Original article

Reassessment of species distribution and occurrence of mud crab (*Scylla* spp., Portunidae) in Malaysia through morphological and molecular identification

Darlina Md. Naim [a,*], Siti Azizah Mohd Nor [b], Shahid Mahboob [c]

[a] *School of Biological Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia*
[b] *Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia*
[c] *Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

A B S T R A C T

This study utilized genetic and morphometric approaches to assess the molecular and morphometric differentiation among commercially important species of mud crab. Molecular investigations were derived from 542 bp mitochondrial DNA COI on 249 individuals within genus *Scylla* from nine states in Malaysia represents four marine regions; South China Sea, Sulu Sea, Straits of Singapore and Straits of Malacca. Four specimens were obtained from Indonesia to give a robust analysis in this study. For species delimitation, Automatic Barcode Gap Discovery (ABGD) method on a web interface was employed. Analysis on phylogenetics was implemented utilizing Neighbour joining (NJ) and Maximum Parsimony (MP) methods. The inter- and intraspecies genetic distances (D_s) was computed using Kimura 2-parameter distance and executed in MEGA version 5.05. All samples were genetically and morphologically identified and clustered into four distinct species. Among the species, *S. olivacea* was the most abundant ($n = 111$), on the other hand the occurrence of *S. paramamosain* in Malaysia was very low ($n = 29$). No single individual of *S. serrata* from Malaysia was recorded in this study. Both genetic distance and phylogenetic approaches exhibited a correlative monophyletic association among all specimens analysed. This present study is crucial as it reports the reassessment of all species within genus *Scylla* in Malaysia, eventually could be employed as a reference source for subsequent research mainly on mariculture and other conservation efforts for the species.

© 2019 The Authors. Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Scylla (Portunidae) is a mud crab that occupies an immense dispersion running from the Indo Pacific area to eastern and southeastern of Africa. Since the first description of this species by Forskål (1775), mud crabs were considered as an exclusive member within genus *Scylla.* The weakness of species identification systems and a small sample size constrains are major contributors to the assumption that only a single species exist in *Scylla*. Afterward, Estampador (1949) perceived that other species could be a member of the genus after some modification of the morphological keys was used to identify the genus. Nonetheless, an overlapping traits and/or phenotype and morphological homogeneity among species have created a huge taxonomic uncertainty.

Several comprehensive research has been carried out with the objective to delineate the species within genus *Scylla* (see. Overton et al., 1997) based on traditional approaches (morphological identification technique), conversely, the taxonomic abstruseness had continued to exist, until the molecular identification technique prior to the plausibility of more than one species within the genus (Keenan et al., 1995; Fuseya and Watanabe, 1996). The main traits that can distinguish individual within *Scylla* spp. are the pattern and also height of the spines at a frontal lobe and the occurrence of spines at propordus and carpus of the cheliped (Keenan et al., 1998). Taking into account all the studies that have utilized diverse molecular methods such as both mitochondrial and nuclear genes and also allozyme electrophoresis, all complied with the fact that there are four different species belong to the

genus, specifically *S. paramamosain*, *S. olivacea*, *S. serrata* and *S. tranquebarica* (Keenan et al., 1998).

There is significant research has been conducted and discussed on the appropriation and scientific classification of *Scylla* occupying the Malaysian waters (see, e.g. Ikhwanuddin, 2001; Ikhwanuddin et al., 2010; Mohammad Zaidi et al., 2011; Rosely et al., 2013). The occurrence of *Scylla serrata* has been recorded in the Matang Mangrove Forest Reserve, off the Straits of Malacca by Kosuge (2001), however, other taxonomic specialists neglected to recognize any. However, the latter group put together their perceptions with respect to the East Coast of Peninsular Malaysia and no studies were conducted near the area visited by Kosuge (2001). This vagueness has led to significant concern to the aquaculture and wild fisheries administration of mud crab. This article presents a reassessment of the distribution and species occurrence of *Scylla* spp. from Malaysia stem from taxonomic keys (morphological characteristics) and partial COI gene sequences.

2. Materials and methods

2.1. Sample collections

Scylla spp. can be found mostly in muddy habitats at estuarine and mangrove areas. Thus, before the selection of suitable locations for sampling activities, several field trips have been conducted across mangrove areas in Peninsular Malaysia including two locations from the east Malaysia (Borneo; Sabah and Sarawak). Sample collections was conducted for consecutively seven days every month except during monsoon season. Sample collection was accomplished from year 2010 and 2013. Mud crabs were discovered utilizing 20 baited crab traps with fish or chicken head set up in estuarine and mangrove areas (dispersed around 50 m separated from one another). After each collection of samples, the trap will be re-baited every 24 h. The South China Sea region was represented by populations sampled from Sarawak and eastern Peninsular, while the Straits of Malacca region was epitomized by groups from the western part of Peninsular Malaysia. Mud crabs originated from Sandakan, Sabah were obtained from the Sulu Sea while the Straits of Singapore was represented by population from Johor.

About 15 g of muscle tissues from each individual of mud crab was excised (from claw or abdomen) then put into a sterile microcentrifuge tube that contains 600–800 μL of 97% alcohol. The alcohol solution was frequently replaced every day to maintain the state of the samples. All microcentrifuge containing mud crab tissues were put at room temperature preceding DNA extraction.

2.2. Morphological recognition of mud crab

All specimens obtained from sampling locations, including three samples from Sulawesi and one sample from Pulau Jawa were examined and identified as specified by the taxonomic keys substantiate by Keenan et al. (1998) for primary discrimination before initiating DNA verification (Table 1). Mud crab samples from Sulawesi and Pulau Jawa were collected by colleagues currently conducting a research at the area.

2.3. Total genomic isolation and PCR amplifications

The high salt protocol (Aljanabi and Martinez, 1997) was used to isolate genomic DNA from all samples with some modifications. The PCR amplifications were applied utilizing the Cytochrome Oxidase Subunit I (COI) as a barcode marker. The constitution of isolated DNA was examined using 100 V electrophoresis on a 1.0% agarose gel approximately 30 min. The product was photographed using an ultraviolet light transilluminator after been stained using ethidium bromide (EtBr).

