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Mitochondrial *COI* gene Sequence of giant freshwater prawn, *Macrobrachium rosenbergii*: An assessment of a community-based stock enhancement programme in Petagas River, Sabah, Malaysia

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Abstract
In order to offset the decline in the population dynamics of fisheries of the Petagas River, Sabah, a community-based stock enhancement project was initiated by the Borneo Marine Research Institute (BMRI) of University Malaysia Sabah to increase the population of giant freshwater prawn, *Macrobrachium rosenbergii* and its natural spawning in the wild. A total of 38,000 of hatchery reared *M. rosenbergii* juveniles have been released in the Petagas River from year 2012 to 2015. In order to assess the success of stock enhancement, sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene of *M. rosenbergii* reared in the BMRI hatchery and the samples caught in Petagas River were analyzed. In the present study, a total of 46 individuals (18 adults and 28 juveniles) were collected from both BMRI shrimp hatchery and Petagas River. *COI* sequences of 600 to 657 bp were successfully obtained. Sequence similarity analyses showed that all samples from BMRI shrimp hatchery were identified as *M. rosenbergii* (89% to 99% sequence similarity) while samples from Petagas River were identified as *M. rosenbergii* (89% to 95%), *M. mammilodactylus* (83% to 99%), *Macrobrachium* sp. (84% to 86%), *Caridina gracilipes* (91% to 92%), *Caridina* sp. (81% to 87%), *Litopenaeus stylirostris* (83%) and *Metapenaeus ensis* (99%). The phylogenetic tree of *COI* gene produced three clusters of *Macrobrachium* genus, separated with its closely related species. Genetic distances between *M. rosenbergii* from BMRI shrimp hatchery and *M. rosenbergii* caught in Petagas River ranged from 0.00% to 13.8%. It suggested that the *M. rosenbergii* released in stock enhancement programme were closely related to the *M. rosenbergii* caught. Abundant individuals of *M. mammilodactylus* were also caught in the river indicating that the species is the dominant species and *M. rosenbergii* has yet to be established in its population following the stock enhancement practice. This molecular analysis of *Macrobrachium* sp. will provide baseline information on existing species in Petagas River as well assessing the effectiveness of this stock enhancement programme.

Keywords: Stock enhancement, giant freshwater prawn, *Macrobrachium rosenbergii*, Sabah

1. Introduction
Stock enhancement is defined as the release of hatchery-reared juveniles into wild populations to enhance the natural stock of harvestable fish and overcome recruitment problem [1-3]. Stock enhancement has been attempted in various type of water body globally, however the effectiveness of stock enhancement of hatchery-cultured organisms, though, has been hindered by lack of scientific, institutional, and fisheries-management perspective focusing in planning, design, implementation, and evaluation of enhancement programs [4, 2, 5-13]. Stock enhancement practices must be followed by genetic management in order to avoid harmful genetic effects on wild stocks [2, 3] i.e potential disturbance of spatial population structure, negative effects on fitness and diversity of wild stocks [15, 16].
The Palaemonoidea (Rafinesque, 1815) is the largest family of the order Decapoda which comprised of 2 subfamilies and 102 genera [16]. They are distributed widely in marine, estuarine and freshwaters. Freshwater prawns of the genus *Macrobrachium rosenbergii* commonly known as Giant Freshwater Prawn is one of the largest prawns in the family of Palaemonoidea which belongs to genus *Macrobrachium*. *M. rosenbergii* lives in tropical freshwater environments that are subjected to brackish water [17].

Their morphology classification was doubted because they appear to be morphologically highly conservative [18, 19]. The most common morphological characters used in taxonomy of the genus *Macrobrachium* are the second pereiopod and the rostrum [20]. Molecular genetic approaches were used to clarify the systematic relationships of different species [21].

As the rivers are important reservoirs of this *Macrobrachium* species, it is essential to conserve their genetic diversity especially in stock enhancement programme. There is a positive correlation between the genetic diversity and evolutionary potential and fitness of a population [22, 23]. Estimation of the distribution of subpopulations based on the population genetic structure may help researcher to protect weaker populations through regulating harvest based on their genetic properties [24]. Genetic characterization also serves as a useful tool for evaluating the direction and magnitude of changes in genetic structure of species over time especially in stock enhancement programme.

