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RECONFIRMING THE SPECIES OF MUD CRAB GENUS SCYLLA (DE HAAN, 1833) IN BALIKPAPAN, EAST KALIMANTAN PROVINCE, INDONESIA BASED ON MITOCHONDRIAL 16S rRNA

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ABSTRACT

Taxonomy of mud crab species under the genus *Scylla* has been misidentified for several years due to their high morphological similarity. In Indonesia, some reports concerning mud crab have been published with misleading identification results where the species under the genus *Scylla* all named as *Scylla serrata*. The study was conducted to reconfirm the validity of species in the mud crab genus *Scylla* collected from Balikpapan mangrove, East Kalimantan, Indonesia and to analyze the genetic variation of the first generation (G-1) offspring, based on mitochondrial 16S rRNA sequence. The animal test used for species identification was a representative sample of mud crab. Ten of the G-1 crablet were randomly sampled for genetic variation analysis. Fragment of the 16S rRNA gene was isolated by PCR technique and purified for sequencing purpose. The mtDNA sequences were analyzed using Genetyx, BLAST-N, and DnaSP to get a consensus sequence, similarity index, haplotype and sequence diversity, and the number of haplotypes. The results showed that the 16S rRNA gene was successfully isolated with a single band in size of approximately 600 bp. The mud crab morphologically identified as *Scylla tranquebarica* was genetically confirmed as a species of *S. tranquebarica.* High haplotype diversity (0.9254) and low nucleotide diversity (0.1256) were revealed in the G-1 mud crab population, while the number of haplotypes was 7.5.

KEYWORDS: 16S rRNA; genetic variation; mitochondrial DNA; mud crab; species reconfirmation

INTRODUCTION

Mud crab belonging to the genus *Scylla* is a commercially valuable crab resource for fisheries and aquaculture, both for local and international markets. Before the 1990s, most of the taxonomical works on the genus *Scylla* referred only to species of *S. serrata* (Mandal *et al.,* 2014a; Sarower *et al.,* 2016). The classification of genus *Scylla* has been controversial for a long time. Estampador (1949) claimed that based on the morphological characteristics, *Scylla* consisted of three species and one subspecies (four taxa) including *S. serrata, S. oceanica, S. tranquebarica,* and *S. serrata var. paramamosain*. However, the gene replacement at the three loci and the genetic distances between species suggested that the genus *Scylla* included at least three species (Fuseya & Watanabe, 1996).

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Furthermore, Keenan *et al*. (1998) extensively revised the taxonomy of genus *Scylla* using integrative approaches (allozyme electrophoresis with sequencing cytochrome oxidase-I and 16s RNA). They divided this genus into four distinct species, including *S. serrata, S. olivacea, S. tranquebarica,* and *S. paramamosain*. The taxonomic status of genus *Scylla* based on morphological and molecular approaches, as proposed by Keenan *et al*. (1998), is widely accepted. The latest publication from Fazhan *et al.* (2020) hypothesized that the additional taxa in the genus *Scylla* with intermediate characteristics could be presumed hybrids. However, this work needs to be validated at the molecular level. Up to the present, misidentification of the species in genus *Scylla* is still a big problem related to developmental stage, size difference, and close similarity.

Species identification is a starting point in bio-ecological studies, and correctly-determined species is fundamental to develop a breeding program. The high morphological diversity and plasticity of mud crabs due to developmental stages and environment

have made it difficult to accurately identify a species of mud crabs based only on morphological characteristics (Balasubramanian *et al.*, 2014; Mandal *et al.,* 2014a, 2014b; Sarower *et al.,* 2016).

DNA-based identification techniques have been developed and proven to be analytically robust. A short DNA sequence of the mitochondrial genome area could be taken as a DNA barcode for species identification. A gene of 16S rRNA is one of the most common mitochondrial DNA (mtDNA) gene markers used to identify species because it belongs to the most conservative gene among the mtDNA protein-coding genes (Yang *et al.,* 2014). Eventhough mitochondrial 16S ribosomal RNA gene regions have been used in genetic studies for various purposes, (Imai *et al.*, 2004; Masaoka & Kobayashi, 2005; Akasaki *et al.*, 2006; Saavedra & Pena, 2004; Srinu *et al.,* 2018), their usage in species identification at a molecular level for mud crab is still very limited. The study aimed to reconfirm the validity of species in the mud crab genus *Scylla* collected from Balikpapan mangrove, East Kalimantan, Indonesia and to analyze the genetic variation of the first generation (G-1) offspring, based on mitochondrial 16S rRNA sequence.