The isolated DNA template was PCR amplified using primer pair of COI: Mtd-10, 5′-T TGA TTT TTT GGT CAT CCA GAA GT-3′ (heavy strand primer) (Roehrdanz, 1993) and C/N 2769, 5′-TT AAG TCC TAG AAA ATG TTG RGG GA-3′ (Gopurenko et al., 1999) (light strand primer). All PCR amplified products were purified utilizing MEGA Spin Total Fragment DNA Purification Kit (Intron Biotechnology INC. Korea). All the purified PCR samples were sent for sequencing to a service provider (First Base Laboratories Sdn Bhd., Malaysia). This was performed using a BigDye® ver. 3.1 Terminator (Applied Biosystems) sequencing kit. Each reaction consists of 30 ng PCR products and 1.6 pmol of forward and reverse primer in a separate tube. Sequenced products were then electrophoresed on an ABI 3100*xl* capillary sequencer following standard protocols.

2.4. Molecular identification of mud crab and sequence alignment

All successfully sequenced samples were contemplated and applied on MEGA version 5.05 (Tamura et al., 2011) for alignment. This includes all four specimens obtained from Indonesia [Sulawesi (n = 3) and Jawa (n = 1)]. Additionally, three COI fragments of *S. serrata* retrieved from Genbank were also included in the analysis; GenBank accession numbers: KC154082.1 (from India), FJ011455.1 (from Australia) and AB600256.1 (from Japan). We also add in our analysis two COI fragments of other *Scylla* species (each from two different countries); *S. olivacea* - AB600270.1 (from Japan) and KC154078.1 (from India); *S. tranquebarica* - AB600266.1 (from Japan) and FJ011461.1 (from Australia); *S. paramamosain* - FJ011462.1 (from Australia) and HQ687256.1 (from China).

All sequences need to be aligned before further analysis and this was performed using ClustalW version 1.6 by Thompson et al. (1994) and implemented in MEGA version 5.05. Aligned fragments with noisy peaks were carefully examined and excised utilizing BIOEDIT version 7.0.9 (Hall, 1999). Afterwards, all aligned sequences were reassessed manually in order to maximize positional homology.

2.5. Molecular identification of mud crab

Both The Basic Local Alignment Search Tool (BLAST) web interface (http://www.ncbi.nlm.nih.gov/blast) and Genbank database

Table 1
Morphological keys identification for adult mud crab genus *Scylla*.

Species	Frontal lobe spines		Cheliped		Polygonal Patterning
	Shape	Height	Carpus spines	Propodus spines	
S. serrata	Blunt point	High	Both obvious	Obvious	Obvious
S. tranquebarica	Blunted	Moderate	Both obvious	Obvious	Weak
S. paramamosain	Triangular	Moderately high	Inner absent, outer reduced	Obvious	Weak
S. olivacea	Rounded	Low	Inner absent, outer reduced	Reduced	Absent

Modified from: Keenan et al., 1998. A revision of the genus *Scylla* De Haan, 1833 (Crustacea: Decapoda: Brachyura: Portunidae). *The Raffles Bulletin of Zoology*. Table 4, pp. 241.

were utilized to blast all aligned sequences in order to assign each individual into its respective taxon. Results from the blast search were compared with the morphological data to discern any disparity with the morphology and genetic data obtained. The locality of parsimony and variable sites was identified and implemented in MEGA version 5.05 (Tamura et al., 2011).

2.6. Phylogenetic analysis

In order to reveal the phylogenetic correspondence among all sequences analysed, Maximum Parsimony (MP; Faris, 1983) and Neighbor - Joining (NJ; Saitou and Nei, 1987) were employed in MEGA version 5.05 (Tamura et al., 2011). Off all the samples obtained in this study, only 51 sequences (inclusive of all sequences resolved from Genbank) were employed to generate the MP and NJ trees. These correspond to all the samples obtained in this study.

Maximum parsimony analysis was performed using the heuristic search algorithm with close - neighbour - interchange (CNI) on random tree branch swapping and 10 random sequence addition replicates (Nei and Kumar, 2000) as implemented in MEGA version 5.05 (Tamura et al., 2011). To resolve the most relevant DNA substitution's model prior to reconstruct mud crab's interrelations, the Akaike's Information Criterion (AIC) that correspond with the Bayesian's Information Criterion (BIC) was used. This test was also employed in MEGA version 5.05 (Tamura et al., 2011). Subsequently, Tamura 3-parameter model (T92 + G + I; Tamura, 1992) was chosen to be the best model for the substitution pattern because the BIC scores was very low among all models suggested. The nonparametric resampling procedure with Tamura 3-parameter model was conducted for NJ analysis with 10,000 replicates.

Johora singaporensis (Genbank accession no.: AB290641.1) and *Stoliczia chaseni* (Genbank accession no.: AB290645.1) are indigenous portunids and were used to root the NJ and MP tree regenerations in this study. Tamura-Nei genetic distance (Tamura and Nei, 1993) was used to compute the inter and intraspecies genetic distances (D_s; Nei, 1972) of mud crabs and implemented in MEGA version 5.05.

2.7. Species partitioning based on DNA barcodes

The Automatic Barcode Gap Discovery approach (ABGD, Puillandre et al., 2012) contemplates a COI segment as hypothetical species automatically based on the barcoding gap. This model engages a two-phase structure that firstly fractionates sequences into OTUs based on a statistically hypothesized barcode gap (e.g. primary partitioning), and thereafter engage a second cycle of splitting (e.g. recursive partitioning). There are three crucial variables in ABGD namely (1) X, relative gap width estimate, (2) minimum and (3) maximum values of prior intraspecific divergence, P that are important to determine barcode gap. ABGD analyses were performed at the web interface (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) on 12 December 2013 utilizing a customary value of relative gap width (X = 1.5) and JC69 (Jukes and Cantor, 1969) together with P-distance. The JC69 distance method was used in the analysis as it is a simple model assumes that substitutions occur with equal probability among the four nucleotide types of DNA (Jukes and Cantor, 1969).