Stock enhancement of *M. rosenbergii* has been embarked by Borneo Marine Research Institute (BMRI), University Malaysia Sabah, Malaysia by releasing hatchery-reared post larvae and juveniles of giant freshwater prawn into Petagas River. The programme was launched in 2012 and aims to develop method for natural spawning and boost the population of *M. rosenbergii* followed by a genetic evaluation of the stocking species. A total of 38,000 of *M. rosenbergii* post larvae and juveniles which were bred in the BMRI hatchery were released into the Petagas River, Sabah from year of 2012 until 2015 in a community-based stock enhancement programme for the benefit of local community [25]. Stock enhancement is one of the aquaculture practices associated to the production of *M. rosenbergii* in Malaysia [25] and this practice was expected to yield the desired outcome from a natural body of water where natural productivity is high [9] and its role in rebuilding depleted populations.

A preliminary study on the distribution and population abundance on *M. rosenbergii* in Petagas River was carried out from 2013 to 2015 and found that, there were two morphologically different species of giant freshwater prawn occurred in the Petagas river. Although this genus is potentially suitable for aquaculture, nothing is known of the genetic variability of the *M. rosenbergii* within and among natural populations in Petagas River, or the phylogenetic relationships between species caught. Previous studies suggested that DNA barcoding using a standardized mitochondrial cytochrome oxidase subunit I (*COI*) sequence was proposed as species identification system, and as a method for detecting putative species [26, 27]. We hypothesized that the two morphologically different species were from same genus of *Macrobrachium* in Petagas River.

Thus, this research was to report the findings of mitochondrial *COI* use in taxonomy and phylogenetic relationships among genus *Macrobrachium* as well as determination of the genetic distance between *M. rosenbergii* from BMRI shrimp hatchery and *M. rosenbergii* caught in Petagas River. Genetic data in local populations of genus *Macrobrachium* in Petagas River will be necessary for defining appropriate future management and monitoring of stock enhancement and will establish as a baseline data thus assessing the effectiveness of the stocking programme.

2. Materials and Methods

2.1 Sampling

A total of 46 individuals, i.e., 2 adult brood stocks and 6 juveniles that were involved in stock enhancement programme, were collected from BMRI shrimp hatchery, University Malaysia Sabah and 16 adults and 22 juveniles were captured from Petagas River (latitude 05º54.847' and 05º53.889' North and longitude 116º02.744' and 116º04.644' East of Sabah). All samples were transferred in 95% ethanol to the laboratory and stored at -20 °C until used. Henceforth, the adult brood stocks and juveniles from BMRI shrimp hatchery were abbreviated as BS and JH respectively. Based on the distinctive morphology of the adult prawns caught from the Petagas River, they were abbreviated as BA (Light blue carapace and extended rostrum) and WA (Light brown carapace and shorter rostrum) respectively. The samples of juvenile prawns from the river could not be differentiated easily thus, they were labeled as JS.

2.2 DNA extraction, PCR amplification and sequencing

DNA was extracted from the abdominal or pereiopod muscle tissue of the 46 specimens using Qiagen DNeasy Blood and Tissue (Germany) following manufacturer protocols. Extracted DNA was checked using 1% agarose electrophoresis, then diluted to appropriate concentration (50-100 ng/µl) for PCR amplification. Amplification reaction of the *COI* gene was in 50 µl volume consisting of 1 x TopTaq PCR buffer, 1 x Q-solution, 200 µM of dNTPs, 10 µM of each primer, 1.25U of TopTaq DNA polymerase (Qiagen, USA), DNA template (50-100 ng), with MilliQ sterilized water added to make up to the final volume to 50 µl. Thermal cycling was performed as follows: initial denaturation for 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The primer pair used was COIa (AGT ATA AGC GTC TGG GTA GTC) and COIf (CCT GCA GGA GGA GGA GAC CC [28]. The size and quality of the PCR products were visualized on 1.5% agarose gels. The purified PCR products were then sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems), using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems).