MATERIALS AND METHODS

Sample Collection

Mud crabs (n = 30) with a weight of 235.7 ± 88.4 g were collected from a mangrove area surrounding brackish water ponds along the coastline of Balikpapan Bay, Balikpapan, East Kalimantan. The mud crabs were morphologically identified as a species of *S. tranquebarica* (Gunarto *et al.,* 2017). One individual sample weighed 228.3 g was randomly taken to re-identify its species using the molecular technique. The other mud crabs were continuously reared to produce the first generation (G-1) offsprings via individual spawning. The gonadal maturation of broodstock was performed based on the standard operating procedure (SOP) established in mud crab hatchery of Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) in Barru, South Sulawesi (see Gunarto *et al.,* 2016). After hatching, the larvae from each parent were reared separately in a conical fiber tank until reaching the crablet stage and then harvested at crablet D-10. Furthermore, the crablets were reared in a concrete tank until reaching crablet D-30 with a weight range of 1-2 g. Ten individual crablets were randomly collected from six parents for genetic analysis purposes.

Genomic DNA Extraction

The genomic DNA of adult and mud crab crablet (G-1) was isolated using the phenol-chloroform extraction method developed by Parenrengi *et al*. (2000). The purity of genomic DNA was estimated by measuring in a GeneQuant. The DNA concentration and purity were quantitatively determined from the ratio between the reading of absorbance at 260 nm and 280 nm (OD_{260}/OD_{280}) (Linacero *et al*., 1998). DNA purity was qualitatively observed through the appearance of the band on the agarose gel.

Isolation and Sequencing of 16S rRNA Gene

A gene encoding mtDNA 16S rRNA of mud crab was isolated according to the method of Tenriulo *et al.* (2009). The method has been applied to green tiger prawn *Penaeus semisulactus* by using a pair of specific primers, i.e., 16SrRNA-F: 5'- cgc ctg ttt aac aaa aac at -3' and 16SrRNA-R: 5'- ccg gtc tga act cag atc atg t -3'. PCR reaction consisted of PureTaq Ready-To-Go PCR Beads kit, 1 μL for each primer (50 ρmol), and sterile water until the final volume of 25 μL. PCR was processed in the 2720 Thermal Cycler with the following program: initial denaturation of 15 min at 55°C and 2 min at 94°C; followed by 40 cycles of denaturation 15 sec at 94°C, annealing 30 sec at 60°C, and extension 1 min 15 sec at 68°C; and final extension of 5 min at 72°C. The success of mtDNA isolation was determined by running the PCR product at 0.8% agarose gel to visualize the single fragment result. The good PCR product was purified using commercial Kit and DNA concentration was measured by using GeneQuant before the PCR products were sent to the First Base for reading nucleotide sequences by ABI sequence scanner.

Data Analysis

The mtDNA sequence results were analyzed by using Genetyx Version 7.0, BLAST-N (basic local alignment search tool–nucleotide) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and DnaSP Version 5.0 (Librado & Rozas, 2009). The Genetyx program was applied to obtain the consensus of forward-reverse sequences and determine nucleotide variation of intra-population. BLAST-N was used to confirm species identification by sequence analysis of similarity index among crustacean species deposited at GenBank. DnaSP was performed to get the haplotype variation and number of mud crab larvae.

RESULTS AND DISCUSSION

Genomic DNA and 16S rRNA Isolation

Genomic DNA of mud crab was successfully extracted using the conventional method in a concentration of $34.5 \pm 11.4\ \mu g\ mL^{-1}$ with a high purity level of 1,873 ± 0,099. Linacero *et al*. (1998) recommended the DNA purity for molecular analysis in the range of 1.8-2.0. This method could also produce a high purity of genomic DNA on tiger shrimp (Tenriulo *et al.*,

2012; Parenrengi *et al*., 2017) and rabbitfish (Lante *et al.,* 2011). A partial 16S rRNA gene was successfully isolated from the genomic DNA of mud crab with a single band in size of approximately 600 bp (in range of 500-750 bp) (Figure 1). This range of DNA fragment position of the mtDNA gene was also observed in green tiger prawn *P. semisulcatus* (Tenriulo *et al*., 2009).