3. Results

3.1. Sampling data

There are 249 mud crabs were randomly caught from 24 mangrove swamp areas across Malaysia and represents nine states in Malaysia that involved four oceanic regions (Fig. 1). All samples
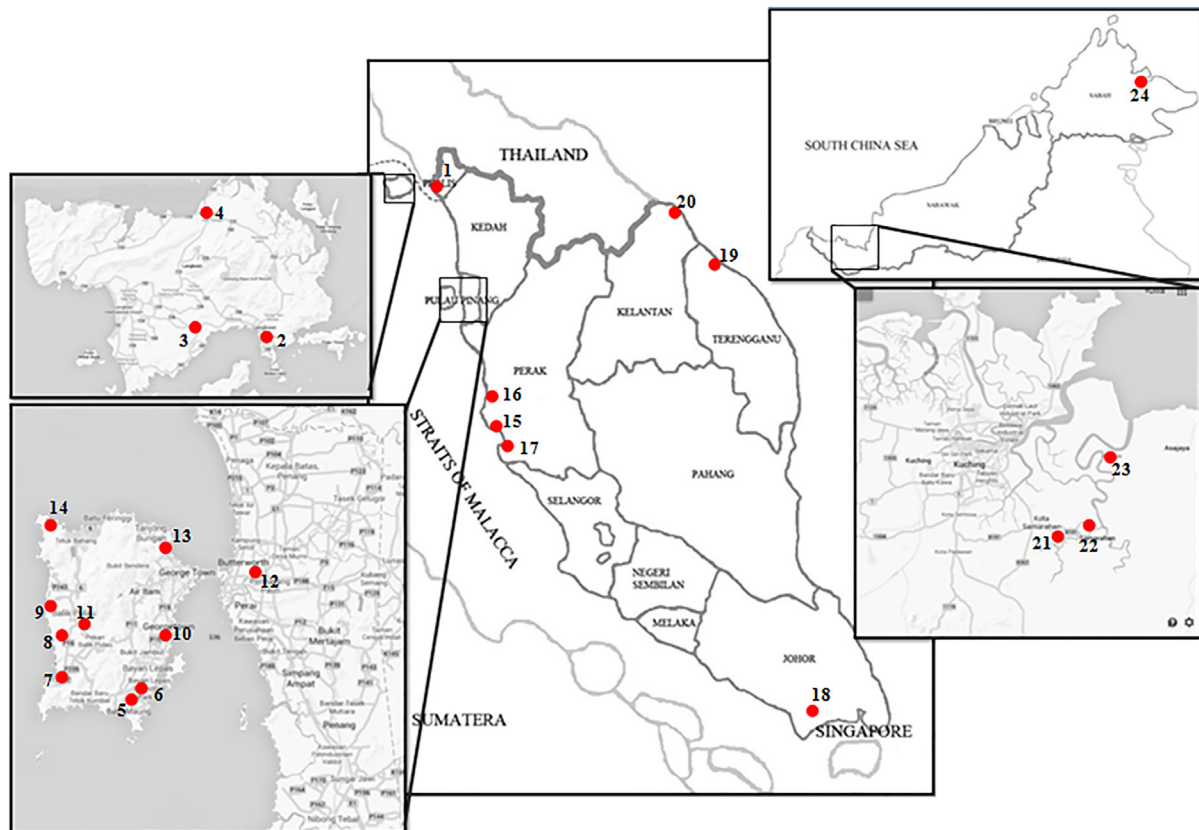

Fig. 1. Map of sampling locations. The numbering refers to the respective sampling sites as stated in Table 2.

were collected from two states in the East Peninsular Malaysia (Terengganu, $n = 21$; Kelantan, $n = 18$), four populations represent the West Peninsular Malaysia (Langkawi, Kedah, $n = 18$; Penang, $n = 40$; Perlis, $n = 15$; Perak, $n = 49$), and a single population from the southern part of Peninsular Malaysia (Johor, $n = 15$). Additionally, one population each from Sabah ($n = 38$) and Sarawak ($n = 35$) were also obtained (Table 2).

3.2. Species identification and distribution

Off the 249 mud crabs sampled from Malaysia, approximately 81% individual mud crabs were undoubtedly designated to three putative taxa derived from morphological characteristics; _S. tranquebarica_, _S. olivacea_ and _S. paramamosain_. No _S. serrata_ was recorded in this research. However, there was a potential occurrence of _S. serrata_ in our sampling locations based on an initial investigation on immature specimens, but this is just an assumption and cannot be accounted for. We experienced a great intricacy in distinguishing among _S. tranquebarica_ and _S. serrata_ based on their morphological characteristics.

BLAST assessment of the partial COI sequence divulged the number of individuals for each species; _S. olivacea_ ($n = 111$), _S. tranquebarica_ ($n = 61$) and _S. paramamosain_ ($n = 29$). We also found that there are no evidences of _S. serrata_ in our data sets. Most of the sequences (99%) matched to their correspond sequence in Genbank (data not shown) demonstrating that our technique on species delineation of adult mud crab was valid. The most dispersed spe-

cies in this study with 111 individuals is _S. olivacea_ where they can be found in all sampling locations with the exceptions of Sabah and the southern part of Peninsular Malaysia (Johor). There are 61 individuals of mud crab has been distinguished as _Scylla tranquebarica_ and this species occurs in most of the locations but absent in Penang and Langkawi. Our data shows that the number of _S. paramamosain_ obtained is very low compared to other species which only 29 individuals recorded. This species was also absent in Johor, Perlis and Sabah.

3.3. Sequence information and genetic distance

There are 165 and 146 variable and parsimonious informative sites respectively in the 542 bp COI sequences of mud crab (Table 3). The strong number of parsimonious informative regions demonstrate that the gene is an appropriate and useful marker for species delineation and phylogenetic studies (Kamarudin et al., 2011). This marker is also beneficial for population disparity and genetic configuration studies. The between species genetic distances (D_s) were significantly higher (0.100 – 0.218), manifesting that all the member of _Scylla_ were genetically unrelated (Table 4). In contrast, within species genetic distances (D_s) were significantly low, ranging from 0.003 to 0.006 signifying the exclusion of cryptic species (Table 4).

The species' numbers defined by the ABGD ranged with the change prior thresholds from 0.0010 to 0.1 prior intraspecific divergences (P) as shown in Fig. 2. The lowest threshold values

Table 2
Sampling locations with coordinate, oceanic region, and sample size (_N_) of _Scylla_ species.