2.3 Data Analysis

Partial *COI* sequences of each individual were checked and analyzed using BioEdit 5.0.9 software (Hall, 1999). A consensus sequence was obtained for each DNA segment and later the sequences were aligned using ClustalW ver. 1.6 [29]. Then, the alignment was checked again by the BioEdit 5.0.9 software [30] and corrected manually where necessary. The absence of stop codons in COI sequences was checked using software BioEdit. Apart from that, sequence similarity was determined by comparing with existing databases in the NCBI Genbank using the Basic Local Alignment Search Tool (BLAST) algorithm [31]. To clarify the taxonomic status of *Macrobrachium* species in this study, *COI* gene sequences of reference species were retrieved from GenBank and were used to generate a phylogenetic analysis (Table 1).

The phylogenetic reconstruction was inferred using the Neighbor-Joining (NJ) method [32]. Before that, Maximum likelihood (ML) [33] analysis was carried out to find best-fit DNA substitution model. In the NJ analysis, levels of branch support were assessed using the bootstrap test [34] with 1000 replicates to evaluate the reliability of the inferred topologies and only confidence values >50% were reported. Inter- and intraspecific genetic distances of the sequences were calculated using the Kimura (1980)-2-parameter model [35]. Both phylogenetic analysis and genetic distance were performed using the MEGA 7.0.14 [36].

3. Results

3.1 Sequence Characteristics and Similarity Analysis

The mitochondrial *COI* sequence amplified by the COIa and COIf primer pair varied from 600 to 657 bp in length (Table 2). No stop codon was revealed when the *COI* sequences were translated into amino acids. All COI sequences determined in this study were deposited in the Genbank data bases under accession numbers KX151823-KX151868 (Table 2). Based on sequence similarity analysis in BLAST, the sequences of individual prawn samples showed varied degree of similarity with existing data in the NCBI database (Table 2).

COI gene revealed that brood stock (BS) and juvenile (JH) prawns from BMRI hatchery showed high similarities (89 to 99%) to *M. rosenbergii* to sequences from GenBank. Two morphologically different species of adult prawns (BA and WA) doubted at the early stage of the study were identified as similar to *M. rosenbergii* (89 to 95%) and *M. mammillodactylus* (83 and 99%) respectively. Remarkably, three species of *Macrobrachium* were identified from sequence similarity in this study *viz*. *M. rosenbergii, M. mammillodactylus* and undescribed juvenile of *Macrobrachium* species. Juvenile prawns (JS) caught in Petagas River recorded similarity of 99% to *M. mammillodactylus*, 84 – 86% to *Macrobrachium* sp. and other species of prawns were also found in this study, *Caridina gracilipes* (91-92 %), *Caridina* sp. (81 – 87%), *Litopenaeus stylirostris* (83%), *Metapenaeus ensis* (99%) (Table 2).

Table 1: Additional mitochondrial *COI* sequences of prawn and outgroup used in this study obtained from GenBank

Order	Family	Genus	Scientific name	Accession no.
Decapoda	Palaemonidae	*Macrobrachium*	*M. rosenbergii*	HG779438
			M. rosenbergii	KJ652338
			M. rosenbergii	AB235295
			M. rosenbergii	AY569990
			M. rosenbergii	KM593101
			M. rosenbergii	AB235295
			M. mammillodac-tylus	AB235282
Decapoda	Atyidae	*Caridina*	*C. gracilipes*	KM023648
		Caridina	*Caridina* sp.	DQ478464
			Caridina sp.	EF432627
			Caridina sp.	AM747790
Decapoda	Penaeidae	*Litopenaeus*	*L. stylirostris*	EU517503
Decapoda	Penaeidae	*Metapenaeus*	*M. ensis*	KF192817
		Metapenaeus	*M. ensis*	KP637170
Decapoda	Portunidae	*Scylla*	*S. serrata*	AB114216
		Scylla	*S. paramamosain*	AY750935

Table 2: Sequence similarity analyses of prawns used in this study i.e. samples from BMRI shrimp hatchery and samples caught in Petagas River. Sample abbreviations, sample localities, maximum identity (%) based on BLAST and Accession numbers are provided.