Confirmation of Mud Crab Species Identification

A consensus sequence in the length of 568 bp (Figure 2) was used as a reference to confirm the species identification of mud crab understudy at the molecular level by BLAST-N online. At least 116 mtDNA sequences of crustacean species at GenBank exhibited a high similarity in the range of 93-100% with the species in the present study. Twenty representatives of mtDNA genes of mud crab with high similarity were used as references and listed in Table 1. The mud crab sample had an identical sequence of mtDNA genes (100% similarity) to *S. tranquebarica* isolate of P-ST-01, a partial sequence of 16S rRNA gene (accession number of KT921342.1). So, it is argued here that the mud crab sample in this study was unmistakenly confirmed as a species of *S. tranquebarica.* This species identification was accurately supported by the high similarity (identical sequence) and the rate of maximum scoring (total score). The sequence similarity of the 16S rRNA gene at GenBank was very close to species of *S. tranquebarica*, followed by *S. olivacea* (99%), *S. paramamosain* (95%), and *S. serrata* (93%). The highest scoring value was obtained by species of *S. tranquebarica* (1,022);

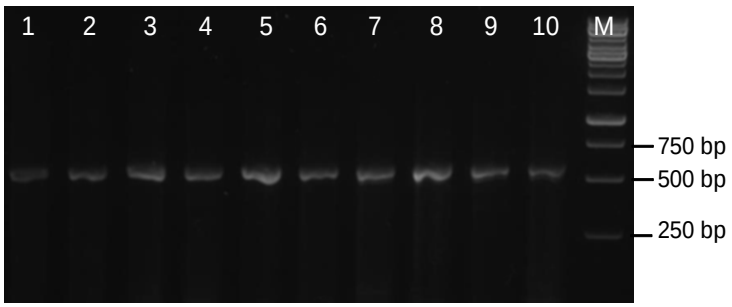

Figure 1. The single band of mitochondrial 16S rRNA gene isolated from the G-1 offspring of mud crab on 0.8% agarose gel (M = DNA marker and 1-10 = crablet samples).

10	20	30	40	50	60
TTAAATCGCC	TGTTTTACAA	AAACATGTCT	ATTTGAAATT	ATATATAGTC	TAGCCTGCTC
70	80	90	100	110	120
ACTGACAAAG	ATGTTAAATA	GCCGCGGTAT	TTTGACCGTG	CAAAGGTAGC	ATAATCATTA
130	140	150	160	170	180
GTTTTTTAAT	TGGAAACTTG	TATGAATGGT	TGGACAAAGA	AAAAGCTGTC	TCTTTTATTA
190	200	210	220	230	240
AAATTGAAAT	TAACTTCTAA	GTGAAAAGGC	TTAGATTTTC	TAGAGGGACG	ATAAGACCCT
250	260	270	280	290	300
ATAAAGCTTT	ATAAATTAAG	TAAGTTAGAA	CAAATTTTAT	AGAATAAAGG	TAAATTTAAT
310	320	330	340	350	360
ATATTTATTT	GGTTGGGGCG	ACAATGGTAT	AAATTAAATA	ACTGCTATTA	AAATTAAACA
370	380	390	400	410	420
ATTATATTTG	ATTAAAATTA	TAATTGATCC	TTATTATGGA	TTAAAAGATC	AAGTTACTTT
430	440	450	460	470	480
AGGGATAACA	GCGTAATTTC	TTTTAAGAGT	TCTTATCAAA	GAAGAAGTTT	GCGACCTCGA
490	500	510	520	530	540
TGTTGAATTA	AAATGTCTTT	ATAGTGCAGA	AGCTATAAAA	GAAGGTCTGT	TCGACCTTTA
550	560	570			
ATTTTTTACA	TGATCTGAGT	TCAGACCG			

Figure 2. Partial nucleotide sequences of gene encoding the mitochondrial 16S rRNA isolated from mud crab.

Table 1. Sequence similarity and total score of mitochondrial 16S rRNA gene isolated from mud crab