No.	Sampling Sites	Oceanic Region	Code	N	Coordinates
1	Kuala Perlis, Perlis	Straits of Malacca	KP	15	6°40′ N, 100°13′ E
2	Kuah, Langkawi, Kedah	Straits of Malacca	KL	5	6°31′ N, 99°85′ E
3	Kuala Muda, Langkawi, Kedah	Straits of Malacca	KML	7	6°32′ N, 99°78′ E
4	Bohor Merah, Langkawi, Kedah	Straits of Malacca	BML	6	6°42′ N, 99°80′ E
5	Teluk Tempoyak, Penang	Straits of Malacca	TTP	11	5°27′ N, 100°28′ E
6	Batu Maung, Penang	Straits of Malacca	BM/BMP	7	5°27′ N, 100°28′ E
7	Pulau Betong, Penang	Straits of Malacca	PB	1	5°30′ N, 100°19′ E
8	Sg. Burung, Penang	Straits of Malacca	SB	1	5°32′ N, 100°19′ E
9	Kuala Jln Baru, Penang	Straits of Malacca	KJB	6	5°35′ N, 100°19′ E
10	Gelugor, Penang	Straits of Malacca	GPP	1	5°36′ N, 100°31′ E
11	Sg. Pinang, Penang	Straits of Malacca	SP	1	5°39′ N, 100°21′ E
12	Permatang Pauh, Penang	Straits of Malacca	PMP	1	5°42′ N, 100°41′ E
13	Tanjong, Penang	Straits of Malacca	TPP	4	5°45′ N, 100°31′ E
14	Muka Head, Penang	Straits of Malacca	n/a	7	5°47′ N, 100°19′ E
15	Kuala Sepetang, Perak	Straits of Malacca	KS	30	4°83′ N, 100°62′ E
16	Kuala Gula, Perak	Straits of Malacca	KGU	13	4°93′ N, 100°46′ E
17	Manjong, Perak	Straits of Malacca	MNJ	6	4°32′ N, 100°66′ E
18	Gelang Patah, Johor	Straits of Singapore	J	15	1°44′ N, 103°60′ E
19	Kg. Dalam Rhu, Terengganu	South China Sea	RHU	21	5°84′ N, 102°53′ E
20	Kg. Dal, Kelantan	South China Sea	DAL	18	6°20′ N, 102°26′ E
21	Sg. Stakan, Kuching	South China Sea	ST	10	1°43′ N, 110°45′ E
22	Muara Tuang, Kuching	South China Sea	MT	15	1°44′ N, 110°48′ E
23	Asajaya, Kuching	South China Sea	AJ	10	1°54′ N, 110°51′ E
24	Sandakan, Sabah	Sulu Sea	S	38	5°84′ N, 118°09′ E

Table 3
Summary of sequence information and pairwise Tamura-Nei genetic distance (among and within species) for all collected specimens. N = sample size, _N_hap = number of haplotypes, Ti = transition; Tv = transversion.

Species	Variant	_S. olivacea_	_S. tranquebarica_	_S. paramamosain_	_S. serrata_
N		111	61	29	4
_N_hap		66	12	16	3
G + C (%)		42.71	21.19	29.87	26.93
Ti		40	10	12	2
Tv		12	2	3	0
Number of sites	Variable	81	55	27	2
	Conserved	492	530	527	540
	Parsimonious informative	56	26	9	1

Table 4
Pairwise of inter- and intraspecific genetic distance (D_s) of *Scylla* spp. based on Tamura-Nei statistic.

Species	S. tranquebarica	S. paramamosain	S. olivacea	S. serrata
S. tranquebarica	0.003			
S. paramamosain	0.100	0.006		
S. olivacea	0.172	0.209	0.002	
S. serrata	0.183	0.112	0.218	0.004
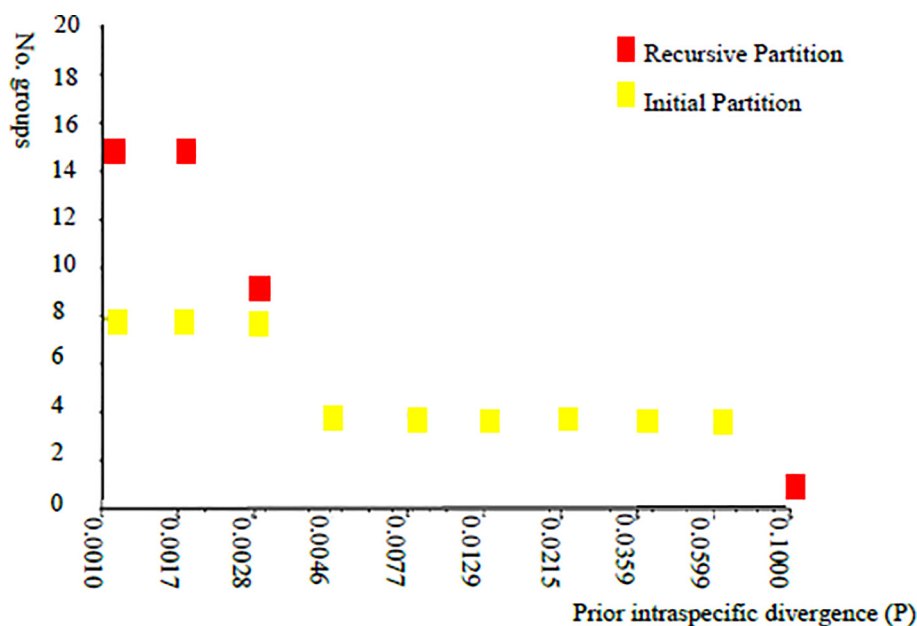
Fig. 2. Barcode gap analysis of COI sequences performed by ABGD shows the number of partitions obtained in each prior threshold.

varied from 0.0010 to 0.0017 and grouped nearly all haplotypes as a different species (15 species). Threshold values ranged from 0.0046 to 0.0599 partitioned only four species. This has resulted in the same grouping as shown by MP and NJ phylogenetic tree model of all species. Thus, the four species clusters that were demarcated by the ABGD approach via the high prior threshold for COI marker are supported by the outcome of NJ and MP analysis (Fig. 3).

Four monophyletic groups analogous to *S. serrata, S. tranquebarica, S. olivacea* and *S. paramamosain* as inferred by BLAST (with an additional of Genbank sequences for each species) were established from MP and NJ phylogenetic trees. All clustered groups were supported by a high percentage of bootstrap values (Fig. 3). Yet, the non-appearance of *S. serrata* from Malaysian waters is highlighted. All three *Scylla* species putatively assigned based on their morphological characteristics were grouped with *S. serrata* (sequences retrieved from Genbank) and forming the sister taxon in as demonstrated by the NJ phylogenetic tree (Fig. 3A). The MP tree (Fig. 3B) revealed that *S. tranquebarica* and *S. olivacea* were nearly homologous species, while *S. paramamosain* was genetically distant from the group. The basal group was formed by *S. serrata*.

4. Discussion

This current research not only successfully established COI data for four distinct species within *Scylla* spp. but also gave initial signals on species structuring within the Malaysian waters. Overall, all species showed congruency with taxonomic boundaries investigated within current study.

