (Yoshiyasu et al., 2004), while in Trinidad, *P. viridis* cultivation began in 1990 (Agard et al., 1992). The species of *P. Perna* cultivation started in 1992 in along Venezuelan coast (de Bravo et al., 1998), and began in 1990, 1998 and 1999 in the Gulf of Mexico, Jamaica and Florida respectively (Hicks, 2001; Holland, 2001).

In Asia, the cultivation of *P. viridis* occurs in Thailand, Philippine, Malaysia, Singapore, as well as India and some African countries (Mohamed, 2008; Cao et al., 2013). While in Indonesia, the introduction of *P. viridis* cultivating technology began in the late 1970s (Evans et al., 1995), such as in Jakarta Bay (Jalius et al., 2008), Sunda Strait and Surabaya at the Java Strait (Sudharyanto et al., 2005), and in Pelabuhan Ratu Coast (Arfin et al., 2012).

In Lampung Bay, the culture activity located in the Pasaran coastal water due to the availability of natural seeds. *P. viridis* cultivation since 2012 involves local people as the main actors by adopted the raft technology of cultivation (Noor, 2015; Noor et al., 2016).

CONCLUSION

Identification of green mussel species from the coastal waters of Pasaran Island, Lampung Bay conducted by morphological and molecular method showed that the specimens analyzed were species of *Perna viridis* LC360888. This finding is supported by the phylogenetic position which is placed in a clade containing *P. viridis* with a high bootstrap value (99%).

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NMN designed and conducted the research, data collection and analysis also wrote the manuscript. HN, MSW, and YR designed experiments and reviewed the manuscript. All authors read and approved the final version.

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