Description	Total score	Similarity (%)	Accession
Scylla tranquebarica isolate P-ST-01 16S ribosomal RNA gene, partial sequence; mitochondrial	963	100	KT921342.1
Scylla tranquebarica 16S ribosomal RNA gene, partial sequence	1.022	99	AF109320.1
Scylla tranquebarica mitochondrion, complete genome	1.02	99	FJ827759.1
Scylla tranquebarica voucher DLSU.MI.00032 16S ribosomal RNA gene, partial sequence; mitochondrial	953	99	KM258653.1
Scylla tranquebarica voucher DLSU.MI.00033 16S ribosomal RNA gene, partial sequence; mitochondrial	950	99	KM258654.1
Scylla olivacea isolate SOPB4 16S ribosomal RNA gene, partial sequence; mitochondrial	935	99	KU306796.1
Scylla olivacea isolate SOLG5 16S ribosomal RNA gene, partial sequence; mitochondrial	935	99	KU306746.1
Scylla olivacea mitochondrion, complete genome	824	93	FJ827760.1
Scylla olivacea 16S ribosomal RNA gene, mitochondrial gene for mitochondrial RNA, partial sequence	821	93	AF109321.1
Scylla olivacea voucher NBFGR-CHN-Scylla1 16S ribosomal RNA gene, partial sequence; mitochondrial	813	93	KC154070.1
Scylla paramamosain 16S ribosomal RNA gene, partial sequence	893	95	AF109319.1
Scylla paramamosain mitochondrion, complete genome	891	95	MG197997.1
Scylla paramamosain mitochondrion, complete genome	891	95	JX457150.1
Scylla paramamosain mRNA sequence	891	95	HM217900.1
Scylla paramamosain mitochondrion, complete genome	891	95	FJ827761.1
Scylla serrata mitochondrion, complete genome	824	93	HM590866.1
Scylla serrata 16S ribosomal RNA gene, mitochondrial gene for mitochondrial RNA, partial sequence	815	93	AF109318.1
Scylla serrata mitochondrion, complete genome	813	93	FJ827758.1
Scylla serrata voucher NBFGR-CHN-Scylla10 16S ribosomal RNA gene, partial sequence; mitochondrial	811	93	KC154088.1
Scylla serrata voucher NBFGR-CHN-Scylla8 16S ribosomal RNA gene, partial sequence; mitochondrial	808	93	KC154086.1

followed by *S. olivacea* (935); *S. paramamosain* (893); and *S. serrata* (824).

Prior to this study, Imai *et al*. (2004) had reported species identification for four species of mud crabs using RFLP (*restriction fragment length polymorphism*) mtDNA marker, especially for enzyme restriction of *Dra*-I and *Hin*d-III. Species confirmation of mud crab conducted by Sarower *et al.* (2016) using the mtDNA marker showed that mud crabs collected in Bangladesh were identified as a species of *S. olivacea.* Their result challenged erroneous species identification results from other studies that mistakenly identified the species as *S. serrata*. The mud crab *S. serrata* commonly mentioned in India's literature was, in fact, *S. olivacea* when identified using RAPD and PCR-RFLP markers (Mandal *et al.* 2014a) and sequencing of COI gene (Mandal *et al.,* 2014b). In addition, a comprehensive DNA sequence analysis of mud crabs in India revealed that the species commonly reported as *S. tranquebarica* was, in fact, *S. serrata* (Balasubramanian *et al.*, 2014). Based on the evidence from this present study, we argue that the mud crab of *S. serrata* commonly reported in Indonesia's past studies is likely to be a species of either *S. olivacea* or *S. tranquebarica*.

Genetic Variation of The G-1 Mud Crab *Scylla tranquebarica*

The reading of mtDNA 16S rRNA nucleotide sequences of mud crabs was in the range of 573-624 bp. The sequence alignment of ten mud crab larvae showed that the reading of forward and reverse sequences was not started in the same position, while the variation of nucleotide was in sequence position of 26, 27, 30, 39, 516, 558, 574, and 595 bp (Figure 3).