The GC content in the COI gene composition and complete mitochondrial genome is considered a very important factor in

the analysis of related molecular studies (Min and Hickey, 2007). A high GC-content is helpful for understanding nucleotide diversity and evolutionary processes (Romiguier et al., 2010). The overall mean GC content of 30.18% (Table 3) was observed across the species of genus *Scylla* which was slightly lower than the GC content in marine teleosts (47.1%) as reported by Lakra et al. (2011). The assessment of GC frequencies in commercial fish is useful in understanding the nucleotide diversity and further in assessment of evolutionary lineages for interpreting mutation pressures on taxa by choosing substitution models (Figuet et al., 2014). For example, the more GC content the more nucleotide diversity and there will be more chances of mutations in a population.

Both the DNA barcode data demonstrated by ABGD analysis (Fig. 2) and species relatedness exhibited by both trees of maximum parsimony (MP) and neighbour-joining (NJ) (Fig. 3) were congruent (four distinct groups) with the previous research performed by Keenan et al. (1998) that showed a similar phylogenetic sister-species relatedness of mud crab using COI gene markers. This was further corroborated by the genetic relatedness analysis among all the *Scylla* spp. (Table 4). The topography of both phylogenetic trees is same in which *S. olivacea* formed the terminal taxon, while *S. serrata* formed the basal group in spite of the fact that the support was not very high. Undoubtedly, this research has successfully demonstrated that the utilization of the morphological keys proposed by Keenan et al. (1998) (Table 1) in the delineation of genetic relatedness of a species was beneficial, especially to distinguish between *S. paramamosain* (Fig. 4) and *S. olivacea* (Fig. 5), even in their juvenile stage. Nevertheless, the morphological abstruseness between *S. serrata* and *S. tranquebarica* was apparent compared to the other species. The fact that some species of mud crab are dominant in certain area (for example *S.*
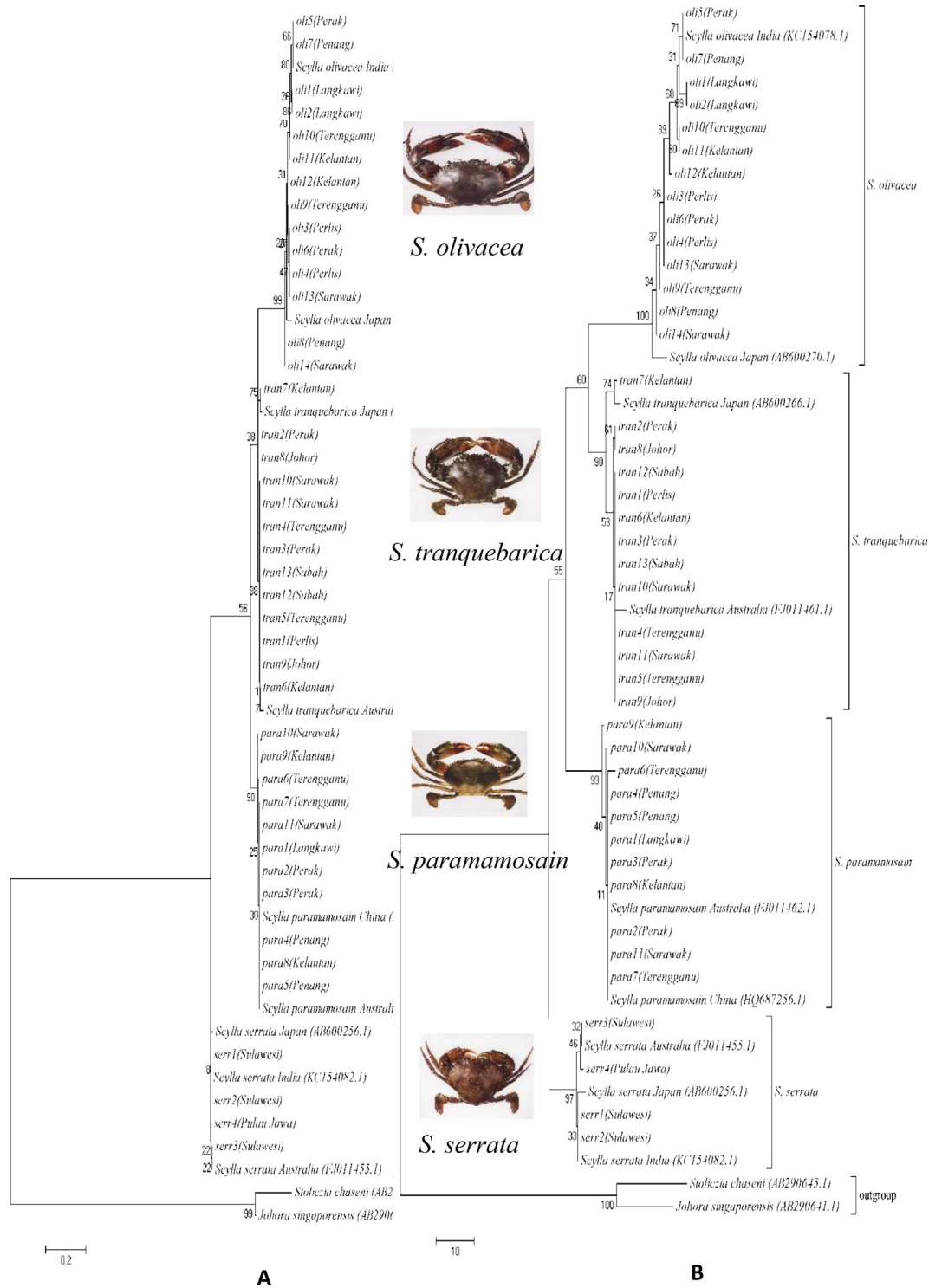
Fig. 3. Neighbour-joining (NJ) phylogenetic tree (A) and Maximum Parsimony (B) demonstrating the genetic relatedness among all *Scylla* species based on COI (COI partial segment of *S. serrata* retrieved from GenBank was included).

paramamosain in Johor) is probably due to the suitability of habitat conditions for the crab's survivability. This may also cause by the presence of abundant food and the absence of predators that will reduce the population of crabs in the region.