Sample abbreviation	Sequence similarity	Sample locality	Maximum Identity (%)	Accession no.
BS01	*M. rosenbergii*	BMRI, shrimp hatchery	99	KX151830
BS02	*M. rosenbergii*	BMRI, shrimp hatchery	92	KX151831
JH01	*M. rosenbergii*	BMRI, shrimp hatchery	91	KX151832
JH02	*M. rosenbergii*	BMRI, shrimp hatchery	89	KX151833
JH03	*M. rosenbergii*	BMRI, shrimp hatchery	92	KX151834
JH04	*M. rosenbergii*	BMRI, shrimp hatchery	92	KX151835
JH05	*M. rosenbergii*	BMRI, shrimp hatchery	92	KX151836
JH06	*M. rosenbergii*	BMRI, shrimp hatchery	92	KX151837
BA005	*M. rosenbergii*	Petagas River	91	KX151823
BA006	*M. rosenbergii*	Petagas River	92	KX151824
BA007	*M. rosenbergii*	Petagas River	90	KX151825
BA010	*M. rosenbergii*	Petagas River	89	KX151826
BA011	*M. rosenbergii*	Petagas River	95	KX151827
BA012	*M. rosenbergii*	Petagas River	90	KX151828
BA013	*M. rosenbergii*	Petagas River	92	KX151829
WA001	*M. mammillodactylus*	Petagas River	99	KX151860
WA002	*M. mammillodactylus*	Petagas River	99	KX151861
WA003	*M. mammillodactylus*	Petagas River	99	KX151862
WA004	*M. mammillodactylus*	Petagas River	99	KX151863
WA005	*M. mammillodactylus*	Petagas River	99	KX151864
WA006	*M. mammillodactylus*	Petagas River	99	KX151865
WA007	*M. mammillodactylus*	Petagas River	99	KX151866
WA008	*M. mammillodactylus*	Petagas River	99	KX151867
WA009	*M. mammillodactylus*	Petagas River	99	KX151868
JS01	*M. mammillodactylus*	Petagas River	99	KX151838
JS04	*Macrobrachium* sp.	Petagas River	86	KX151839
JS06	*Litopenaeus stylirostris*	Petagas River	83	KX151840
JS07	*Metapenaeus ensis*	Petagas River	99	KX151841
JS08	*Litopenaeus stylirostris*	Petagas River	83	KX151842

JS09	*Litopenaeus stylirostris*	Petagas River	83	KX151843
JS10	*Litopenaeus stylirostris*	Petagas River	83	KX151844
JS11	*Metapenaeus ensis*	Petagas River	99	KX151845
JS12	*M. mammillodactylus*	Petagas River	99	KX151846
JS13	*M. mammillodactylus*	Petagas River	99	KX151847
JS14	*M. mammillodactylus*	Petagas River	99	KX151848
JS15	*M. mammillodactylus*	Petagas River	99	KX151849
JS16	*M. mammillodactylus*	Petagas River	99	KX151850
JS18	*Macrobrachium* sp.	Petagas River	86	KX151851
JS23	*Macrobrachium* sp.	Petagas River	84	KX151852
JS24	*M. mammillodactylus*	Petagas River	99	KX151853
JS25	*Caridina gracilipes*	Petagas River	92	KX151854
JS26	*M. mammillodactylus*	Petagas River	99	KX151855
JS27	*Caridina gracilipes*	Petagas River	91	KX151856
JS28	*Caridina* sp.	Petagas River	87	KX151857
JS29	*Caridina* sp.	Petagas River	81	KX151858
JS37	*Caridina gracilipes*	Petagas River	92	KX151859

3.2 Phylogenetic analyses of all prawn samples used in the study

Based on results from Model test, the best-fit model of the ML analyses was T92+G (Tamura, 1992), with a correction for the among-site rate variation (G) of 0.28 and no correction proportion of invariable sites (I). A total of 62 nucleotide sequences of prawn species, comprised of 46 samples of prawn analyzed in this study, 2 species sequences of the outgroup and 14 sequences available from GenBank (Table 1). The phylogenetic tree (Figure 1) was constructed using the 62 *COI* gene sequences showed evident separation clades from all species found. Each sample species of *M. rosenbergii*, *M. mammillodactylus* and *Macrobrachium* sp. which were morphologically close to each other formed a strongly supported monophyletic subclade by 71%, 92% and 100% bootstrap value respectively together with the *COI* sequences retrieved from GenBank (Table 1). The *M. rosenbergii* of the stocking species from BMRI clustered together with the *M. rosenbergii* caught from the river with relatively high bootstrap value by 57%. Other species of prawn samples found from the Petagas River also formed monophyletic clades with *COI* gene sequences from GenBank.

