Molecular phylogenetic analysis of commercially important Asian monsoon scallop, *Amusium pleuronectes* (Linnaeus 1758) from Indonesia

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Abstract

The Asian monsoon scallop, Amusium pleuronectes, is a key member of shellfish communities that are the most commercially harvested in Asia. Materials were obtained from four sites in Indonesia: Semarang, Bali Island, Maros and Buru Island. Nucleotide sequence analysis was performed on PCR-amplified mitochondrial DNA 16S rRNA. In total, 556-558 base pair nucleotide sequences were obtained from 8 individuals. Molecular analyses revealed that the samples belong to the A. pleuronectes species. Phylogenetic analyses were carried out by comparing the gene sequences of A. pleuronectes haplotypes in Indonesia with gene sequences of the same base pair length and with the of A. japonicum which was used as an outgroup. The phylogenetic trees were consistent and indicated in the two sub-clades. The presence of A. pleuronectes and the phylogenetic status of the A. pleuronectes Indonesian haplotype were reported. Clarifying the phylogenetic status of ecologically important species provides basic information for biosecurity studies for possible future conservation and control programs.

Keywords: *Asian monsoon scallop – Indonesia – mitochondrial DNA – phylogenetic analyses.*

INTRODUCTION

Asian monsoon scallop, *Amusium pleuronectes*, is a bivalve species which belongs to the Pectinidae. The species has very wide distribution from central Ryukyu Island, South China Sea to the South-East Asia, and Australia (Habe, 1964; Morton, 1980), at a depth of 18 to 40 m (Minchin, 2003) and temperatures ranging 28 to 29°C (Del Norte 1986; Cabacaba *et al*., 2020). *A. pleuronectes* lies recessed in the substrate, the white colored valve positioned lying on the seabed while brown colored valve is on top and the umbonal region is submerged in the substrate (Morton 1980).

Asian monsoon scallops are often a major component of the catch of fishing vessels. In Indonesia, the species is of significant commercial value, with about 500 to 1100 metric tons/year harvested during 1994–1999 (Suprijanto and Widiowati, 2006). However, the recent catch statistics showed that production declined to 100 to 300 metric tons/year between 2000 and 2003 (FAO, 2006). Uncontrolled fishing activities may kill large numbers of stocks, leading to a serious threat to this species as a whole. Assessment of its populations in Indonesian revealed a high exploitation ratio due to the high demand and overfishing. Continuous fishing pressure may ultimately lead these marine species to a high risk of genetic and population bottlenecks if preventive action is not taken (Hardianto *et al*., 2022a; Hardianto *et al*., 2022b). Thus, there is a need to establish baseline data to understand the current stock status from this species.

DNA barcoding combined with a molecular phylogenetic tree is a system designed for precise and accurate identification of a species using a short and standardized gene region (Hebert *et al*., 2003). Research on DNA Barcode and phylogenetic tree has been carried out on marine organism like fish (Khansa *et al*., 2023; Nursalim *et al*., 2022; Syaifudin *et al*., 2021), shellfish (Beden and Karahan, 2020; Praipue *et al*., 2021), crustacean (Irwani *et al*., 2020; Vella and Vella, 2022;) and coral reef (Wijayanti *et al*., 2017a; Wijayanti *et al*., 2018). The most promising benefit of DNA barcoding and phylogenetic tree for species authentication lies in the ability to identify early stages that cannot be

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done by using morphological descriptions and inter species connections. It has been proved to be effective for identifying species in juvenile, larvae and adult organism.

This research is conducted to amplify nucleotide sequences of the mitochondrial 16S ribosomal RNA, to identify species, determine genetic distances and analyze phylogenetic relationships of Asian monsoon scallop from Indonesia. Thus, the identification of the species using the 16S rRNA is an effort to develop genetic information that can be used as the basis for developing better conservation strategies for the target species in the future.