The diagnostic traits between *S. tranquebarica* (Fig. 6) and *S. serrata* (Fig. 7), namely the frontal lobe spines and both carpus and propodus spines were identical and difficult to distinguish even though several reassessment of the samples has been done sub-

stantially in juveniles. In all likelihood, this may be due to patterning and irregular coloration as exhibited by mud crabs in reciprocation to their surrounding (Keenan et al., 1998), hence, induced misidentification of species especially among non-expert individuals in field identification. Our study is in agreement with the research conducted by Mohammad Zaidi et al. (2011) in which reported the absence of *S. serrata* in Malaysia (derive from morphological studies) though the presence of this species has been
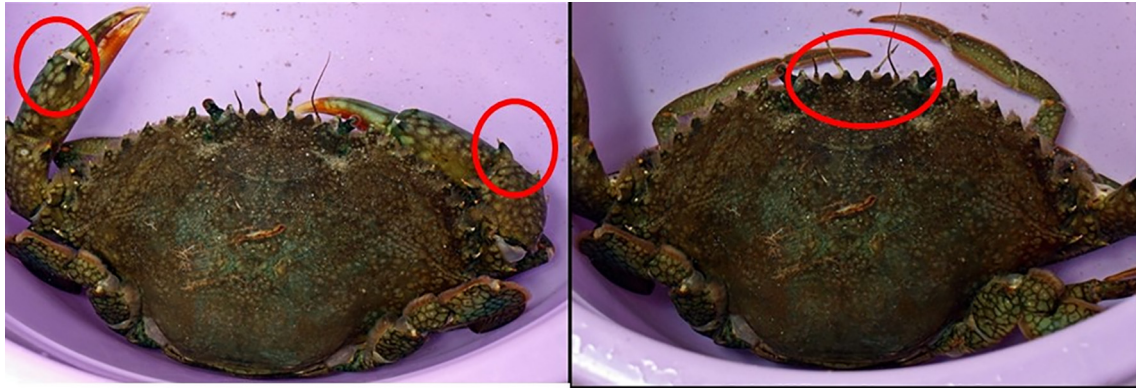
Fig. 4. Carpus spines and propodus spines (left, circled area) and also the triangular, moderately high frontal lobe spines (right, circled area) of *S. paramamosain*.

Fig. 5. Carpus spines and propodus spines (left, circled area) and also the rounded and low frontal lobe spines (right, circled area) of *S. olivacea*.


Fig. 6. Carpus spines and propodus spines (left, circled area) and also the blunted moderate frontal lobe spines (right, circled area) of *S. tranquebarica*. It looks similar to *S. serrata* except for the patterning which was absent on both chelipeds and weak on the legs.

Fig. 7. Carpus spines and propodus spines (top, circled area) and also the blunt point, high frontal lobe spines (bottom, circled area) of *S. serrata* (specimens obtained from Sulawesi, Indonesia). The patterning is also very obvious and clear on both chelipeds and all legs.

recorded by Najiah et al. (2010). Likewise, the four individuals of *S. serrata* from Indonesia (Sulawesi, *n* = 3; Pulau Jawa, *n* = 1) have furnished irreversible proof that none of the specimens collected from Malaysia was *S. serrata* (Table 2). For that reason, the exclusion of *S. serrata* in this study was not because of identification fallacy.

The fact that no *S. serrata* was identified in our study has raised a scepticism as most previous researchers declared the profusion of *S. serrata* in Malaysia and neighbouring waters (see, e.g. Overton et al., 1997; Keenan et al., 1998; He et al., 2010). Thus the morphological characteristics of *S. serrata* was relooked and compared with
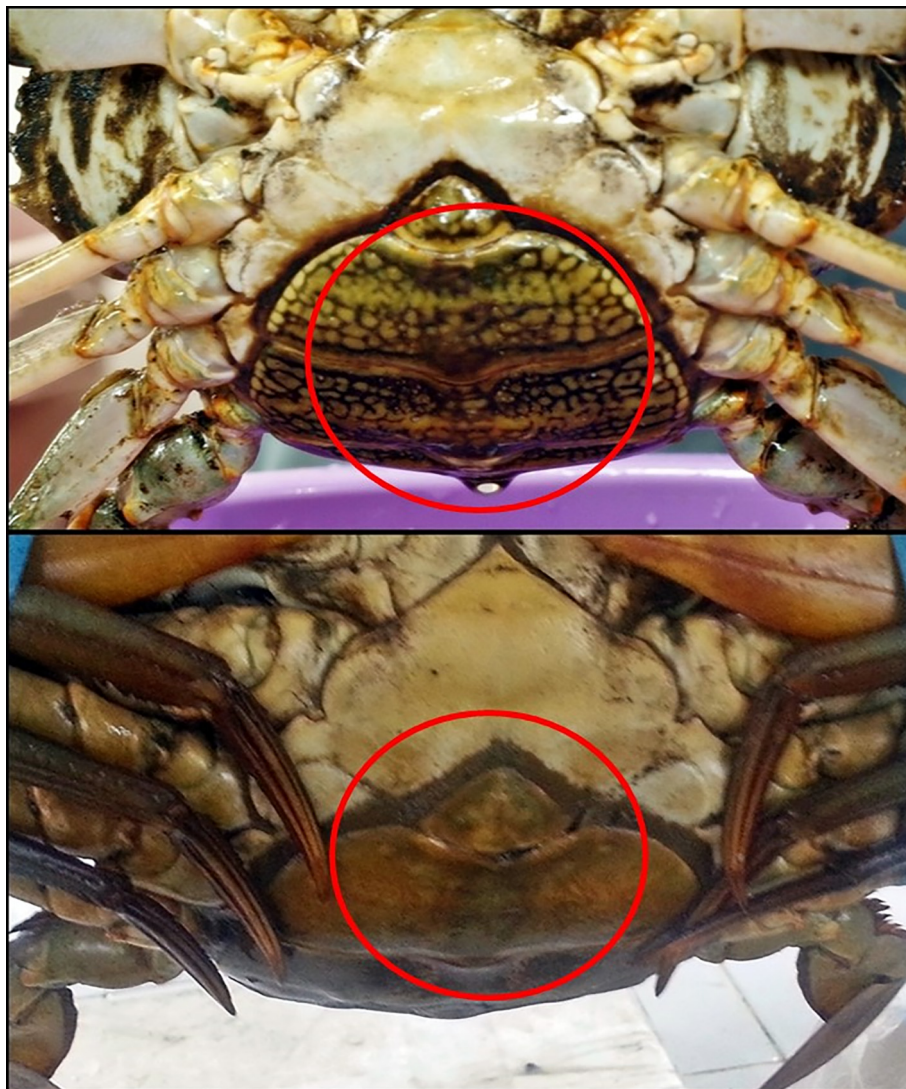
Fig. 8. The female abdomen of *S. serrata* (top, circled area) with obvious and clear polygonal patterning compared to *S. tranquebarica* (bottom, circled area) with absent patterning.

the other three species. We found that a trait that possibly mysterious in mud crab identification is the polygonal patterning that could be observed in the species particularly the *S. serrata - S. tranquebarica* species delineation complex. The polygonal patterning is clear on the chelipeds and all the legs in *S. serrata* meanwhile it is poorly determined on the chelipeds and the first two pairs of legs for *S. tranquebarica*; however, on the last two pairs of legs the patterns quite discrete (Keenan et al., 1998). The patterning on the female abdomen of *S. tranquebarica* is poorly viewed when compared to *S. serrata* (Fig. 8).