It is worth noting that, juvenile samples of prawn caught in Petagas River, *L. stylirostris, C. gracilipes* and *Caridina* sp. were clustered in the same clade with *Macrobrachium* genus, though their sister taxon pairs have weak bootstrap support. The species of *M. ensis* formed a monophyletic clade and separated from *Macrobrachium* genus, *L. stylirostris, C. gracilipes* and *Caridina* sp. at 57% bootstrap value. The outgroup species *S. Serrata* and *S. paramamosain* were genetically distinct and formed a strongly supported monophyletic group distinct from all the prawn clade with high bootstrap support value by 100%.

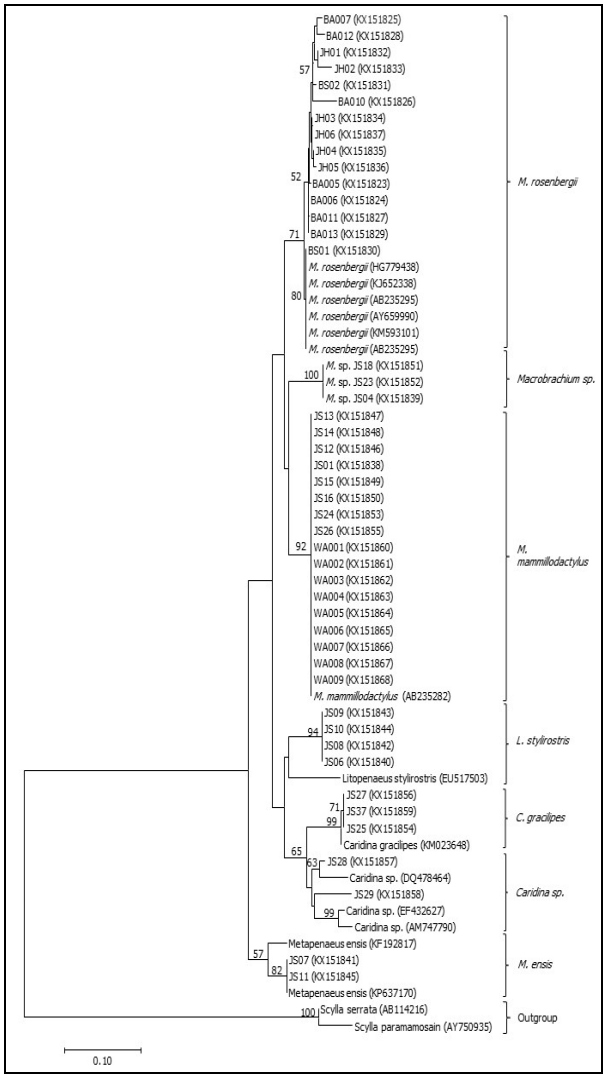

Fig 1: Molecular phylogenetic tree of prawn samples based on cytochrome oxidase subunit I (*COI*) sequences generated by neighbor-joining (NJ) method. Percentage of bootstrap values above 50% after 1000 replications are shown above the line of each node.

3.3 Genetic distances of COI gene of all *M. rosenbergii* samples from BMRI hatchery (stocking species) and Petagas River

Genetic divergence among the individual samples of brood stock and juvenile *M. rosenbergii* from BMRI ranged 0.00% - 13.8%. Populations of *M. rosenbergii* from different locality (Petagas River) showed genetic divergence ranged 0.6% - 10.1%. The lowest intraspecific divergences ranged between *M. rosenbergii* from BMRI hatchery and *M. rosenbergii* from the river (box in Table 3) were 0.6% (JH04 – BA005), followed by 0.9% (JH03 – BA005, JH06 – BA005, JH04 – BA013). Lowest values of genetic distance between individuals reflect closest intraspecific level of the population between both localities of BMRI hatchery and Petagas River. The highest intraspecific divergences were also found in this study with value >10%. The ranges were exceeded the usual value, and might therefore reflect intraspecific differences and come from population of different locality. Such situations were found in samples between BS01 – BA010 (15.8%), BS01 – BA007 (12.6%), BS01 – BA012 (11.7%) and BS01 – BA005 (10.4%).