METHODS AND MATERIALS

Samples were collected in 2022 from the ocean at the deep 10-30 m from a large proportion of the natural distribution of this species in Indonesia from four different Islands (Java Island; Semarang, Bali Island; South Denpasar, Sulawesi Island; Maros, and Buru Island). Individual *A. pleuronectes* samples were fixed in 95% ethanol on site and then transported to the laboratory until used for genetic analysis. Muscle tissue (10 mg) from the samples was excised from near the shell using scissors and forceps. DNA was extracted using the Realpure Genomic DNA Extraction Kit (Durviz S.L) following the manufacturer's manual procedure.

The partial mitochondrial 16S ribosomal RNA gene was amplified via polymerase chain reaction (PCR) using universal primers 16sar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16sbr-H (5'-CCGGTTTGAACTCAGATCACGT-3') (Palumbi *et al*. 1991). All reactions were carried out in a 25 µl total volume and the following reagents were added to each PCR microtube: 1 µl of template DNA, 25 pmol of each primer, 12.5 µl of Bioline master mix Taq DNA polymerase. Each sample was adjusted to 25 µl with distilled H_2O. PCR conditions consisted of a hot start (94°C, 180 s) followed by 30 cycles of denaturation at 94°C (30 s), annealing at 48-50°C for 30 s, extension at 72°C (90 s), and final extension at 72°C (420 s) using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Verification of PCR was performed via electrophoresis in a 1% agarose S gel. After electrophoresis, gels were stained with ethidium bromide and products checked on a transilluminator (Advanced Scientific Products Pty Ltd., Queensland, Australia). PCR products were purified using a PCR product pre-sequencing kit (USB Co., USA). Amplified DNA was sequenced on an ABI 3730xl DNA analyzer using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

Sequence data were aligned using muscle alignment software, implemented in MEGA X (Kumar *et al*., 2018) using default alignment parameters, and the sequences were adjusted manually to avoid mismatches. The sequences were analyzed their identity using BLAST (Basic Local Alignment Search Tool) in NCBI (National Center for Biotechnology Information). We also conducted sequence data to calculate Kimura's two-parameter (K2P) distance with 1,000 bootstrap replications, to analysis the pairwise genetic difference, nucleotide composition and to constructed phylogenetic tree by the Neighbor Joining (NJ) methods using MEGA. Haplotype network analysis between the species also constructed by the analysis of minimum spanning network (MSN) using PopART (Leight and Bryant, 2015).

Figure 1. Asian monsoon scallops, *Amusium pleuronectes* (Linnaeus 1758)

RESULTS AND DISCUSSION

In the sequencing results, we obtained a 556–558 bp mtDNA 16S rRNA sequence. The sequence was obtained after the alignment of eight specimens of *A. pleuronectes* from four localities across Indonesia (Figure 2).

Nucleotide sequences of all haplotypes were deposited in the DNA Databank of Japan under the accession numbers LC761945–LC761942 (Table 1). BLAST analysis of the sequence results (LC761945–LC761942) has a match range of 97% to 99% with *A. pleuronectes* haplotypes from other DNA data banks (Table 2). The mean genetic distance in pairwise analysis was calculated to range from 0.01 to 0.04 (Table 3). The maximum genetic distance was observed between SEM-2 and HM630501.1 Australia at 0.04 (Table 3). Result of the haplotype network analysis showed that not many gaps between localities investigated (Figure 2). The most appropriate model was determined as K2+G+I as a result of base substitution model analysis. Bases ratios were calculated: Adenine ratio: 24.7%, Thymine ratio: 30%, Guanine ratio: 27.8%, and Cytosine ratio: 17.5% (Table 2). Phylogenetic analyses indicated that our specimen was evolutionary closer among locality observed despite a two sub clade difference. Neighbor Joining and Maximum Likelihood trees showed the same topology and similar results, and they proved to be consistent (Figure 3).