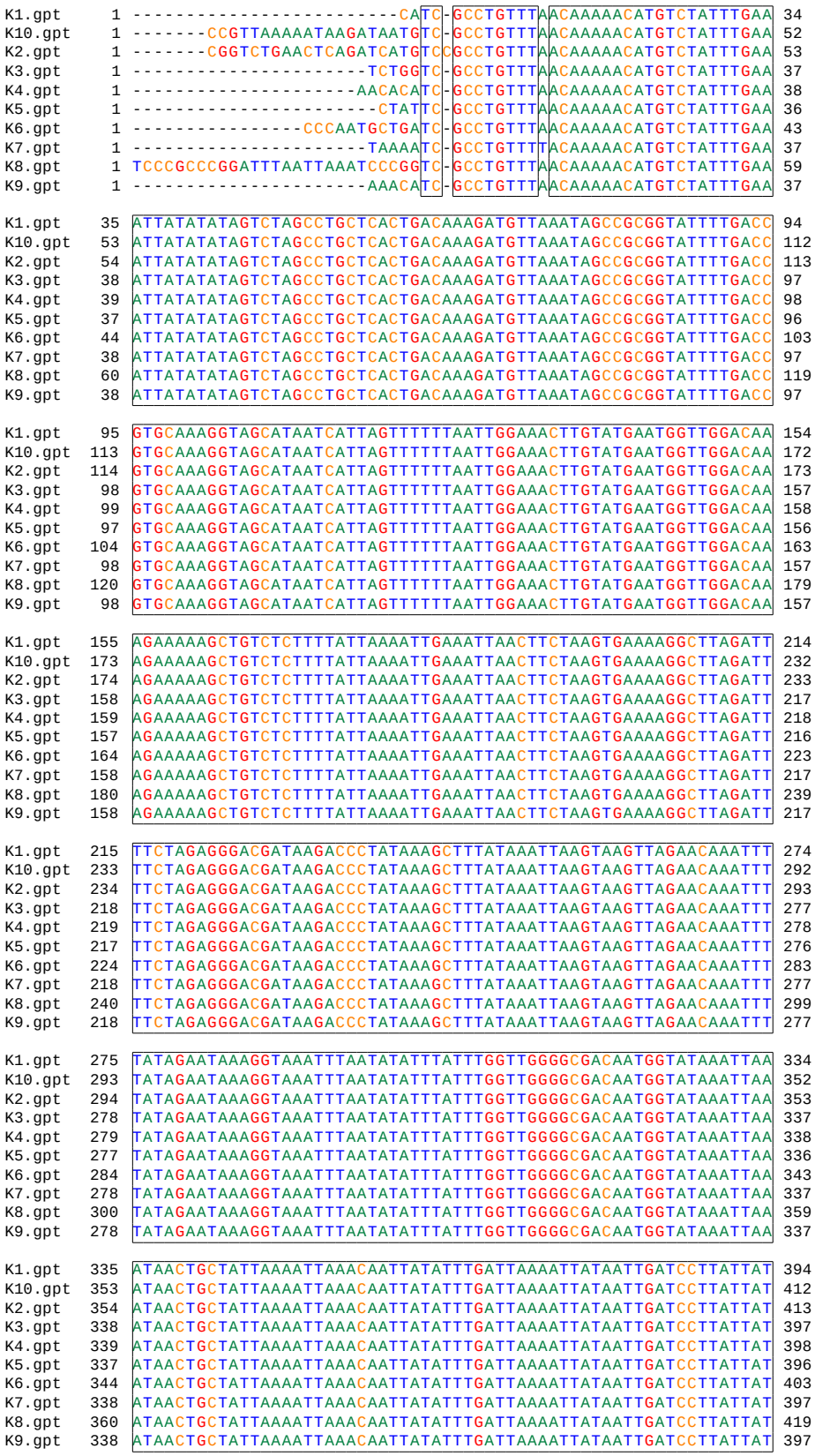

Figure 3. Alignment of mtDNA 16S rRNA sequence of mud crab on 1st generation (F1) from Balikpapan broodstocks (A= adenine; T= thymine, G= guanine, C= cytosine, and without box indicating the nucleotide variation).