In contrast, the divergence between two morphologically distinct species of *Macrobrachium* found in the study (BA and WA), *M. rosenbergii* and *M. mammillodactylus* showed higher value which reflects interspecific differences between species with range 21.4% - 30.4%. This pattern was also found in the undescribed species of genus *Macrobrachium* revealed in this study. They were confirmed from phylogenetic analysis (Figure 1) to be genetically distinct from other species, with interspecific divergences of 23.3% - 33.2%. They were referred to as *Macrobrachium* sp. (JS18, JS23 and JS04). No value of intraspecific divergence was found to be within these ranges in this study, thus supporting the fact that they were from different species of the genus. Genetic divergence between *M. rosenbergii* and outgroup species *S. serrata* ranges between 196.4% - 252.7% showed extreme distinct distance between different family of decapoda.

Table 3: Genetic distances of the cytochrome oxidase subunit I (*COI*) mitochondrial gene between *M. rosenbergii* from BMRI shrimp hatchery (BS and JH) and *Macrobrachium* (WA and JS) with other prawn species caught from Petagas River following the stock enhancement programme including outgroup using Kimura-2-parameter pairwise distance.

		***M. rosenbergii* (BMRI shrimp hatchery)**								***M. rosenbergii* (Petagas River)**							***M. mammillodactylus***		***Macrobrachium* sp.**
		BS01	**BS02**	**JH01**	**JH02**	**JH03**	**JH04**	**JH05**	**JH06**	**BA005**	**BA006**	**BA007**	**BA010**	**BA011**	**BA012**	**BA013**	**WA001**	**JS01**	**JS18**
***M. rosenbergii* (BMRI shrimp hatchery)**	BS01																		
	BS02	0.092																	
	JH01	0.111	0.020																
	JH02	0.138	0.045	0.030															
	JH03	0.103	0.020	0.012	0.042														
	JH04	0.104	0.017	0.014	0.039	0.003													
	JH05	0.103	0.020	0.023	0.048	0.012	0.009												
	JH06	0.103	0.020	0.012	0.042	0.000	0.003	0.012											
***M. rosenbergii* (Petagas River)**	BA005	0.104	0.023	0.020	0.045	0.009	0.006	0.012	0.009										
	BA006	0.097	0.017	0.026	0.052	0.014	0.012	0.012	0.014	0.006									
	BA007	0.126	0.033	0.017	0.045	0.023	0.026	0.036	0.023	0.033	0.039								
	BA010	0.158	0.068	0.068	0.100	0.068	0.072	0.082	0.068	0.079	0.082	0.062							
	BA011	0.063	0.033	0.049	0.069	0.043	0.039	0.039	0.043	0.040	0.033	0.062	0.101						
	BA012	0.117	0.036	0.026	0.058	0.033	0.036	0.045	0.033	0.042	0.048	0.026	0.065	0.062					
	BA013	0.093	0.014	0.017	0.042	0.012	0.009	0.014	0.012	0.009	0.009	0.030	0.072	0.030	0.039				
M. mammillodactylus	WA001	0.214	0.255	0.279	0.304	0.257	0.262	0.255	0.257	0.257	0.246	0.283	0.297	0.241	0.289	0.258			
	JS01	0.214	0.255	0.279	0.304	0.257	0.262	0.255	0.257	0.257	0.246	0.283	0.297	0.241	0.289	0.258	0.000		
***M.* sp.**	JS18	0.233	0.282	0.307	0.340	0.297	0.302	0.301	0.297	0.297	0.285	0.317	0.332	0.255	0.311	0.285	0.244	0.244	
S. serrata		2.433	2.338	2.319	2.319	2.231	2.274	2.274	2.231	2.181	2.292	2.366	2.266	2.223	2.248	2.223	1.964	1.964	2.527

4. Discussion

The BLAST analysis was a primary analysis for prawn identification and used widely in the studies of *Macrobrachium* and other genus of freshwater prawn [35]. The identification of freshwater prawn using *COI* gene marker was used widely in aquaculture and fisheries to clarify the phylogenetic relationships and genetic divergences between species [37, 38, 35, 39, 40]. The mitochondrial *COI* is noted as an ideal molecular marker to identify freshwater prawn population at the level of species and have been particularly helpful analyzing crustacean phylogeny at the species level [41-43].