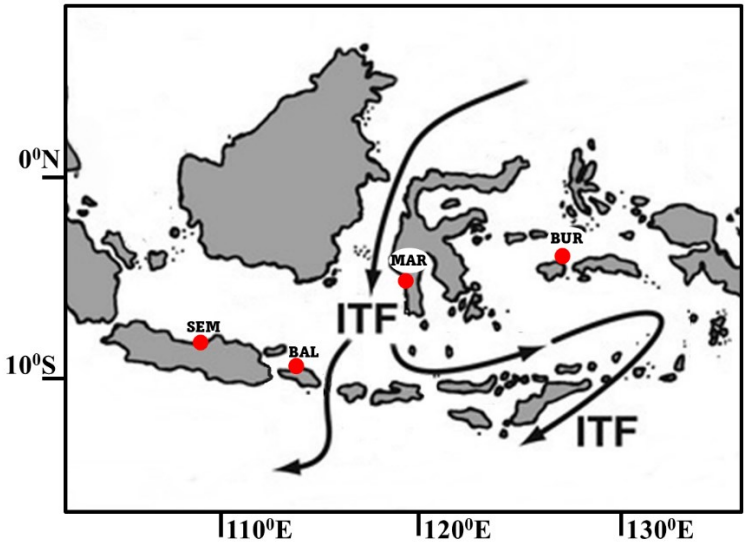

Figure 2. Map of *Amusium pleuronectes* sampling sites in Indonesia. ITF, Indonesian Throughflow; SEM, Semarang; BAL, Bali Island; MAR, Maros; BUR, Buru Island.

Table 1. Details of four sampling sites, number of samples collected (n) and accession number for 16S rRNA sequence

Location site	n	Collecting date	Sample code	DDBJ accession number
Genuk, Semarang City, Central of Java	2	October 20, 2022	SEM-1 SEM-2	LC761945 LC761946
Kedonganan, Badung Regency, Bali Island	2	December 10, 2022	BAL-1 BAL-2	LC761947 LC761948
Maros, South Sulawesi	2	November 12, 2022	MAR-1 MAR-2	LC761949 LC761950
Buru Island, North Maluku	2	December 10, 2022	BUR-1 BUR-2	LC761951 LC761951

Although the presence of *A. pleuronectes* has been recorded in different countries of Asia and in Australia, studies on its phylogenetic status are limited in the literature especially the phylogenetics status in Indonesia. In the previous study reported by Mahidol *et al*. (2007b), only Thailand *A. pleuronectes* were phylogenetically studied. Salvi *et al*. (2010), compared the phylogenetic status of Pectinidae, but also only include the *A. pleuronectes* from Thailand. Mynhardt *et al*. (2014), studied shell shape convergence and systematically status of family Pectinidae including *A. pleuronectes* from China, Thailand, the Philippines dan Australia without Indonesia. Determining the phylogenetic status of an organism provides an important pool of information for biosecurity studies. *A. pleuronectes* is a species that provides important information on environment quality and plays a role in the food chain of marine ecosystems (Mahidol *et al*., 2007; Cabacaba *et al*., 2020). If the phylogeny of *A. pleuronectes* is fully clarified, basic information will be supplied to the studies to be carried out in order to protect the balance of marine ecosystems.

The result of BLASTn analyses in Table 2 denoted that nucleotide sequences of the from four localities observed in Indonesia were identical, showing ranged 97% to 98.69% matched to *A. pleuronectes* from Thailand (DQ640841.1 and DQ640830.1), the Philippines (KC879122.1, KC879126.1 and KC879114.1), China (AF362387.1), Vietnam (AJ571616.1) and Australia (HM630501.1, JF339128.1 and HM630497.1). Sequence similarity higher than 97% was the criterion for authentication at the species level (Wong & Hanner, 2008) and a similarity lower than that was used for recognition at the genus level. This result denoted that distinguishing species using DNA barcoding is very accurate. The 16S rRNA is effectively used as species authentication method because intraspecific variation is low, but has high interspecific variation values especially in adjacent taxa (Ward *et al*., 2005; Feng *et al*., 2011; Lumogdang *et al*., 2022). DNA barcoding validates to recognize species for international trading, either certifying fisheries products consumption or ornamental fish trade (Dahruddin *et al*., 2016; Feng *et al*., 2011; Lumogdang *et al*., 2022). This method is very useful for sustainable fishery resource management.