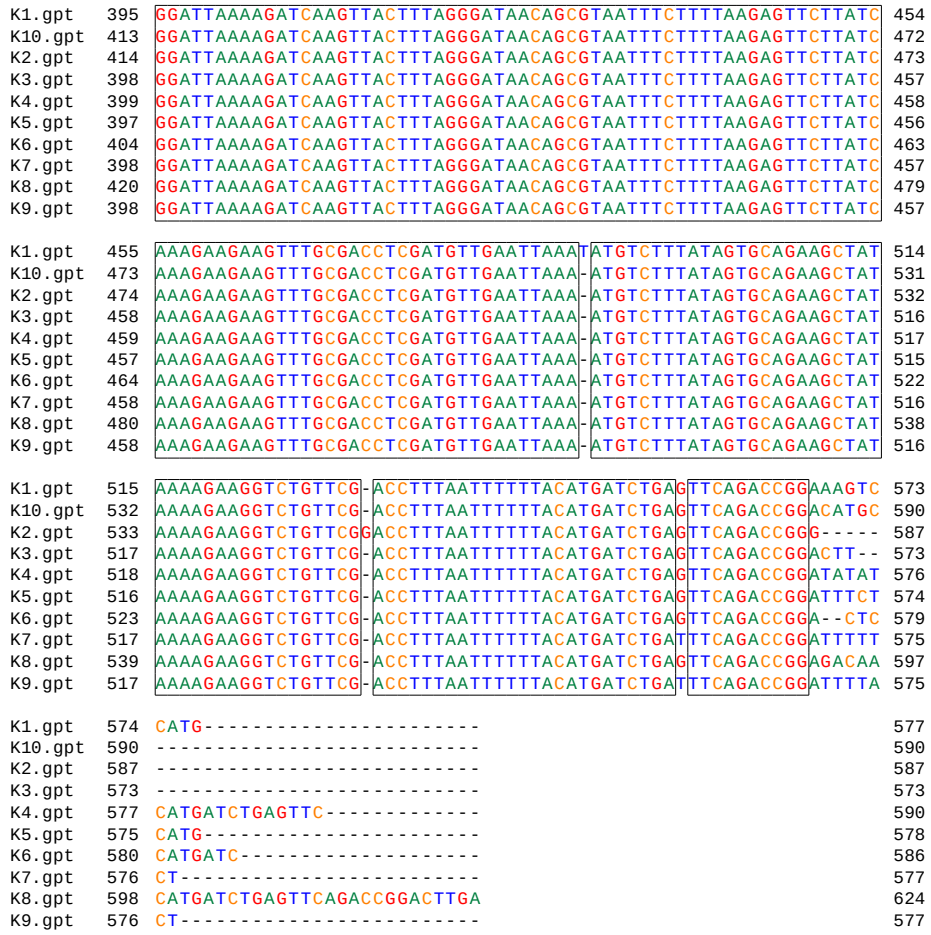

Figure 3. Continued

The genetic variation analysis of the mud crab larvae population revealed haplotype variation as much as 0.9254, and the number of haplotypes was 7.5, with a nucleotide variation of 0.1256. The high haplotype diversity observed in this present study indicated the high genetic variation of mud crab *S. tranquebarica* population in Balikpapan, in which these data are useful information for mud crab breeding program in the future. In contrast, Sharif *et al.* (2016) reported the lower haplotype variation of the mtDNA CO-I gene in *5 populations of* mud crab *S. tranquebarica in* Sabah, Malaysia, where the averages of haplotype variation and nucleotide were of 0.5564 and 0.0038, respectively. Different approaches using microsatellite markers on mud crab, *S. paramamosain* in China, had reported high heterozygosity of 0.62-0.67 (Ma *et al.,* 2012).

Other studies also reported similar findings regarding the high haplotype and low nucleotide variations in mud crab *S. olivacea*. Rosly *et al.* (2013) reported that high haplotype diversity (0.968) and low nucleotide diversity (0.120) were displayed in mud crab populations of the east and west coast of Malaysia Peninsular. A haplotype is one of the biological indicators which can be used to analyze the genetic variation of a population. The high haplotype variation of mud crab in the present study offers a significant advantage to develop a breeding program of the crab species in the future. Imai *et al.* (2002) have successfully used D-loop mtDNA haplotype as an indicator to measure the stocking affectivity of wild populations of mud crab *S. paramamosain*. The mtDNA marker could act as a DNA barcode for animal species identification due to more straightforward isolation procedures and good resolution results for animal species identification. The other markers, such as RAPD, have been used to assess the genetic variation of three mud crab species, *S. serrata*, *S. oceanica*, and *S. tranquebarica* mud crab. Klinbunga *et al.* (2000) reported that the percentage of polymorphic bands within these populations ranged from 47.92% to 77.59%, and species-specific RAPD markers were also observed and used to construct a molecular diagnostic key in these taxa.

Knowledge of genetic diversity is an essential aspect of ensuring sustainable exploitation of any commercial fish species. The findings from the present study are arguably important not only in providing baseline information on taxonomic identification and genetic diversity of *S. tranquebarica* species but also in the overall management of mud crabs for fisheries and aquaculture uses considering the increasing market demand for this species.

CONCLUSION

This study found that the mud crab understudy was a species of *S. tranquebarica* confirmed through its 16S rRNA gene, which was identical to the mtDNA sequence (100%) of the same species at GenBank. The haplotype variation of 0.9254, sequence nucleotide variation of 0.1256, and haplotype number of 7.5 were observed in the populations of G-1 mud crab *S. tranquebarica*. The high haplotype diversity of the mud crabs suggested a potential quantitative trait for the breeding program of this species. However, the proper species identification should be performed before developing the mud crab culture in the future.

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