The index of genetic divergence obtained in interspecific and intraspecific level comparisons allowed us to describe the actual genetic structure of these *Macrobrachium* populations. The genetic divergence values between *M. rosenbergii* in BMRI are lower and very similar to the values of most samples from Petagas River. This trend could also be observed in *M. mammillodactylus* samples from Petagas River. This result reflects probable alterations in the genetic structure of these populations [44]. Besides, the lowest genetic divergence can be observed in samples from same species with genetic values of 0.00% could be attributed to the long-term captivity, which makes the population genetically homogenous [44].

However, in term of stock enhancement programme, the genetic distances analysis between the stocking species, *M, rosenbergii* from BMRI shrimp hatchery and Petagas River was the key to the effectiveness of the stock enhancement programme. Lowest genetic distances were observed in samples, 0.6% (JH04 – BA005), followed by 0.9% (JH03 – BA005, JH06 – BA005, JH04 – BA013), reflects close relationship between samples. The most accepted hypothesis is that these samples caught from Petagas River were probably originally from the stock enhancement project which already matured and reproduced in the wild. Reported that crustacean in the British Isles have shown a substantial number of released matured lobsters and reproduced in the wild [45-47]. Thus, the high divergence values can refute the hypothesis that the samples came from BMRI shrimp hatchery.

Molecular studies did not support the close relationship of adult *M. rosenbergii* and *M. mammillodactylus* (with genetic distances of 21.4% to 30.4%) (Table 3) as indicated by close morphological similarities. The dataset was analyzed and these two different species formed evident different subclades from the same genus (Figure 1). This reflects the interspecific differences between the species. Abundant of *M.*

mammillodactylus found in Petagas River illustrates that the species was the dominant species in the river compared to the stocking species, *M. rosenbergii* [48]. This is consistent with earlier finding suggesting that *M. rosenbergii* and *M. mammillodactylus* are genetically distinct by using *COI* gene [35].

Three monophyletic subclades formed from phylogenetic analysis comprised of adult and juvenile stages of *M. rosenbergii*, *M. mammillodactylus* and *Macrobrachium* sp., with high bootstrap support and showed interspecific levels of genetic divergences (21.4% - 33.2%). They showed minor morphological differences of intraspecific variations. This suggests that the use of morphological characters alone is insufficient to establish species groups of *Macrobrachium* accurately [18, 49, 19, 35]. According to prior study, *Macrobrachium* species share some morphologic characteristics and they are syntopic, which complicates their taxonomic identification [50]. Different morphotypes in this species also increases doubts about the taxonomic identification [51].

COI gene can differentiate among the closest species, corroborating the utility of the COI gene as a good marker to separate close and sibling species. Moreover, within the *M. rosenbergii* and *M. mammilodactylus* clades illustrated in the phylogenetic analyses, there were an obvious different genetic structure fixed in both two populations. Therefore, the observed morphological variability as mentioned in the problem statement of this research must be not a phenotypic plasticity. They are closely related and there are a small number of differences morphological between them. As reported by Liu et al., 2007, by using *COI* gene, *M. olfersii* showed no genetic structure and no haplotype fixed in a single population and thus proving that the species experienced phenotypic plasticity [35].

At the specific level, all species of *M. rosenbergii* represented by individuals from distant locations (Table 3) form well supported monophyletic lineages. There was an unusually large level of divergence between the two *M. rosenbergii* samples ranged 0.117-0.158, which correlates high divergence of genetic distance. However, the range of the divergence was not much and greater than that *M. mammillodactylus* from across a similar geographical range.

The lack of sister-group relationships among areas i.e BMRI hatchery and Petagas River that share a common geological history proves that large-scale events have not been important in shaping the evolutionary history of *Macrobrachium* [52, 53]. Instead, it appears that the population's genetic structure can be modified and attributed by human interference [44]. It has been reported that, with the rapidly growing population in Putatan, which is more than 50,000 residents [54], housing and building projects are increasing fast. With the absence of proper sewerage and water treatment system, discharge of untreated wastes released into the river contributes to river pollution [55] and might disturb the population's genetic in aquatic species.