Table 2 BLAST Identity percentage of nucleotide of *Amusium Pleuronectes* based on 16S rRNA

No.	Species	Origin	Accession number	Identity (%)
Sample code: SEM-1 and SEM-2				
1.	*Amusium pleuronectes*	Thailand	DQ640841.1	98.32
2.	*Amusium pleuronectes*	Guus Island, the Philippines	KC879122.1	98.19
3.	*Amusium pleuronectes*	Roxas city, the Philippines	KC879126.1	98.03
4.	*Amusium pleuronectes*	China	AF362387.1	97.67
Sample code: BAL-1 and BAL-2				
1.	*Amusium pleuronectes*	Vietnam	AJ571616.1	98.69
2.	*Amusium pleuronectes*	Thailand	DQ640830.1	98.51
3.	*Amusium pleuronectes*	Guus Island, the Philippines	KC879122.1	98.38
4.	*Amusium pleuronectes*	Roxas city, the Philippines	KC879126.1	98.20
Sample code: MAR-1 and MAR-2				
1.	*Amusium pleuronectes*	Roxas city, the Philippines	KC879126.1	97.84
2.	*Amusium pleuronectes*	Calbayog,samar, the Philippines	KC879114.1	97.84
3.	*Amusium pleuronectes*	Queensland, Australia	HM630501.1	97.67
4.	*Amusium pleuronectes*	Queensland, Australia	JF339128.1	97.65
Sample code: BUR-1 and BUR-2				
1.	*Amusium pleuronectes*	Queensland, Australia	HM630501.1	98.32
2.	*Amusium pleuronectes*	Queensland, Australia	JF339128.1	98.14
3.	*Amusium pleuronectes*	Roxas city, the Philippines	KC879126.1	97.39
4.	*Amusium pleuronectes*	China	AF362387.1	97.39

The genetic distance in this research was also used to determine the genetic relationship between *A. pleuronectes* observed from Indonesia with other samples from DNA data bank. Genetic distance is varied in the ranged of 0.01 to 0.04. Hubert *et al*. (2014) stated that a genetic distance difference of less or equal to 3% indicates molecularly identical species. The smaller the genetic distance between individuals in a population, the more uniform the population is. Conversely, the greater the genetic distance of an individual in a population, the more diverse the population will be. The largest genetic distance (0.16 to 0.17) was found between *A. pleuronectes* from Indonesia and species outgroup *A. japonicum* from Japan (HM622707.1). Similar to the genetic distance analysis, the haplotype network analysis showed not to big different gap within species analysis (Figure 2). It is clearly denoted that within *A. pleuronectes* barcode variation was low in compare to the sequence variation between species in genus *Amusium*. Genetic distance indicates the ratio of a genetic distinction between species or populations (Dogan & Dogan, 2016). Therefore, a smaller genetic distance value creates a more indistinguishable appearance partial sequence of 16S rRNA gene compared between the two species (Mahidol *et al*., 2007b).

The phylogenetic tree of Asian monsoon scallop was presented in Figure 3. This study determined the level of evolution and kinship of a species, where there are separated become 2 sub cluster. *A. pleuronectes* was separated from *A. japonicum* (HM622707.1) with bootstrap value/bv of 100%. Within species, *A. pleuronectes* from Indonesia was separated into two sub-clusters (bv of 68%). The first sub-cluster consisted of *A. pleuronectes* from this studi (SEM-1, SEM-2, BAL-1 and BAL-2) with China (AF362387.1), Vietnam (AJ571616.1), Thailand (DQ640841.1, DQ640830.1) and the Philippines (KC879122.1, KC879126.1, KC879114.1). The second sub-cluster consists of *A. pleuronectes* from this study (MAR-1, MAR-2, BUR-1, BUR-2) and Australia (HM630501.1, JF339128.1).