Furthermore, *S. serrata* is known for their bluish black colouration, but *S. tranquebarica* has green-brown colour. This phenomenon is probably because of their habitat and environment where *S. serrata* is more oceanic. Having said that, the identification of mud crab will become more problematic if the patterning level and colouration are consecutive, overlap and morphologically plastic, thus may further confound the observer.

It becomes visible that genetic identification was a more practical approach than traditional methods for a more accurate species discovery, in our case *Scylla* spp. Individual in Fig. 8 represent *S. serrata* and *S. tranquebarica* that were conclusively determined, however, as formerly discussed, many individuals collected in this study failed to show such clear distinction for species divergence.

Without being affected by the broad geographical area across Malaysia, we found that no *S. serrata* was obtained from all sampling locations in this study (Fig. 1), though the occurrences of S. *serrata* has been reported previously (see Overton et al., 1997; Keenan et al., 1998; Gopurenko et al., 1999; He et al., 2010; Fratini et al., 2010) in Malaysia and its surrounding. A plausible elucidation is overharvesting may serve as a powerful agent in reducing the number of mud crab in Malaysian waters. Ironically, mud crabs have not yet been considered as an endangered species. Notwithstanding, based on our current knowledge and research, there might be other phenomena that caused this to occur and may not be realized.

Previous research by Kosuge (2001) publicized that no *S. tranquebaria* and *S. paramamosain* were recorded during their sampling activities in Matang Mangrove Forest Research, off the Straits of Malacca. However, *S. serrata* was the dominant species occurred within the area (60%, n = 81) followed by *S. olivacea* (40%). Research by Kosuge (2001) is extensively diverged with this current study. However, most of the specimens examined by Kosuge (2001) were juvenile and this could lead to misidentification. In order to disentangle the confusion on the abundance of *S. serrata* in Malaysia, the region near the original sampling by Kosuge (2001) was revisited (Fig. 9). Based on the information from the local community, there are two distinct categories for mud crabs within the area; mangrove crabs (sampled at the X-marked location) while Z-marked location is the area where the marine crabs usually can be found (Fig. 9). Although we managed to collect both categories as described by the local communities, however, *S. serrata* was still failing to discover. Likewise, based on morphological characters, Ikhwanuddin et al. (2010) and Mohammad Zaidi et al. (2011) declared that *S. serrata* absent on the continental coast of the South China Sea in spite of the fact that they are prevalent in the Indo-Pacific Ocean (Keenan et al., 1998). Thus, the use of other DNA marker as well as the expansion of the area for sampling is very crucial to support the results claimed by Kosuge (2001).
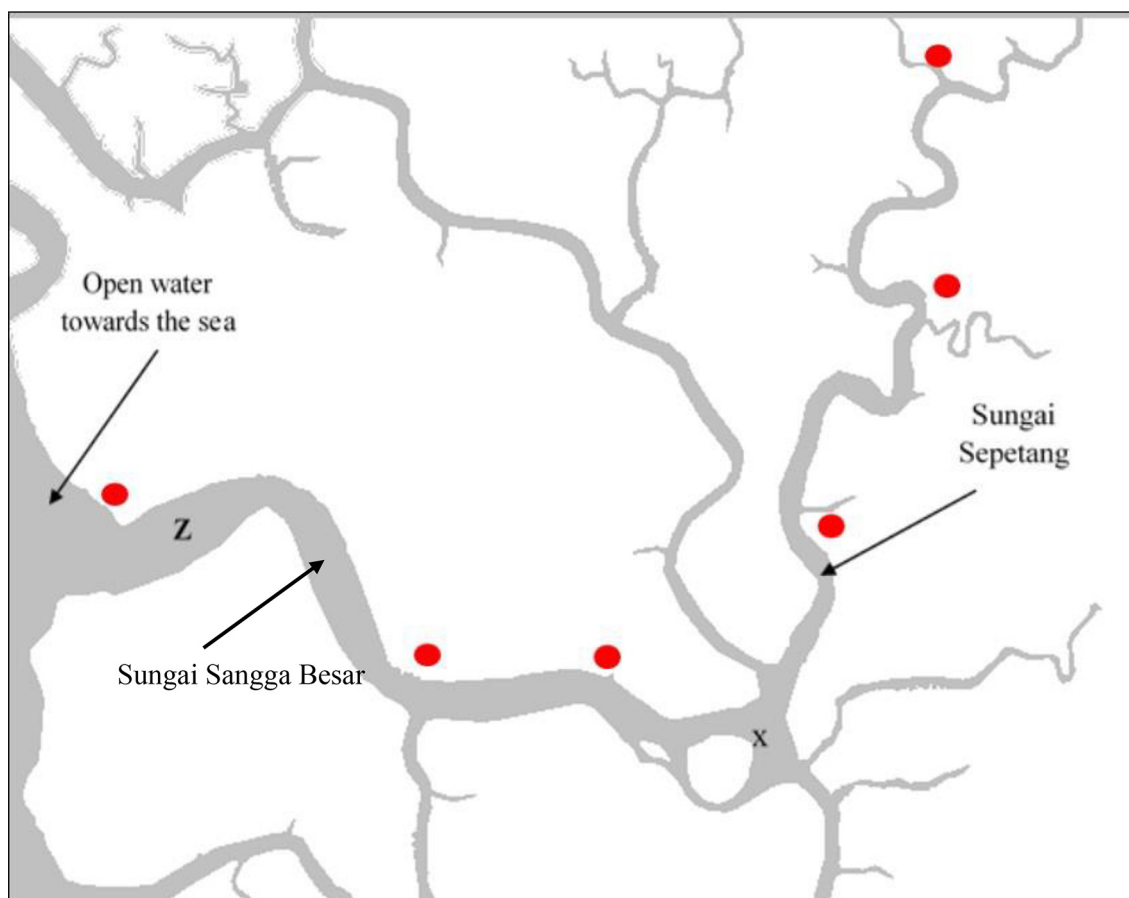
Fig. 9. Sampling areas in Kuala Sepetang, Perak. Red filled circle were the original sampling areas done by Kosuge (2001). The re-sampling area in this current research was conducted along the Sungai Sangga Besar (from X to Z).