The *Macrobrachium* sp., *L. stylirostris*, *C. gracilipes*, *Caridina* sp. and *M. ensis* found in this study (Figure 1) suggests that the use of molecular techniques using COI marker will be a significant help in delimiting species and understanding their relationships [56-58, 35]. *C. gracilipes* is a common atyid shrimp (Family Atyidae) lives in the fresh and brackish waters [59]. Its life histories can be classified into amphidromous and landlocked types [60-61]. The presence of the *C. gracilipes* and *Caridina* sp. in this study is acceptable because Petagas River covers freshwater and estuaries. The samples of this species were caught at both upstream and downstream of the river. According to the divergence and phylogenetic tree (Figure 1), *C. gracilipes* and *Caridina* sp. formed a monophyletic subclade separated with other genus of prawn evidently. According to Liu et al. (2007), the family of Atyidae tend to become established in freshwater resulted from selective pressures and adaptive convergence [35].

In contrast, *L. stylirostris* and *M. ensis* were found in this study at the estuary part of Petagas River. These prawns breed only in tropical marine habitats and spend their larval, juvenile, in coastal estuaries. They are marine shrimps of the superfamily Penaeoidea represent approximately one third of the world's commercially important shrimp species and account for over 80% of the wild catch [62]. From the *COI* phylogenetic tree analysis (Table 1), *L. stylirostris* and *M. ensis* formed separated clade and subclade. This is due to their distinct groups in the family of Penaeidea. In distinguishing Penaeidae, the family is divided into five groups : group 1 (*Penaeus*), group 2 (*Penaeopsis*), group 3 (*Atypopenaeus, Trachypenaeopsis*, *Metapenaeus*), group 4 (Parapenaeus, Parapenaeopsis, Trachypenaeus), and group 5 (Metapenaeopsis) [63, 64]. This would explain the species separation occurred in the phylogenetic tree analysis in this study.

The genetic structure of a population is not a stable phenomenon and subjected to modification over time. The magnitude and trend of the modification mostly depend on the intensity of natural and human interventions [65]. This happens as the parent prawn and PL are harvested from the rivers and the sizes of the existing populations are determined by the intensity of catch and recruitment that occur in the river. Apart from that, genetic diversity of smaller populations declines at a faster rate than that of a larger population. Eventually, a loss of genetic variation might happen and this loss of genetic variation is considered to be the loss of fitness of a population [65].

Since the stocking species, *M. rosenbergii* seems to be distributed continuously along the Petagas River, the consequent question is would it be sufficient the amount of stocking juveniles released in the Petagas River and the sampling sample sizes to assess the effectiveness of the stock enhancement of the *M. rosenbergii* in the Petagas River. One example of the most successful stock enhancement of prawn in Asia was carried out in Sri Lanka with a total of more than 100,000 juveniles of Penaeus prawns were released in Rekawa lagoon, subsequently yielded an outstanding return [66] and 70 million of *M. rosenbergii* post larvae were released into rivers in Thailand [67]. Not to forget the effectiveness of stock enhancement of rare flatfish in, Fukushima Japan. According to Lorenzen (2005) the level of enhancement must be high relative to the natural recruitment capacity of the depleted stock, therefore the rebuilding stock will be effective [68].

In summary, mitochondrial *COI* gene worked effectively to characterize two morphotypes of *Macrobrachium* found in this study and evidently showed two distinct species viz *M. rosenbergii* and *M. mammillodactylus*. The genetic distance of *M. rosenbergii* sample showed relatively close to the stocking species of *M. rosenbergii* from BMRI shrimp hatchery. This result indicates that currently, the stocking species of *M. rosenbergii* need to be boosted continuously *via* enhancement programme until it can suppress and compete with the established species population of *M.*

mammillodactylus in the Petagas River as it is supposed to be the dominant species in the river. To further studies, a mass and sufficient sampling sample sizes would help to represent the whole population of *Macrobrachium* primarily *M. rosenbergii* in Petagas River to further investigate the evolutionary history of the genus and its genetic distances with the purpose of assessing the effectiveness of the stock enhancement programme.

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