Table 3 Base composition (%) of 16S rRNA gene amplified for each location

Sample	First codon				Second codon				Third codon				Overall			
code	T	C	A	G	T	C	A	G	T	C	A	G	T	C	A	G
SEM-1	29	13.4	26.9	30.6	29	23.7	25.3	22.0	35	13.5	24.9	27.0	30.9	16.9	25.7	26.6
SEM-2	29	13.4	26.9	30.6	29	23.7	25.3	22.0	34	14.1	23.9	27.7	30.8	17.1	25.4	26.8
BAL-1	30	12.9	26.3	31.2	30	23.7	24.7	22.0	35	13.5	24.9	27.0	31.2	16.7	25.3	26.8
BAL-2	30	12.9	26.3	31.2	30	23.7	24.7	22.0	35	13.5	24.9	27.0	31.2	16.7	25.3	26.8
MAR-1	28	13.4	25.8	32.3	29	22.6	25.3	23.1	35	12.9	25.8	26.3	30.8	16.3	25.6	27.2
MAR-2	28	13.4	25.8	32.3	29	22.6	25.3	23.1	35	12.9	25.8	26.3	30.8	16.3	25.6	27.2
BUR-1	28	13.4	25.8	32.3	28	25.1	24.6	22.5	34	12.9	26.9	26.3	30.1	17.2	25.8	27.0
BUR-2	28	13.4	25.8	32.3	27	25.1	25.1	22.5	34	12.9	26.9	26.3	29.9	17.2	25.9	27.0
Overall	29	13.3	25.1	32.3	27	24.7	24.2	24.2	34	14.4	24.7	26.8	30.0	17.5	24.7	27.8

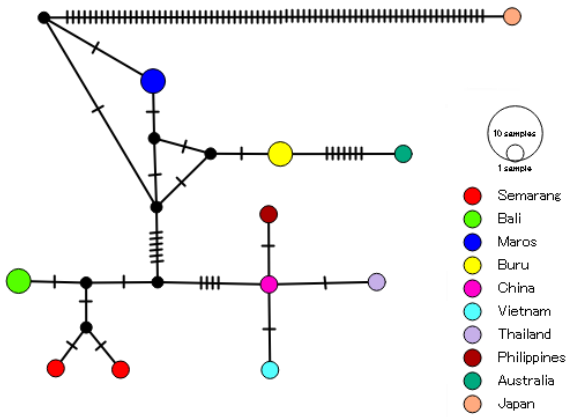

Figure 3. Haplotype network (implemented in PopART) of *Amusium pleuronectes* based on 16S rRNA sequences using minimum spanning network analysis.

Table 4. Pairwise genetic distances of *Amusium pleuronectes* each location sites including out of groups (*A. japonicum*) and DNA data bank data of mitochondrial DNA 16S rRNA based on K2P defined in this study

No	Species and sample code	Genetic distances												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	SEM-1													
2	SEM-2	0.00												
3	BAL-1	0.01	0.01											
4	BAL-2	0.01	0.01	0.00										
5	MAR-1	0.02	0.02	0.02	0.02									
6	MAR-2	0.02	0.02	0.02	0.02	0.00								
7	BUR-1	0.02	0.02	0.02	0.02	0.02	0.01							
8	BUR-2	0.02	0.02	0.02	0.02	0.01	0.01	0.00						
9	KP900978.1-China	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02					
10	AJ571616.1-Vietnam	0.02	0.02	0.01	0.01	0.03	0.03	0.03	0.03	0.00				
11	DQ640841.1-Thailand	0.01	0.01	0.01	0.01	0.03	0.03	0.03	0.03	0.00	0.00			
12	KC879126.1-Philippines	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.03	0.00	0.00	0.00		
13	HM630501.1-Australia	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0.01	0.03	0.04	0.04	0.04	
Out of group														
14	HM622707.1-Japan	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.17	0.16	0.17	0.16	0.16