5. Conclusion

It is shown in this study that the mtDNA COI gene is proficient in species delimitation and genetic relatedness, substitute the traditional approach of distinguishing a species that is based on morphological traits which results are frequently debatable. Ironically, this research has failed to recognize any *S. serrata* despite the fact that there is evidence on the occurrences of the species in Malaysia. Either this phenomenon is due to inadequate efforts of sample collections or a correct indication of the mud crab distribution in Malaysia, continue to be puzzled. Thus, further research needs to be conducted in greater depth by expanding sampling location to cover wider area. The findings in this study has elucidated the significance of COI in taxonomy and undoubtedly will give insight into the species complex.

Acknowledgements

Author contribution

DMN established the theoretical formalism, performed the analytic calculations and numerical simulations. Both SAMN and SM contributed to the final version of the manuscript.

Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Estampador, E.P., 1949. Studies on *Scylla* (Crustacea: Portunidae), I. Revision of the genus. The Philippine J. Sci. 78 (1), 95–109.

Faris, J.S., 1983. The logical basis of phylogenetic analysis. Adv. Cladist. 2, 1–36.

Figuet, E., Ballenghien, M., Romiguier, J., Galtier, N., 2014. Biased gene conversion and GC-content evolution in the coding sequences of reptiles and vertebrates. Genome Biol. Evol. 7 (1), 240–250.

Fratini, S., Ragionieri, L., Cannicci, S., 2010. Stock structure and demographic history of the Indo-West Pacific mud crab *Scylla serrata*. Est. Coast. Shelf Sci. 86 (1), 51–61.

Fuseya, R., Watanabe, S., 1996. Genetic variability in the mud crab genus *Scylla* (Brachyura: Portunidae). Fisherie Sci. 62 (5), 705–709.

Gopurenko, D., Hughes, J.M., Keenan, C.P., 1999. Mitochondrial DNA evidence for rapid colonization of the Indo-West Pacific by the mud crab *Scylla serrata*. Mar. Biol. 134 (2), 227–233.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Series. 41, 95–98.

He, L., Zhang, A., Weese, D., Zhu, C., Jiang, C., Qiao, Z., 2010. Late Pleistocene population expansion of *Scylla paramamosain* along the coast of China: a population dynamic response to the Last Interglacial sea level highstand. J. Exp. Mar. Biol. Ecol. 385 (1), 20–28.

Ikhwanuddin, A.M., 2001. The Biology and Fishery of Mud Crab, genus Scylla in East Malaysia. Degree of Master of Science (Aquatic Science) thesis, Universiti Malaysia Sarawak.

Ikhwanuddin, M., Bachok, Z., Hilmi, M.G., Azmie, G., Zakaria, M.Z., 2010. Species diversity, carapace width-body weight relationship, size distribution and sex ratio of mud crab, genus *Scylla* from Setiu Wetlands of Terengganu coastal waters, Malaysia. J. Sustain. Sci. Manag. 5 (2), 97–109.

Jukes, T.H., Cantor, C.R., 1969. Evolution of Protein Molecules. Academic Press, New York, pp. 21–132.

Kamarudin, K.R., Rehan, A.M., Hashim, R., Usup, G., Ahmad, H.F., Anua, M.H., Idris, M.Y., 2011. Molecular phylogeny of *Holothuria* (*Mertensiothuri*a) *leucospilot*a (Brandt 1835) as inferred from cytochrome c oxidase I mitochondrial DNA gene sequences. Sains Malaysiana 40 (2), 125–133.

Keenan, C.P., Davie, P.J.F., Mann, D.L., 1998. A revision of the genus *Scylla* de Haan, 1833 (Crustacea: Decapoda: Brachyura: Portunidae). Raff. Bull. Zool. 46, 217–246.

Keenan, C. P., Mann, D. L., Lavery, S., Davie, P., 1995. Genetic relationships, morphological identification and taxonomy of mangrove crabs, genus Scylla, from throughout the Indo-Pacific. ACIAR Project Report, QDPI, Brisbane.

Kosuge, T., 2001. Brief assessment of stock of mud crabs *Scylla* spp. in Matang Mangrove Forest, Malaysia and proposal for resources management, Japan. Agr. Res. Quart. 35 (2), 145–148.

Lakra, W.S., Verma, M.S., Goswami, M., Lal, K.K., Mohindra, V., Punia, P., Gopalakrishnan, A., Singh, K.V., Ward, R.D., Hebert, P., 2011. DNA barcoding Indian marine fishes. Mol. Ecol. Res. 11 (1), 60–71.

Min, X.J., Hickey, D.A., 2007. Assessing the effect of varying sequence length on DNA barcoding of fungi. Mol. Ecol. Res. 7 (3), 365–373.

Mohammad Zaidi, Z., Hilmi, M.G., Ikhwanuddin, M., Bachok, Z., 2011. Population Dynamics of Mud Crab, *Scylla olivacea* from Setiu Wetland Areas, Terengganu, Malaysia. UMTAS, 34–38.

Najiah, M., Nadirah, M., Sakri, I., Shaharom-Harrison, F., 2010. Bacteria Associated with mud crab (*Scylla serrata*) from Setiu Wetland, Malaysia with emphasis on antibiotic resistances. Pak. J. Biol. Sci. 13 (6), 293–297.

Nei, M., 1972. Genetic distance between populations. Am. Nat. 106 (949), 283–292.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press.

Overton, J.L., Macintosh, D.J., Thorpe, R.S., 1997. Multivariate analysis of the mud crab *Scylla serrata* (Brachyura: Portunidae) from four locations in Southeast Asia. Mar. Biol. 128 (1), 55–62.

Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD Automatic Barcode Gap Discovery for primary species delimitation. Mol. Ecol. 21, 1864–1877.

Roehrdanz, R.L., 1993. An improved primer for PCR amplification of mitochondrial amplification in a variety of insect species. Insect Mol. Biol. 2 (2), 89–91.

Romiguier, J., Ranwez, V., Douzery, E.J.P., Galtier, N., 2010. Contrasting GC-content dynamics across 33 mammalian genomes: relationship with life-history traits and chromosome sizes. Genome Res. 20 (8), 1001–1009.

Rosely, H.A.-A.M., Mohd. Nor, S.A., Yahya, K., Naim, D.M., 2013. Mitochondrial DNA diversity of mud crab *Scylla olivacea* (Portunidae) in Peninsular Malaysia: a preliminary assessment. Mol. Biol. Rep. 40, 6407–6418.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4 (4), 406–425.

Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C content biases. Mol. Biol. Evol. 9 (4), 678–687.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10 (3), 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28 (10), 2731–2739.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc. Acids Res. 22 (22), 4673–4680.