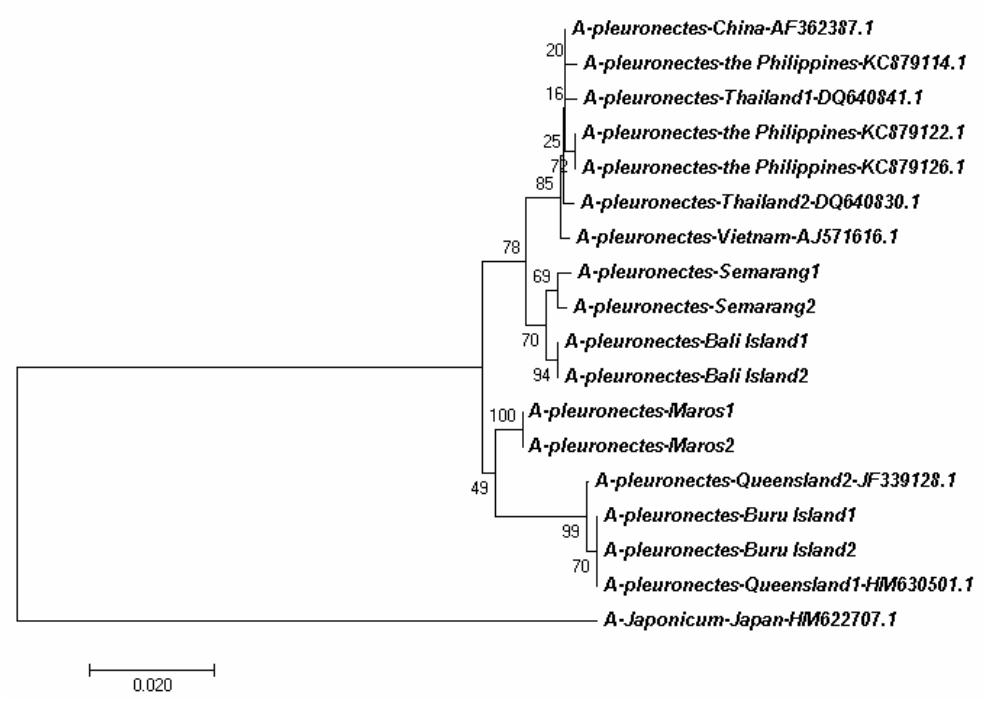

Figure 4. Phylogenetic relationships of Pectinidae inferred by Neighbor Joining analysis (NJ) of 16S rRNA gene regions.

The results showed that the samples SEM-1, SEM-2, BAL-1, and BAL-2 were closely related to the *A. pleuronectes* from Asia, while MAR-1, MAR-2, BUR-1 and BUR-2 were more closely related to Australia. Indonesia is geographically separated into two regions by the Wallace Line which has been shown to have a large impact on genetic differences in many marine taxa between east to west region (Wainwright *et al*., 2017; Hardianto *et al*., 2022a; Hardianto *et al*., 2022b). A previous study using sequence data of four molecular markers (two mitochondrial and two nuclear) indicated the similar results (Mynhardt *et al*., 2014). A bootstrap value greater than 70% indicates that the data is relatively stable (Lemey *et al*., 2009). The phylogenetic tree construction resulted in a scale bar of 0.02. According to Wardani *et al*. (2017), a phylogenetic tree with a 0.01 scale bar shows a genetic distance with a change in nucleotides 1 time every 100 bp. So that the phylogenetic constructs obtained indicate a genetic distance with nucleotide changes 2 times in every 100 bp. DNA barcoding technology has been utilized and validated for many aquatic species with detected more variation among congeneric species than among conspecific individual (Ward *et al*., 2005; Syaifudin *et al*., 2021). Therefore, it can effectively use to distinguish a complex of morphologically distinct species in the Indo-Pacific (Last *et al*., 2005; Khansa *et al*., 2023).

CONCLUSION

In terms of species identification, molecular identification by phylogenetic analysis and DNA barcoding is more reliable and accurate than morphological identification. In the present work, the aim was to produce a definite species identification of the specimens obtained in Indonesia by phylogenetic analysis and DNA barcoding to clarify their phylogenetic position in order to give fundamental data for future biosecurity investigations. The analyses confirmed that the samples originate from the *A. pleuronectes* species and are most closely related to the GenBank-registered haplotypes KP900978.1, AJ571616.1, DQ640841.1, KC879126.1, and HM630501.1. In the light of these results, it was decided that the programs to be established in the places where phylogenetically linked haplotypes are identified can be included into the research in Indonesia.

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